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

and *Escherichia coli* QM B1457), yeasts (*Saccharomyces cerevisiae* ATCC 7753), actinomycetes (*Streptomyces aureofaciens* ATCC 10762), and higher fungi including *Mucor murorum* QM 776, *Penicillium chrysogenum* Wisc. Q176, and *Neurospora crassa* ATCC 9683. Additional experiments are in progress. It is hoped that this technique may be used to study hyphal fusion, cytoplasmic inheritance, and genetic recombination as well as the more common developmental processes. Other applications may be expected to become apparent as the technique is explored further.

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A Quantifiable Behavioral Correlate of Psychotogen and Tranquilizer Actions

Abstract. A representative psychotogen, lysergic acid diethylamide (LSD-25), in doses small enough to be devoid of gross effects, increases response latency in rats to a tone indicating the availability of water reward; this effect is greatly reduced by prophylactic administration of a representative phenothiazine tranquilizer, chlorpromazine (CPZ), in doses that per se do not affect performance. The nature of the chlorpromazine action and its competition with lysergic acid diethylamide is revealed by the effects of chlorpromazine in larger doses.

It was felt that the significance of the cerebral synaptic inhibitory action of psychotogens and the competition by tranquilizers established in these laboratories (*1*) could be understood further and a beginning made in assessing their role in behavior, if quantitative behavioral correlates could be found. One correlate that has proved valuable has been the lever-pressing of thirsty rats for a water reward, when a tone indicates its availability. This conditioned approach behavior, monitored by measuring the stimulus-response latency (length of upper vertical line, with corresponding reward achievement indicated by the marks on the lower line of each strip in Figs. 1 and 2), shows clear inhibition after intraperitoneal injection of a moderate dose of lysergic acid diethylamide (strip 3). As seen in an example (Fig. 1), the latency exceeds the 20-second period within which reward is possible and recycling of the trial takes place after a randomly timed interval. In the doses used, the inhibitory action is selective and not due to general sedation. A preventive intraperitoneal injection of chlorpromazine, in a dose that per se produces no change in the same animal (strip 2), readily gives protection as shown by the abbreviated effect of subsequent lysergic acid diethylamide (strip 4) (*2*).

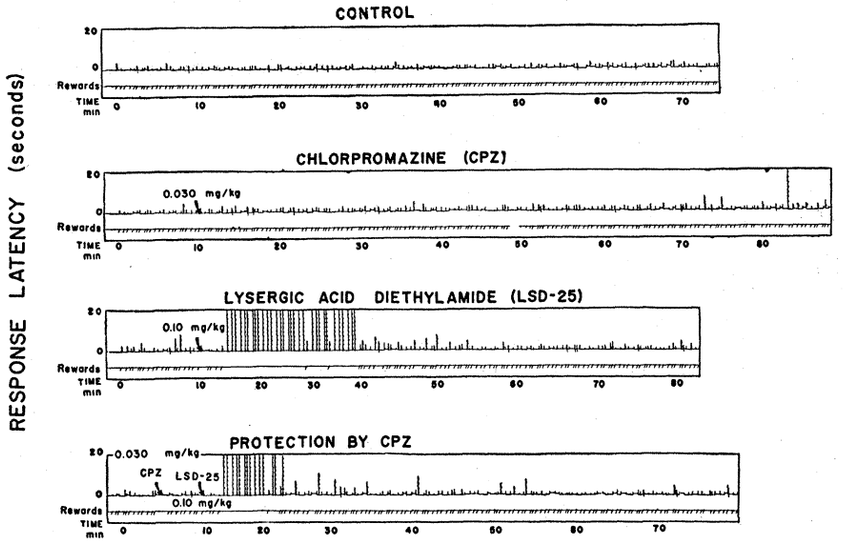

Fig. 1. Lysergic acid diethylamide (LSD-25) inhibition and chlorpromazine (CPZ) protection on approach behavior in rat.

The nature of the antagonism of lysergic acid diethylamide and chlorpromazine to each other is revealed by the effects of larger doses (Fig. 2). Thus, the larger, but nondepressant dose of chlorpromazine enhances instead of reducing lysergic acid diethylamide inhibition, while a still larger dose produces a depression of approach behavior that resembles the effect of lysergic acid diethylamide, but is distinguished from it by a considerable degree of sedation. The observed antagonism and summation are the characteristics that mark the competition between two substances with like actions but very different potencies. Because of this the weaker agent can, when it has gained access to the site of action (receptor) in moderate quan-

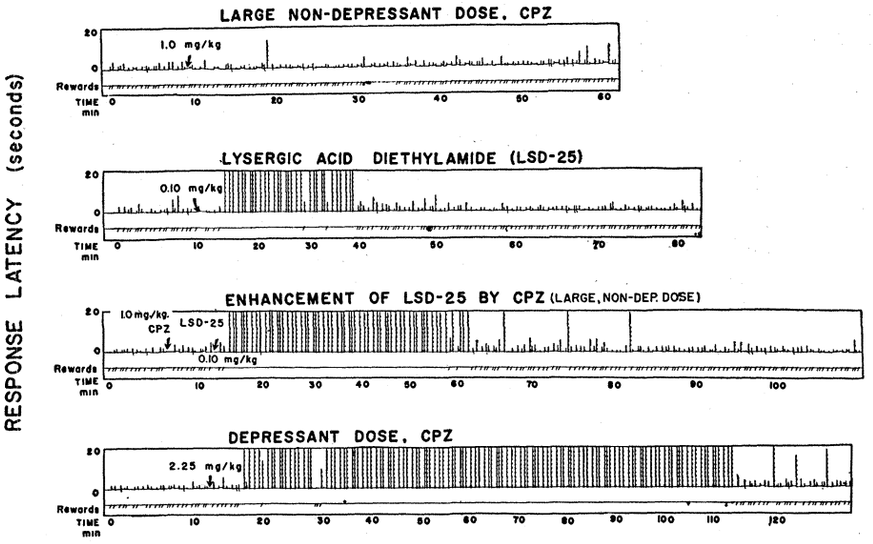

Fig. 2. Effects of larger than protective doses of chlorpromazine (CPZ) on approach behavior in rat.