primary purpose of the article. One of the major problems in attempting to unravel the mechanism of freezing in either the botanical or zoological areas has been the confusing variability in response to freezing by different individual cells and tissues. This variability has often obscured the underlying physical phenomena that are common to all. It was the objective of the article to discuss the basic phenomena and to indicate, using soft tissue cells as a principal example, some of the responses to be expected.

The quotations which Levitt has selected serve quite well to demonstrate some of the specific differences in the responses of plant cells to freezing, and it is indeed unfortunate that the existence of such dissimilarity was not made clear in the original article. However, these specific differences, which are concerned primarily with survival, must not be permitted to obscure the relevance of the underlying general mechanics. To imply, as Levitt does in paragraphs 5, 6, and 7, that dehydration from rapid freezing, recrystallization during thawing, or water binding by glycerine do not apply to plant cells because the cells will have already been killed is to subjugate general principles to a special application. A semirigid cell wall and an essentially water-filled vacuole will, of course, produce a different response to ice crystal formation from that of muscle or liver, just as these tissue responses differ from those of bone or tendon. Despite this variability, crystal growth, dehydration, recrystallization, and all the other physical events attending freezing and thawing inevitably take place regardless of the point in the sequence of events at which the cell is killed. Water binding by glycerine always reduces the dehydration from freezing, and whether or not the cell derives any benefit thereby has no influence on the validity of this fact. It was my experience that a study of the myriad responses of different cells to freezing led to a most confusing assemblage of inconsistent conclusions until the fundamental mechanics of the process had been outlined and a physical basis for interpretation had been provided. It was primarily to provide a hypothesis for this physical basis from which others could interpret their own special applications that this article was written.

Levitt also touches on one point that surely troubles many scientists in many fields: the practical unavailability of knowledge of common value derived by workers in other disciplines. The exchange of reprints cannot, in itself, be an answer, for this presupposes a knowledge of the other man or his work. Much of the responsibility must lie with the individual investigator to evaluate those aspects of his own work that may be of general interest beyond his immediate field and to present them, as much as possible out of their specialized context, in a journal of not too highly specialized nature. The bias of his interests will inevitably show through but, it is hoped, not so strongly that it frightens away the potential beneficiary.

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Carbon-14 Tetrachloride Produced by Neutron Irradiation of Aniline- and Pyridine-CCl_4 Solutions

The recoil method of synthesis for C^{14}-labeled compounds involving neutron irradiation of nitrogen-containing compounds dissolved in other solvents or in a pure state has been discussed by a number of investigators (*1, 2*). This method of synthesis depends on the high recoil energy (approximately 40,000 ev) of the C^{14} atom that is acquired following the $N^{14}(n,p)C^{14}$ reaction. The recoil energy of the C^{14} atom produced by the emission of the proton is dissipated by collisions with neighboring molecules, which often result in bond breaking of the struck molecules. In the recombination of these molecular fragments, the C^{14} atom may be included, and thus a C^{14}-labeled molecule is produced. This study (*3*) reports on the production of $C^{14}Cl_4$ from the neutron irradiation of solutions of aniline ($C_6H_5NH_2$) and pyridine (C_5H_5N) in CCl_4. Although the carbon tetrachloride produced was of a low specific activity, the simplicity of the procedure recommends it over chemical synthesis.

Five milliliters of a 10 mole percent solution of pyridine in carbon tetrachloride was sealed in a partially evacuated quartz capsule held at Dry Ice temperature. Five milliliters of 10 mole percent of aniline in carbon tetrachloride was prepared in a similar way. The samples were irradiated for 1 month in the Oak Ridge graphite reactor at an average neutron flux of 3×10^{11} neutrons cm^{-2} sec^{-1} and gamma ray flux of 5×10^5 r hr^{-1}. The solution of pyridine in CCl_4 following irradiation was a dark liquid with large particles present. When the capsule was opened in a manner previously described (*4*), there appeared to be little or no build-up of pressure in the sample. The aniline-CCl_4 solution appeared to be solid and almost opaque. When it was opened, it was apparent that pressure had built up in the capsule, and the sample became a liquid after release of the pressure. It was similar in appearance to the pyridine-CCl_4 solution with one exception. A tough, dark polymer covered the liquid surface and reacted slowly with added CCl_4 with a hissing noise similar to the sound of a small piece of sodium in water.

Because the neutron irradiation of CCl_4 produces considerable quantities of S^{35}, certain procedures were necessary to obtain pure fractions of CCl_4 and to measure only C^{14} radiations (*5*). After addition of carrier CCl_4 and removal of 1-ml aliquots for determination of total C^{14} originally present, the amines were extracted with three 10-ml washes in 3*N* HCl and one water wash. The CCl_4 layer was then refluxed for 1 hour with a sodium hypobromite solution to oxidize any sulfur-containing compounds, and then the CCl_4 was distilled into three fractions and a residue.

Fifty-microliter samples of the purified CCl_4 fractions and of the original samples were combusted in a Pregl-type combustion tube. The $C^{14}O_2$ was trapped in a standardized NaOH solution and the percentage of carbon recovered was determined by titration procedures. Silver wire present in the combustion tube filling should absorb any oxides of sulfur still present. After transfer to an apparatus described by Comar (*6*), the NaOH solution with the trapped $C^{14}O_2$ was acidified and heated to boiling; the CO_2 was swept out through a mercury seal into an evacuated 250-ml ionization chamber. The disintegration rate as a function of ionization current was then determined with a vibrating reed electrometer .

Table 1 lists the experimental values found for the various fractions and the originals. About 31 percent of the total C^{14} activity measured was found in CCl_4 from both samples. This would tend to corroborate the earlier report (*2*) that the nature of the carbon hot-atom reactions is a characteristic of the solvent. The residue left after distillation was quite active, although no quantitative

Table 1. Carbon-14 activity of the purified CCl_4 fractions and of the original solution after the addition of carrier CCl_4.

Fraction	No. samples (No.)	Boiling point range (°C)	Activity (disintegrations/sec 50 µl)
	Pyridine-CCl_4 solution		
1	2	70–74	9.6
2	2	74–75	10.5
3	3	75	10.4
Original	2		32.5
	Aniline-CCl_4 solution		
1	3	45–72	7.4
2	2	72–75	6.7
3	2	75	4.5
Original	4		20.0

studies were made on the high-boiling fractions. Neither the acid nor the basic washes contained appreciable quantities of C^{14}-labeled compounds.

It is apparent that $C^{14}Cl_4$ of low specific activity can be produced by the recoil method.

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6 December 1956

Double-Gradient Agar Plates

A considerable portion of the *in vitro* study of the action of antimicrobial compounds consists of the investigation of possible effects of inorganic or organic substances on the potency of the compounds. Such investigations are usually performed with only a single or a few concentrations of each substance and antimicrobial compound. To obtain complete information concerning possible suppressing or enhancing effects, or both, of a test substance on a compound, however, a large number of substance-compound ratios should be screened. Correspondingly large numbers of flasks, tubes, or petri plates are required for such studies if conventional techniques are employed.

A useful and widely adopted method for the establishment of numerous gradually differing concentrations of an antimicrobial compound (or, alternately, of an essential nutrilite) in a single petri plate has been described by Szybalski (*1*). His method may be designated the single-gradient plate technique. Sacks (*2*) has described recently a type of double-gradient plate in which K_2HPO_4 is contained in the medium in the first or lower layer (which is solidified while the plate is in an inclined position) and KH_2PO_4 in the second or upper layer of medium (solidified while the plate is in a level position). Thus a continuous change in the ratio of the phosphates is established across the horizontal axis of the medium, with a resulting series of *p*H reactions from 5.6 to 7.8 over the surface of the agar. Such a plate can be used to determine the influence of *p*H on (i) growth, virus propagation, or other physiological activities of microorganisms and on (ii) the potency of an antimicrobial agent or the utilization of a nutrilite, provided that an appropriate concentration of the particular compound is incorporated in a thin (third) layer that is spread evenly over the surface of the agar medium.

This communication (*3*) describes the inclusion of a test substance (a metallic salt) in the first layer and an antimicrobial compound in the second layer of nutrient agar gradient plates. Thus a continuously graded ratio of test substance/compound is established over the surface of the medium. Preliminary titrations with single-gradient plates are used for determination of the concentrations of test substance or compound that completely inhibit, partially inhibit, or fail to inhibit the microorganisms. In the actual tests, the appropriate single-gradient plates are included to serve as controls. The test organisms are spread evenly over the surfaces of the control and experimental plates; our most consistent results are obtained with an inoculum per plate of not more than 10^3 viable cells contained in 0.05 ml of nutrient broth. An alternate method of inoculation consists of the streaking of cells of several test strains at right angles to the double gradient.

The observations made with the double-gradient plates clearly indicate instances of suppression or enhancement of an antimicrobial compound by a test substance. A diagrammatic representation of some of the results obtained with a strain of *Pseudomonas aeruginosa* is given in Fig. 1. It may be observed that Fe^{++} but not Cu^{++} suppresses the antibacterial activity of oxytetracycline and that the toxicity of neither of the ions is affected by the drug. In contrast, the antibacterial activity of kojic acid is affected by both Fe^{++} and Cu^{++}; certain proportions of metallic ion/compound are more toxic than either the ions or the compound alone, and other proportions are less antibacterial than the compound itself. As with oxytetracycline, however, the toxicity of the metallic ions is not suppressed by kojic acid. These observations are in agreement with results obtained previously with *Pseudomonas* by other methods (*4*).

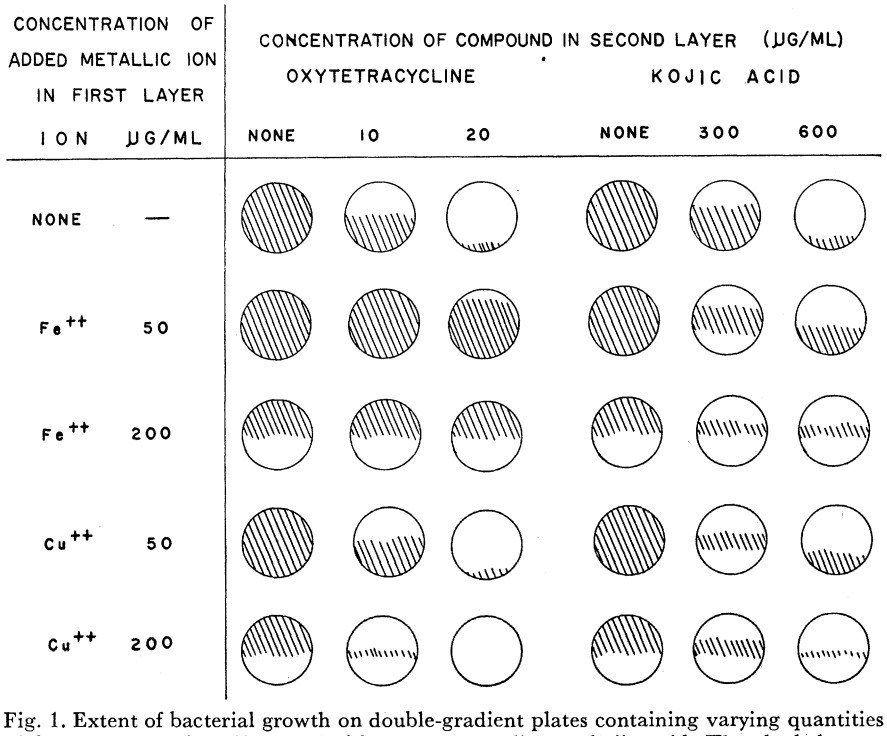

Fig. 1. Extent of bacterial growth on double-gradient plates containing varying quantities of ferrous or cupric sulfate and either oxytetracycline or kojic acid. The shaded areas represent visible growth of *Pseudomonas aeruginosa* after 18 hours at 37°C.

It is believed that use of the double-gradient plate method can facilitate research not only in studies of antimicrobial compounds, but also in certain areas of microbial nutrition, physiology, and virology.

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