which should be plotted for such a process, and, in such a plot, the points in Fig. 1 fall on a curve with a continually increasing slope for lighter δ values, as would be expected (from the vapor pressure data) for precipitation at lower temperatures in high latitudes. The linear relation observed in Fig. 1 simply reflects a coincidence of the effect of the increasing difference in δ and log $(1 + \delta)$ at high enrichments with the effect on the slope of the average temperature decrease for precipitation along a meridian from equator to poles (*6*).

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6. Detailed papers on isotopic variations in meteoric and volcanic waters of specific areas will be published elsewhere. It is a pleasure to acknowledge my gratitude to Harold C. Urey in whose laboratories at the Institute for Nuclear Studies, University of Chicago, most of this work was done, to Mrs. T. Mayeda for her excellent services in the Chicago laboratory, and to G. Boato for many interesting discussions. This research has been supported by the National Science Foundation, the University of California Water Resources Commission, the Office of Naval Research, and the Atomic Energy Commission.

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Germination of Bacterial Endospores with Calcium and Dipicolinic Acid

Abstract. Aerobic and anaerobic bacterial endospores can be germinated if calcium chloride and dipicolinic acid are added to well-washed suspensions. Maximum germination is obtained when the calcium and acid are present in a molar ratio of one or more. This suggests that the 1:1 chelate of calcium and dipicolinic acid is the agent that induces germination.

During experiments concerned with the effect of chelating agents on spore germination, we observed that germination was induced when calcium chloride and dipicolinic acid (2,6-pyridine dicarboxylic acid) were added to suspensions of clean, well-washed bacterial spores provided that the molar ratio of calcium to dipicolinic acid was 1:1 or higher.

The procedure was to dissolve the dipicolinic acid in enough NaOH solution to give a neutral solution of known strength. A standard $CaCl_2$ solution, either with or without tris buffer, was mixed with the solution of dipicolinic acid and NaOH immediately before addition to the spore suspension. In such mixtures of calcium and dipicolinic acid, we have been able to germinate spores of the following organisms: Putrefactive anaerobe 3679 (NCA and h strains), putrefactive anaerobe S_2, *Clostridium perfringens, Bacillus cereus, B. megaterium, B. mycoides, B. subtilis,* and *B. coagulans.* The germination was sometimes incomplete, but in most cases rapid and complete germination took place.

The effect of calcium and dipicolinic acid on germination seemed to be rather specific. None of the following metal ions could be substituted for calcium: Na^+, K^+, Mg^{2+}, Mn^{2+}, Ba^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{3+}. Neither could other chelating agents or any of the other pyridine dicarboxylic acids be substituted for dipicolinic acid.

Table 1 shows that germination with calcium and dipicolinic acid, as measured either by counting residual (heat-resistant) spores or by counting the dark and the refractile spores with a phase-contrast microscope, is quite rapid.

The effect of different ratios of calcium and dipicolinic acid was tested by adding various concentrations of the acid to a germination solution containing 40 mmole of $CaCl_2$. Figure 1 shows the results obtained with *Bacillus megaterium* and putrefactive anaerobe S_2.

Chelation takes place when dipicolinic acid is added to a solution containing calcium ions. The calculated concentrations of the 1:1 chelate of calcium and dipicolinic acid are also plotted on the graph. The relationship between the two curves provides a strong suggestion that this chelate is the active inducer of germination. The calculation of the concentration of the 1:1 chelate of calcium and dipicolinic acid was based on data obtained by titrations of the acid with NaOH in the presence and absence of calcium ions. These titrations showed that two types of chelates with different stability constants were formed. If the molar concentration of calcium equals or exceeds that of dipicolinic acid, a chelate is formed between 1 mole of calcium ion and 1 mole of the acid. If the concentration of dipicolinic acid is increased over that of calcium, the formation of a higher chelate containing 2 moles of the acid per mole of calcium ion takes place. That this 1:2 chelate apparently has little or no germination-stimulating effect is also indicated in Fig. 1. This has been further substantiated by similar experiments with other organisms showing that germination in the presence of optimum calcium (40 mmole) is decreased by excess dipicolinic acid. The addition of excess calcium to a given solution of the acid does not change the concentration of the 1:1 chelate, and such additions have been shown to have little or no influence on the germination rate.

Table 1. Germination of *Bacillus megaterium* and putrefactive anaerobe S_2 spores in 40 mmole of $CaCl_2$, 40 mmole of dipicolinic acid, and 10 mmole of tris buffer at *p*H 7.0.

Incubation (min)	Germination (%) based on Phase microscopy	Germination (%) based on Pasteurized counts
	*B. megaterium**	
0	0	0
5	0	
10	81	100
20	99	100
	Putrefactive anaerobes S_2†	
0	0	0
5	93	98
10	99	100

* Incubation temperature, 25°C. † Incubation temperature, 35°C.

Germination with the 1:1 chelate of calcium and dipicolinic acid took place readily over a *p*H range of 5 to 9, but was generally most rapid at values close to *p*H 7. The concentration of calcium and dipicolinic acid required for rapid germination was between 20 and 40 mmole, which is somewhat higher for the aerobes we have tested than for the anaerobes. The optimum temperature for germination with the 1:1 chelate of calcium and dipicolinic acid was about 45°C for clostridial spores, but most aerobic spores were found to germinate very slowly or not at all at temperatures above 35°C. This was apparently because of the formation of a precipitate of calcium and dipicolinic acid when the concentration of calcium and the acid was higher than about 20 mmole. The rate of precipitation was increased at higher temperatures and

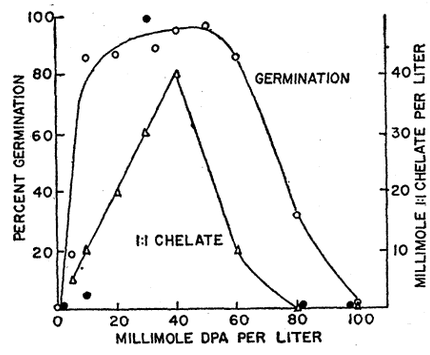

Fig. 1. Germination of spores suspended in 10 mmole of tris buffer, 40 mmole of $CaCl_2$, and varying concentrations of dipicolinic acid (DPA). Open circles: spores of putrefactive anaerobe S_2 incubated at 35°C for 60 minutes. Closed circles: spores of *Bacillus megaterium* incubated at 25°C for 30 minutes. Triangles: the calculated concentration of the 1:1 chelate of calcium and dipicolinic acid.

thus may have prevented germination of aerobic spores which required a concentration of the 1:1 chelate of close to 40 mmole. If the precipitation was retarded by the addition of amino acids or protein (gelatin)—substances which by themselves did not induce germination—to a solution of calcium and dipicolinic acid, germination of *Bacillus* spores also took place at higher temperature.

The literature contains very little information concerning the effect of chelating agents on spore germination. Powell (*1*) reported that the germination of *Bacillus subtilis* spores with L-alanine was inhibited completely by 10 mmole of oxine or 2,3-dimercaptopropanol (BAL). Brown (*2*) observed that the putrefactive anaerobe PA 3679 could be made to germinate in solutions of ethylenediaminetetraacetic acid; however, an optimum amount had to be used. If too much was added, the spores did not germinate. We have been able to reproduce Brown's observations by using putrefactive anaerobe 3679, but none of the other spore formers we tested germinated with ethylenediaminetetraacetic acid. We have also observed that some of the spore suspensions of PA 3679 could be germinated with tripolyphosphate or dipicolinic acid alone. Here, as with ethylenediaminetetraacetic acid, an optimum concentration had to be used. The inhibitory effect of high concentrations could be reversed by the addition of calcium chloride.

It is not yet possible to explain the mechanism by which a mixture of calcium and dipicolinic acid induces germination. Neither is it known whether the calcium and dipicolinic acid naturally present in spores have a stimulatory function when germination is induced by other agents, that is, added amino acids or mechanical abrasion. However, such a function cannot be ruled out. The large amounts of dipicolinic acid (6 to 12 percent of the dry weight) and calcium (1.5 to 3.0 percent of the dry weight) present in spores provide a calcium-to-dipicolinic acid molar ratio of close to one; this could conceivably affect the germination rate, particularly after these materials appear in the suspending medium.

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13 January 1961

Applications of Fluorescent Brighteners in Biological Techniques

Abstract. The visual labeling of various microorganisms has been accomplished with specific fluorescent ultraviolet-absorbing compounds that not only have been bound by the cell but have been transferred by it to subsequent growth. The possibility of application to genetic studies is suggested.

Although the use of fluorescent substances to visualize specimens was initiated in 1914 by Provazek (*1*), this technique has developed slowly. It was not until the early 1930's that the fluorescent dyes acriflavine, fluorescein, primulin, rhodamine, and thioflavin were applied to living tissues (*2*), and it was not until 1940 that the diamino acridines were used as vital stains (*3*). The interaction of the diamino acridines with nucleoproteins has formed the basis of techniques for observations of virus-infected cells (*4*), of the multiplication of bacteriophage in lysogenic cells (*5*), and of the multiplication of bacteria and yeast cells (*6*). In all of these experiments the dye has been used either as a simple stain with or without fixation or has been kept constantly in contact with the cells.

The possible use of fluorescent substances not only as vital stains but also as markers for genetic and developmental studies of microorganisms is reported here. Such compounds should be essentially nontoxic at the concentration employed, efficiently absorbed by the cell, and sufficiently stable for detection in subsequent growth. Attempts to use such well-known vital fluors as acridine orange, fluorescein, rhodamine, and thioflavin were unsuccessful in preliminary trials. Some of the commercially available official bleaching agents or brighteners, however, were found to offer some possibility of successful application. These substances appear to be (i) highly fluorescent, (ii) able to pass through cell walls, (iii) substantive to proteins, (iv) fluorescent at *p*H 5.0 to 8.5, and (v) stable as regards fluorescence when bound.

Among the brighteners tested, those of the diamostilbene class with substantivity for cellulosic fibers were selected for preliminary investigations. These compounds were added to submerged shaker fermentations, either before inoculation or after a period of incubation. Both synthetic and natural media were employed. Although the uptake of these brighteners by the cell wall was essentially instantaneous, distribution within the cell required a longer period of time. It is of significant interest that active growth centers have evidenced the greatest concentration of these fluors, as for example the budding region in yeast cells, hyphal tips, and germ tubes. When fluorescent cells which were washed three times with sterile 0.85 percent NaCl were transferred to liquid or agar medium, subsequent growth fluoresced. Fluorescent spores which were washed three times with sterile 0.85 percent NaCl also gave rise to fluorescent mycelium. Indications are that leaching of the fluorescent compounds into the medium did not occur to an extent sufficient to cause this fluorescence, since other organisms which had previously been observed to fluoresce with these particular substances did not do so when introduced into the medium. There is obviously a point at which this fluorescence may become undetectable by reason of consecutive dilution as growth proceeds.

Observations were made in a dark room with a magnesium fluoride coated microscope with an 85-watt mercury arc lamp and a No. 5840 Corning exciter filter; a set of Wratten barrier filters was fitted in the oculars, and an aluminized mirror was placed over the substage mirror of the microscope. Cargille's immersion oil Type A of very low fluorescence was used. In addition to the usual microscopic observations, hanging drop and agar cover-slip preparations were also employed for growth and reproduction studies.

A specific example of this technique follows. *Penicillium chrysogenum* (Wisconsin Q176) was grown at 28°C on a reciprocal shaker for 1 day in a 250-ml erlenmeyer flask containing 50 ml of a corn steep liquor-glucose medium. At this time 0.01 ml of a 12 percent solution in aqueous Cellosolve of the disodium salt of 4,4′-bis [4-anilino-6-bis(2-hydroxyethyl) amino-S-triazin-2-ylamino]-2,2′-stilbenedisulfonic acid (*7*) was added. On the second day a blue fluorescent differentiation was evident within the cells as well as in the side walls and cross walls of the mycelium; there was marked concentration of fluorescence at the hyphal tips which glowed brightly. That this fluorescence is stable is shown by the observation that refrigerated mycelium has continued to fluoresce strongly for the past 6 months. The fluorescent mycelium was washed three times with sterile 0.85 percent NaCl and was then transferred at a concentration of 1 percent to fermentation shaker flasks containing corn steep liquor-glucose medium. Hanging drop slides were also prepared. Transfers were incubated at 28°C. New growth in both instances showed a blue fluorescence.

The technique of fluorescent labeling with brighteners has been applied to bacteria (*Bacillus subtilis* ATCC 6633