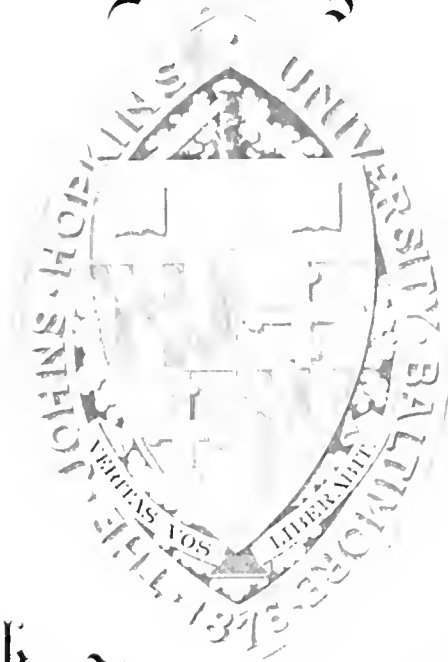


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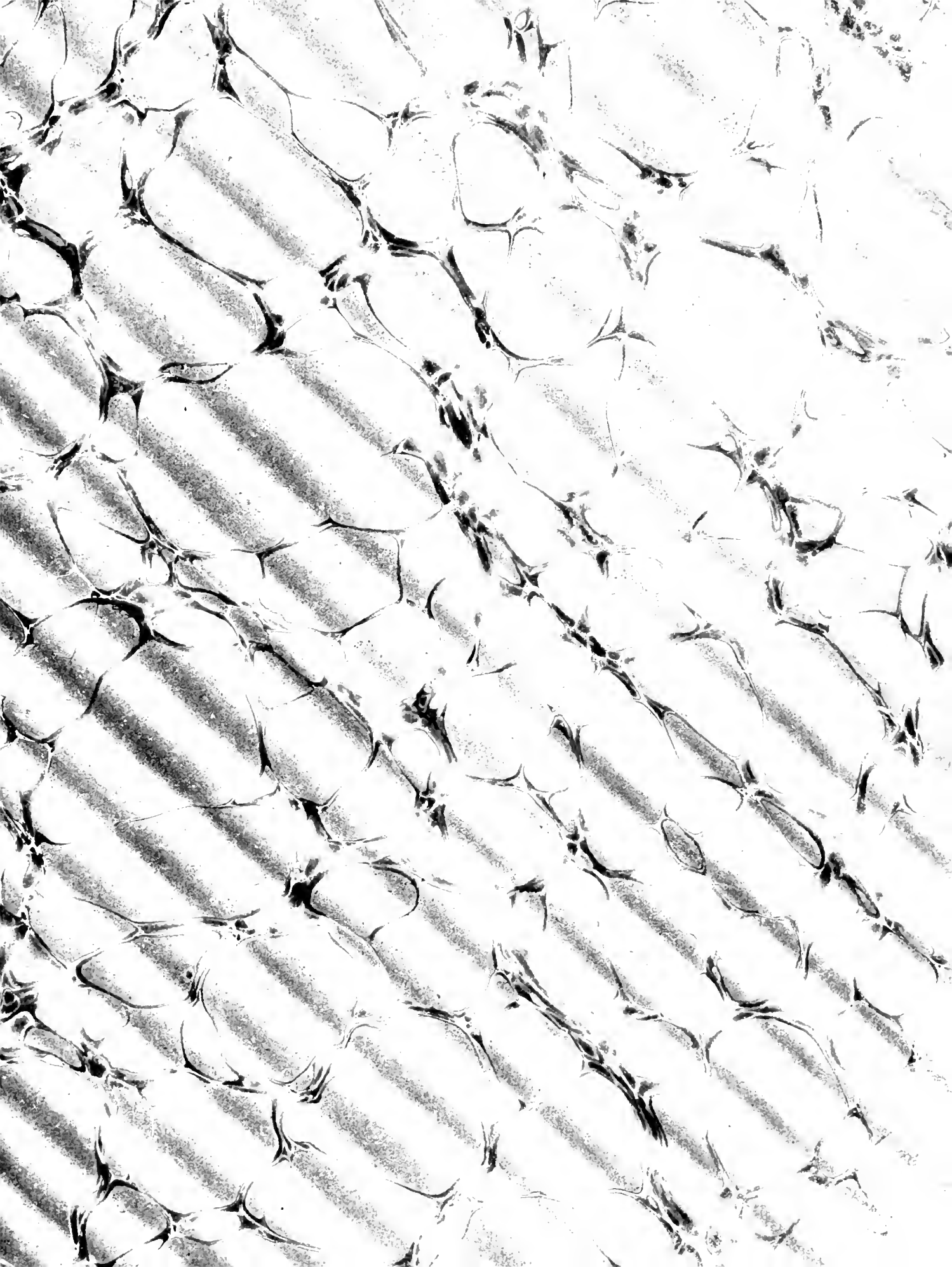


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THE INFLUENCE OF CALCIUM, MAGNESIUM AND POTASSIUM NITRATES
UPON THE TOXICITY OF CERTAIN HEAVY METALS
TOWARD FUNGUS SPORES.

DISSERTATION

Submitted to the Board of University Studies of
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THE INFLUENCE OF CALCIUM, MAGNESIUM AND POTASSIUM NITRATES
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TOWARD FUNGUS SPORES.

ABSTRACT

This study has to do with the influence of one salt upon the toxic effect of another upon fungus spores. It is shown that the toxic effect of $\text{Cu}(\text{NO}_3)_2$ on the germination of Gloeosporium spores can be inhibited or modified by the addition to the medium of $\text{Ca}(\text{NO}_3)_2$ and that the molecular ratio of the quantity of $\text{Ca}(\text{NO}_3)_2$ thus required to the amount of $\text{Cu}(\text{NO}_3)_2$ present increases with the concentration of the latter. This effect is apparently the result of a simultaneous action of the two salts upon the organism and cannot in cases here considered be related either to formation of an undissociated double salt or to depression of the ionization of the toxic salt because of the ion common to the two salts. Potassium nitrate is also effective in inhibiting or modifying the toxicity of $\text{Cu}(\text{NO}_3)_2$. The influence of calcium upon the toxicity of copper is of interest in the problem of fungicides and fungicidal action.

The toxicity of $\text{Pb}(\text{NO}_3)_2$ is similarly influenced by the presence of $\text{Ca}(\text{NO}_3)_2$ and the ratio of the calcium salt to that of lead was found to be constant for the three different concentrations of the toxic salt that were employed. The toxicity of this lead salt is likewise influenced by proper concentrations of $\text{Mg}(\text{NO}_3)_2$. Both $\text{Ca}(\text{NO}_3)_2$ and

$Mg(NO_3)_2$ markedly decrease the toxicity of $Zn(NO_3)_2$, but neither exhibited any effect on the toxicity of $Al(NO_3)_3$ at the concentrations used in these experiments.

The effects produced by the various single salts upon the germinating fungus spores are of interest in that four types of response to the toxic stimulus are clearly discernible. It was usually possible to bring about these four types of response in any one of the compounds used in this study by varying its concentration. If the substances are arranged in the order of their toxicity as evidenced by their inhibition of spore germination, this same arrangement is found also to hold for their effectiveness in bringing about the less final changes which lead to abnormal growth.

THE INFLUENCE OF CALCIUM, MAGNESIUM AND POTASSIUM NITRATES
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Numerous instances have been recorded of the influence of salts on the toxicity exerted by substances upon organisms. This antagonistic action, as it is frequently called, of a salt upon a toxic substance, is of considerable importance in influencing the behavior of organisms in a given environment, or indeed in determining whether or not they may exist at all in certain environments. For example, Loew¹ has shown that the toxic effect on Spirogyra of a 1 percent. solution of magnesium nitrate is inhibited by the presence in the medium of a 0.3 percent. solution of calcium nitrate, while Loeb² has demonstrated that the addition of a small quantity of calcium to an 0.625m solution of NaCl inhibits the toxic effect of the NaCl on the development of Fundulus' eggs. Osterhout³ has shown that a physiologically balanced solution

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1. Loew, C., Ueber die physiologicishen Functionen der Calcium- und Magnesium-salze im Pflanzenorganismus. Flora 75:368-394, 1892.
 2. Loeb, J., Ueber den Einfluss der Werthigkeit, und moglicher Weise der electricischen Ladung fur Ionen auf ihre antitoxisch wirkung Pflugers. Archivs. 88:68-78, 1902.
 3. Osterhout, W.J.V., On the importance of physiologically balanced solutions for plants. Bot. Gaz. 42:127-134, 1903.

of NaCl, MgCl₂, MgSO₄, KCl and CaCl₂ is necessary for the best growth of marine algae. True and Bartlett⁴ in an extended research have brought out the fact that a ratio of one molecule of calcium to 9 molecules of magnesium inhibits the toxic effect of rather high concentrations of magnesium upon roots of Canada field peas. Other cases of antagonistic salt action in combinations of salts of the alkali metals or of the alkaline earths have been demonstrated, and some information has been obtained regarding the influence of these salts on the effect of the heavy metals, which are almost universally toxic. The heavy metals have not received so much attention, however, and it seemed worth while to investigate some of these alone and in the presence of calcium and magnesium, to obtain evidence as to whether the lighter metals may modify in any way the toxicity of the heavy metals.

The investigation described in this paper was accordingly undertaken. The problem here involved will be taken up more in detail after some of the literature pertinent to this investigation has been considered.

One of the earlier investigations on the influence of chemical compounds on the toxicity of the heavy metals

4. True, R. H., and Bartlett, H. H., Absorption and excretion of salts as influenced by concentration and composition of culture solutions, U. S. Dept. of Agr., Bur. Plant Ind. Bulletin No. 251, 1912.

was carried out by Krönig and Paul⁵. These authors tried the

5. Krönig, B., and Paul, Th., Die Chemischen Grundlagen der Lehre von der Giftwirkung und Desinfection. Zeitschrift für Hygiene und Infectious Krankheiten. 25: 1-112, 1897.

effects of various heavy metals in combination with many salts as well as with certain acids and bases. Working mainly with Bacillus anthracis, they regard the influence of other halogen compounds upon the toxicity of mercuric chloride as probably due to a depression of the ionization of the latter salt. In this connection they say: "Die Desinfections-wirkung wässriger Mercurichloridlösungen werden durch Zusatz von Halogenverbindungen der Metalle und von Salzsäure herabgesetzt. Es ist wahrscheinlich, dass diese Verminderung der Desinfectionskraft auf einer Rückdrängung der elektrolytischen Dissociation beruht" (page 111).

Clark⁶ carefully studied the influence of various

6. Clark, J. F., On the toxic value of mercuric chloride and its double salts. Journ. Phys. Chem. 5:289-316, 1901.

concentrations of sodium chloride upon the toxicity of mercuric chloride as regards the process of germination in various fungus spores and seeds and the growth of yeasts and bacteria. He found that the toxicity of the mercuric chloride solution increased with the addition of small quantities

of sodium chloride but was decreased when high concentrations of the sodium salt were used. He explained these phenomena by considering that a double salt of sodium chloride and mercuric chloride was formed, such as Na_2HgCl_4 or some similar combination. He supposed that the dissociation tension of this double salt was probably much lower than that of mercuric chloride, a consideration which might account for the decreased toxicity of the combinations in which high concentrations of sodium chloride were employed. In this connection, he suggests that the HgCl_4 ion present when such a salt as Na_2HgCl_4 dissociated at lower concentrations might be considerably more toxic than the Hg ion, the latter being probably the toxic ion of mercuric chloride.

In a later investigation on the toxicity of copper in combination with various chemical compounds, the same writer⁷ has shown for the spore germination of Oedocephalum

7 Clark, J. F., On the toxic properties of some copper compounds with special reference to Bordeaux mixture. Bot. Gaz. 33:26-48, 1902.

albidum and Rhizopus nigricans that ammonium nitrate, sodium sulphate, potassium sulphate, and potassium chloride all markedly decreased the toxic effect of both copper chloride and copper sulphate. He used relatively high concentrations, in some cases 5 per cent of the alkali and ammonia salts. He considers the decreased toxicity just mentioned as due, probably, to the formation of double salts as in the case of mercuric chloride.

5

Le Renard⁸ studied the comparative toxicity of salts

8 Le Renard, Alf., Influence du milieu sur la résistance du Pénicille Crustacé aux substances toxiques. Ann. de Sci. Nat. Bot. 16: 276-356. 1912.

of many of the heavy metals upon Pencillum, the fungus being grown with various concentrations of nutrient media. He used the acetates of potassium, magnesium and ammonium alone, and the acetates, formates, nitrates, phosphates, and sulphates of these three metals with glucose. In combination with various concentrations of the salts in the nutrient media he used several concentrations of the chlorides and nitrates of zinc, nickel, cobalt, copper, mercuric chloride, silver nitrate, and the sulphate and acetate of copper. The presence in the nutrient media of the lighter metals in higher concentration was usually found to decrease the toxicity of the heavy metals.

True and Gies⁹ showed that calcium modified the

9 True, Rodney H., and Gies, W. J., On the physiological action of some of the heavy metals in mixed solutions. Bull. Torr. Bot. Club, 30:390-402, 1903.

toxicity of various copper salts, zinc sulphate and mercuric chloride in their effect upon the growth of roots of Lupinus albus. In discussing their results these authors say: "The stimulating action of the calcium seems to have operated

against the retarding action of the copper and the result is a marked diminution in the poisonous action of the copper. They thus relate this influence of calcium upon copper to a mutual effect of the two salts on the protoplasm.

Szűcs¹⁰ has recently shown that the toxic effect of copper sulphate on the roots of Cucurbita pepo may be inhibited by certain concentrations of aluminum chloride. In this case he used as index of toxicity the ability of the root to react to a geotropic stimulus after it had been removed from the poisonous solution. He varied the presentation time of the toxic stimulus (copper sulphate) both alone and in combination with aluminum, and found for the shorter time periods that the presence of aluminum inhibited the poisonous action of the copper. However, if such a combination of the two salts was allowed to act for longer periods it also was toxic, in some cases, and the roots lost their ability to respond afterwards to a geotropic stimulus. He used concentrations of copper sulphate varying from 0.001875n to 0.075n in combination with aluminum chloride in concentrations varying from 0.005n to 0.45n. The presentation time of the toxic stimulus ranged from 33 minutes to 26 hours and 50 minutes. This writer also studied the effect upon Spirogyra of quinine hydrochlorid, methyl violet and piperidin in combination with various other substances. The toxicity of quinine

10. Szűcs, Joseph, Experimentelle Beiträge zu einer Theorie der antagonistischen Ionenwirkungen. Jahrb. für Wissenschaftliche Botanik 32:85-113, 1912.

hydrochlorid was altered by various concentrations of other substances, being almost inhibited by aluminum nitrate, markedly decreased by calcium nitrate, and only slightly lessened by potassium nitrate. Thus, the effectiveness of these salts in reducing the toxicity of quinin hydrochlorid diminished with the valency of the cation. Similar results were obtained with the same series of salts in combination with methyl violet. The effect of combinations of various substances with piperidin was in most cases markedly to increase its toxicity. Szűcs apparently considers the antagonistic action in the cases investigated by him to be due to the lowering of the rate of absorption of the toxic ion by the presence in the solution of another ion of similar sign. He concludes in summarizing that:- "Die Ursache der 'antagonistischen Ionenwirkungen' liegt in allen von mir untersuchten Fällen in der gegenseitigen Beeinflussung der Aufnahmegeschwindigkeit zweier im gleichen Sinne geladener Ionen."

From the results obtained in the investigation just considered it is apparent that in some cases at least the toxic effect of the heavy metals on an organism can be modified by the addition of the proper concentration of certain salts. True and Gies, and Szűcs, working with higher plants, attribute this influence of one salt on the effect of another to a simultaneous action of the two salts upon the organism itself, while Clark, working with fungi, relates the inhibition of the toxic effect of heavy metals in combination to some modification of the salts in the solution. The prop-

ortions of salts used in these investigations were widely different and it is of course possible that the different conclusions arrived at may have been due to this feature. Furthermore, as fungi and higher plants so frequently react differently to the same stimulus, it is possible that one of these two explanations might hold for one group of organisms and the other for the other. The present study, in which a fungus was employed, was undertaken partly to throw light on the question just suggested.

It is the purpose of this research to examine the effects of the nitrates of copper, lead, zinc, nickel, and aluminum upon the germination of fungus spores, the salts of the heavy metals being used both alone and in combination with the nitrates of calcium and magnesium, to see whether the presence of the lighter metals in various concentrations may or may not decrease the toxic effect of the heavy ones. It was also considered worth while, in case such decrease was found to occur, to determine as far as possible whether this influence might be related to the direct effect of salts on each other in the solution or was due to a modification of the organism itself. Furthermore the results obtained in these experiments should throw some light on the problem of the comparative toxicity of the various substances here employed when used alone, and thus on the general physiological problem of toxicity.

The investigation was carried out at the Laboratory of Plant Physiology of the Johns Hopkins University, and the writer's sincere thanks are due Professor Eurlon S. Livingston for his many helpful suggestions and valued assistance throughout the progress of the work.

ORGANISM.

The fungus spores used in this research were of the gloeosporium or conidial stage of Glomerella dingulata (Stonem) S. and v. S., the fungus causing the disease of the apple known as "bitter rot". The fungus is parasitic not only upon the apple but according to Shear and Wood¹¹ it is also the cause of disease on other plants. On the apple fruit it produces brown sunken areas usually nearly circular in shape which may be covered with the fruiting bodies of the fungus, the conidia being borne in acervuli. In mass the spores appear orange colored but have a hyaline appearance under the microscope. They are usually ovate or oblong in shape and are 12-16 x 4-6 in diameter. Shear and Wood have shown that certain strains of this fungus may, when grown on the proper artificial media, produce conidia for generation after generation, without the interpolation of the ascogenous form at any time. Cultures of such a strain were secured from Dr. Shear for these experiments, and throughout the investigation, which lasted about eighteen months, conidia only were produced, though some forty or more generations must have passed.

Corn meal agar was used as a medium for the stock cultures. This was prepared by adding 4 teaspoonsful of white corn meal to one litre of distilled water, which was allowed to stand at about 58°C for one hour. After

11. Shear, C. L., and Wood, Anna K., Studies of fungus parasites belonging to the genus Glomerella. U. S. Dept. of Agr. Bur. of Plant Ind. Bull. 252.

filtration 12.5 g of agar flour was added to the infusion, and the mixture was steamed for one and one half hours. It was then filtered again and was ready to tube and sterilize. The tubes were slanted and the stock cultures were inoculated in streaks. On this medium the fungus produces but little mycelium, at or beneath the surface. The conidia are borne in relatively large orange colored masses on the surface of the medium along the streak, these acervuli being visible in from two to five days after inoculation.

Before the spores were used in the experiments, the stock cultures were allowed to grow undisturbed from five to fifteen days, which insured a sufficient quantity of spores from a single tube for the inoculation of an entire series of experimental cultures. Preliminary tests seemed to show that spores from acervuli in the same tube but of different ages were not at all uniform in viability. Direct inoculation of the culture dishes from the spore masses of the stock tubes was found to be unsatisfactory, since it was not only desirable to have all cultures contain approximately the same number of spores, but also that the percentage of viability of the spores in all cultures should be as nearly alike as possible. It seemed desirable to avoid small pieces of agar and bits of mycelium in the liquid cultures, for such contamination might influence the effect of the salts in the solutions on the germination of the spores, as by adsorption or the formation of new chemical compounds. The plan was therefore evolved of

allowing the stock cultures to grow for from ten to fifteen days after which the spore masses were carefully removed with a platinum needle to a clean area on the surface of the agar, from which, after thorough mixing in a little heap, the inoculations to the water cultures were made. This method usually resulted in a satisfactory uniformity in the germination of the spores in the various controls, from which fact it was concluded that all cultures thus made contained an approximately equal number of viable spores, and that any inhibition or modification of germination must have been due to properties of the culture medium rather than to differences in the spores introduced.

In comparing the effect of the various media upon the spores the main criterion was the presence or absence of germination after a period of 18 hours. In many cases, however, germination was more or less modified, as in the production of swollen tubes and other abnormalities, and such modifications of germinal activity needed frequently to be taken into account. As has been indicated, it was seldom necessary to consider the percentage of normal germination which occurred, but in many cases the proportion of abnormal to normal growth was approximately determined.

The spores of *gleosporium* possess several very favorable features for such an investigation as the present. They are readily wetted by water and aqueous solutions, and, being slightly heavier than water, they sink quickly to the bottom of a hanging drop. They germinate readily in

distilled water in from 3-4 hours and produce long filament in eighteen hours, a feature which is of considerable advantage here, for it is quite conceivable the influence of various chemical compounds on each other and on the germinating spores may be considerably altered by the presence of nutrient salts in the solution. In view of these considerations these experiments were carried out without the use of nutrient media.

METHODS

The salts used in these experiments were "Eaker's analyzed chemicals" procured in the original packages. Stock solutions of the different salts, from which the experimental solutions were afterwards prepared were made up in 0.2 m, 0.5 m, or molecular concentration. In preparing the stock solutions the salts were weighed in glass-stoppered weighing bottles directly from freshly opened packages and were dissolved in volumetric flasks. These solutions were then made up to the required volume at a temperature of 15°C. They were stored in Jena glass bottles which had been carefully washed with a saturated solution of potassium dichromate in sulphuric acid, steamed for half a day, again washed with distilled water and then allowed to soak in distilled water for a month or more to remove any soluble matter which might be present. Distilled water from a still with tin lined boiler and condenser was used in making the stock solutions as well as in diluting them for the cultural work. Preliminary tests showed that the spores germinated as well in this still as in the more nearly pure water distilled from potassium permanganate using a hard flask and block tin condenser.

The stock salt solutions were diluted to the concentrations required in making up the solutions for the experiments, by pipetting out a certain amount into a hard glass beaker and then adding the necessary distilled water from a burette. The concentrations of these solutions were so cal-

culated the culture solutions could be prepared without the measurement of less than 0.5 cc. in any case. Thus errors that might have arisen in attempting to read hundredths of a cubic centimetre on a burette graduated only to tenths, were obviated.

In making up a series of cultures the two salt solutions which were to be combined were usually diluted to twice the concentration finally desired and then placed in burettes. From these were prepared, with addition of water as needed, the combinations and concentrations actually used in the experiments. These mixtures, in volumes of 10 cc. or more, were made in small flasks (of about 75 cc. capacity), a flask being provided for each of the different combinations as well as one for the control. The latter solution contained the salt of the heavy metal alone.

From each of the flasks just mentioned a small portion of solution (about a cubic centimeter) was placed in a separate glass dish (2 x 3 cm.) to which spores from a stock culture were then transferred. These inoculations were made in order, beginning with the weakest solution of the lighter metal. A platinum needle was used for this purpose, flamed and washed to clean and sterilize it after each inoculation.

In each case the tip of the needle was dipped a single time in the well mixed mass of spores which had been prepared as already described, and the spores which adhered were washed off in the culture solution. Thus approximately the same number of spores were inoculated into all of the dishes

and the solutions were then ready for the preparation of the hanging drops.

The drop cultures were made after much the same method as that described by Clark¹². Van Tieghem cells were used, small glass cylinders with ground ends, 9 mm. high and 12 mm. in diameter, which were cemented to common microscope slides by means of beeswax. Two cells were affixed to each slide. The culture solutions in the glass dishes, into which spores had been inoculated, were thoroughly mixed with a glass rod, by means of which a drop of the liquid was then placed upon a flamed cover glass.

A small drop of the culture solution from the corresponding flask without spores was placed in the bottom of the Van Tieghem cell and the cover bearing the drop culture was inverted over it. Duplicate drop cultures were made from each concentration of the solutions, both cultures being placed on the same slide. As has been shown by Clark, the presence at the bottom of the culture cell of a small amount of the same solution as that from which the hanging drop is composed, prevents evaporation from the drop and hence obviates marked alteration in its concentration, even if the cultures remain in the thermostat for a considerable time. Without this precaution the solution contained in the



12. Clark, J. F., On the toxic effect of deleterious agents on the germination and development of certain filamentous Fungi. Bot. Gaz. 28:290-327 and 378-404. 1899.

hanging drop is apt to become markedly more concentrated during the period of an experiment, which would lead to erratic results. The covers were sealed in position with vaseline. It was not found necessary to take the precaution recommended by Clark of first allowing the expanding air to escape through a small opening in the vaseline seal; possibly because the temperature of the thermostat here used was only a little above the temperature at which the preparations were made.

For ease in handling the cultures, the slides were placed in sheet metal trays which could be piled one upon another in the thermostat so as to form a rack. These trays were 15 cm. wide and 20 cm. long with vertical flanges at the ends, these flanges extending upward about 1.3 cm. and downward about 0.4 cm. They were so bent that the lower flanges of the upper tray fitted outside the upper flanges of the lower one, when one tray was placed upon another, and many trays could thus be arranged in a compact and rigid pile without any disturbance to the slides. The bottom of each tray was perforated with circular openings about 1.5 cm. in diameter and 0.5 cm. apart, to facilitate circulation of air. Each tray carried 14 slides. The slides were always transferred to and from the thermostat by means of these trays, a whole series of cultures being thus moved together.

The cultures were kept during germination in an electrically heated and automatically regulated thermostat, in

which the temperature was maintained at or near 25°C. As the temperature of the room in which the thermostat was placed sometimes rose above 25°, it was necessary to install apparatus for absorbing heat at such times. To accomplish this several coils of thin-walled copper tubing carrying a continuous stream of tap water were placed at the top of the thermostat, surrounding a small motor-driven fan, to insure air circulation. The air of the chamber then tended to assume a temperature several degrees below that of the laboratory, and the thermostat acted as though standing in a cold room.

GERMINATION

In these studies any renewed activity in the protoplasm of the spore was considered as germination. Several forms of such renewed activity may be exhibited in these spores. Without any alteration in size or shape, a portion of the spore may become nearly or quite opaque, thus appearing dark brown or black by transmitted light. A papilla may be formed at any point on the surface. Such papillae may or may not enlarge to form rounded bodies and may either remain hyaline or become apparently darkened. Papillae may enlarge into irregular shapes or may extend outward as markedly thickened tubes. Lastly, the growing papillae may take the form of slender tubes. The latter type of germination always occurs in distilled water and in a period of 18 hours the tubes attain a length at least twice as great as that of the spore itself. In the following treatment germination will be considered "normal" wherever tubes of the last mentioned type, at least twice the length of the spore, were produced in 18 hours.

In the present section will be considered the various effects upon germination brought about in the presence of the following salts either alone or in certain combinations:-

- | | | |
|-----------------------------------|-----------------------------------|-----------------------------------|
| Ca(NO ₃) ₂ | Pb(NO ₃) ₂ | Ni(NO ₃) ₂ |
| Mg(NO ₃) ₂ | Zn(NO ₃) ₂ | Cu(NO ₃) ₂ |
| KNO ₃ | Al(NO ₃) ₃ | CuSO ₄ |

TONICITY OF SALT COMBINATIONS

Calcium, magnesium and potassium. It requires a relatively high concentration of calcium, magnesium or potassium nitrate to inhibit the germination of the spores. With $\text{Ca}(\text{NO}_3)_2$ normal germination was found in 0.5m solutions and swollen tubes were abundant in 0.6m. At a concentration of 0.7m, however, none of the spores germinated in any of the four series of duplicate cultures which were carried out. The concentration of $\text{Ca}(\text{NO}_3)_2$ which just prevents germination appears then to lie between 0.6m and 0.7m. Magnesium nitrate is similar to that of calcium in its effect. The spores germinated normally at a concentration of 0.4m and local swellings of the spore wall and swollen tubes were produced in a 0.5m solution, while no germination was evident when a 0.6m solution was employed. The concentration at which $\text{Mg}(\text{NO}_3)_2$ just inhibited germination thus seems to lie between 0.5m and 0.6m. In solutions of KNO_3 the spores germinated normally in a concentration of 0.9m and germinated with local swellings of the spore wall in molecular concentration. As this was the highest concentration used, the point at which KNO_3 inhibits germination was not determined.

The exceedingly high concentrations of these salts which were found necessary to inhibit germination made it seem possible that they might be without toxic effect upon these spores; the inhibition of germination which was observed in high concentrations might have been the result

of the high osmotic pressure of the medium. To obtain evidence on this point, the germination of the spores was tested in various concentrations of cane sugar. A 0.2m stock solution of this was made up from granulated sugar which had been pulverized and then desiccated; the different concentrations used in the cultures were prepared from this. The spores germinated normally in 1.4m concentration or below; considerable germination in the form of local swellings (see fig. 1) was found in a 1.6m solution, and no germination occurred in 1.8m concentration. The concentration of cane sugar which just inhibits germination lies then between 1.6 and 1.8 molecular.

It will be observed that a 1.6m solution of sucrose exerts about the same effect upon the germination of these *Gloeosporium* spores as do 0.6m, 0.5m and molecular solutions of calcium, magnesium and potassium nitrates respectively. Calculations¹² of the osmotic pressures of these four

12. Morse, H. N., and Holland, W. W., The osmotic pressure of cane sugar solutions at 25°. Am. Chem. Journ. 41:1-19. 1909

The osmotic pressure of molecular cane sugar was measured directly by these writers and the osmotic pressure of 1.6m cane sugar as given above was calculated from their tables. It is probably low as they found that the ratio of observed to calculated osmotic pressure increased with the concentration. For gas pressure formula by which the osmotic pressure of the three electrolytes was calculated see Landolt and Börnstein, R., Physikalische Chemische Tabellen. 3rd auflage Berlin '05, p.34

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solutions gives 51.36, 29.13, 23.00 and 39.31 atmospheres, from which numbers it becomes obvious that the osmotic concentration of the sugar solution was much greater than that of the other three. It may therefore be concluded, unless the wall and protoplasm of the spores be readily permeable to the cane sugar, (which seems highly improbable), that the inhibiting effects observed with the salt solutions cannot be related primarily to their osmotic properties.

The relatively high osmotic pressure within the spores here dealt with, as shown by the data just given, renders it unnecessary to consider osmotic pressure as a factor in bringing about the modifications and inhibitions of germination which are now to be considered; the concentrations of the various salt solutions employed were always far too low to produce any removal of water from the cells. Similarly, the toxicity of calcium, magnesium and potassium nitrates is so very slight as not to require any consideration in connection with the combinations of these salts with the heavy metals; in these combinations the salts of the lighter metals have never been used in concentrations higher than 0.1m.

The influence of the nitrates of calcium, magnesium and potassium upon the toxicity of salts of the heavy metals will now receive attention.

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Copper. As was to be expected the effect of $\text{Cu}(\text{NO}_3)_2$ upon germination of the spores was found to be widely different from that of $\text{Mg}(\text{NO}_3)_2$ and $\text{Ca}(\text{NO}_3)_2$. No germination was ever found in concentrations higher than 0.00006m. and it was only in an occasional culture that one or two spores were observed producing local swellings at the concentration just mentioned. Local swellings of the spore walls and swollen tubes were frequently found at 0.00004m and at the next lower concentration used above that, 0.000026m; at 0.00002m germination was about as in distilled water.

Copper nitrate was combined with $\text{Ca}(\text{NO}_3)_2$ at several concentrations of the copper salt well above that at which the latter was non-toxic when used alone. In a series of combinations of 0.0001m $\text{Cu}(\text{NO}_3)_2$ with different concentrations of the calcium salt, ranging from 0.00003125m to 0.000625m, the spores germinated readily in those cultures where the concentration of $\text{Ca}(\text{NO}_3)_2$ was 0.0000625m or higher. With the last named concentration many local swellings were found and also short swollen tubes (see Fig. 2) much as in a concentration of 0.00004m $\text{Cu}(\text{NO}_3)_2$ alone. With the next lower concentration of the calcium salt 0.00003125m no germination occurred.

From the above it appears that the toxicity of 0.0001m $\text{Cu}(\text{NO}_3)_2$ is so reduced as to be physiologically equivalent to a 0.00004m solution of the same salt, by the addition to the former solution of five molecules of the $\text{Ca}(\text{NO}_3)_2$ for every eight molecules of $\text{Cu}(\text{NO}_3)_2$ present. The addition of

the lighter metal in this proportion produces the same effect as though the 0.0001m copper solution had been diluted to two and a half times its original volume.

Copper sulphate exhibits about the same toxicity towards spores of *Gleosporium* as does the nitrate. A series of combinations of this salt with $\text{Ca}(\text{NO}_3)_2$, quite similar to the series with $\text{Cu}(\text{NO}_3)_2$ just described, gave no germination in the solutions containing 0.00005125m of the calcium salt, while germination occurred in some of the cultures containing 0.0000625m of $\text{Ca}(\text{NO}_3)_2$. In general, the effect of CuSO_4 , either alone or in combination with $\text{Ca}(\text{NO}_3)_2$, was practically the same as was that of $\text{Cu}(\text{NO}_3)_2$. This furnishes some additional evidence toward the already rather firmly established conclusion, that the toxicity of copper salts is due to the cations and it also indicates the probability that considerations bearing upon this toxicity need to deal only with the cations.

A series of experiments was carried out using 0.0004m $\text{Cu}(\text{NO}_3)_2$ in combination with concentrations of $\text{Ca}(\text{NO}_3)_2$ ranging from 0.00025m to 0.025m. The spores in these combinations germinated readily in the presence of $\text{Ca}(\text{NO}_3)_2$ in concentrations ranging from 0.001m to 0.025m, but no germination was found in combinations with 0.0005m or 0.00025m of the calcium salt. The form and abundance of germination with 0.001m $\text{Ca}(\text{NO}_3)_2$ was much the same as that found in 0.00004m of the copper salt alone. Here a ratio of five molecules of the calcium salt to two of copper, the former occurring in the solution at a concentration of 0.0004m reduces the toxicity

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of the $\text{Cu}(\text{NO}_3)_2$ so as to produce an effect on spore germination equivalent to that exercised by a 0.0004m solution of the copper salt alone. In other words, the addition to a 0.0004m $\text{Cu}(\text{NO}_3)_2$ solution of $\text{Ca}(\text{NO}_3)_2$ in the molecular ratio of five of this to two of the copper salt has the same effect as diluting the $\text{Cu}(\text{NO}_3)_2$ solution to ten times its original volume.

A third series of combinations of the same two salts, but at somewhat higher concentrations, was carried out in a manner somewhat different from that of the preceding series. Here the concentration of the $\text{Ca}(\text{NO}_3)_2$ was the same (0.03m) in all of the cultures while that of the copper salt varied. The concentrations of $\text{Cu}(\text{NO}_3)_2$ varied from 0.00025m to 0.01m and spores germinated in all concentrations except the highest. With a concentration of 0.008m $\text{Cu}(\text{NO}_3)_2$ germination was similar to that found with 0.0004m of the copper salt without the addition of $\text{Ca}(\text{NO}_3)_2$. From this series it appears that the addition to the $\text{Cu}(\text{NO}_3)_2$ solution here used (0.008m), of six molecules of $\text{Ca}(\text{NO}_3)_2$ for each molecule of the copper salt present in the solution, reduces the toxicity of the latter compound in the same way as though the original solution had been diluted to 200 times its volume. It appears as though the presence of $\text{Ca}(\text{NO}_3)_2$, in this molecular proportion of about 6 to 1, altered the relations between spores and solution so that only a two-hundredth part of the copper nitrate actually present was effective to modify or retard the germination processes.

A series of combinations of $\text{Cu}(\text{NO}_3)_2$ with KNO_3 was carried out in which the constant concentration of the potassium salt, 0.05m, was used in combination with $\text{Cu}(\text{NO}_3)_2$ in concentrations ranging from 0.0002m to 0.01m. The spores germinated in concentrations up to and including 0.002m of the copper salt. In the last mentioned concentration the form of germination was quite similar to that found in a 0.0004m solution of the copper salt alone. Thus in a solution containing KNO_3 at a 0.05m concentration and $\text{Cu}(\text{NO}_3)_2$ at a 0.002m concentration (a molecular ratio of 25K to 1Cu), the toxicity of the copper salt is decreased to a magnitude only one fiftieth as great as is that shown by this concentration of the copper salt alone.

The results of the three series of combinations of copper nitrate with calcium nitrate which have been described are summarized below, together with certain other data which are about to be considered.

TABLE I.

| | | : Series | : Series | : Series |
|---|--|-------------------|--------------------|---------------------|
| | | : I | : II | : III |
| Cu(NC ₃) ₂ present in | : Common | : | : | : |
| the medium, | : fraction | : $\frac{1}{125}$ | : $\frac{1}{2500}$ | : $\frac{1}{10000}$ |
| molecular | : Decimal | : | : | : |
| | : fraction | : 0.008 | : 0.0004 | : 0.0001 |
| Ca(NC ₃) ₂ required | : Common fraction | : | : | : |
| to allow same kind | : molecular | : $\frac{1}{25}$ | : $\frac{1}{1000}$ | : $\frac{1}{16000}$ |
| and amount of | : Decimal fraction | : | : | : |
| germination as | : molecular | : 0.05 | : 0.001 | : 0.0001 |
| occurs in 0.00014M | : No. of molecules | : | : | : |
| ($\frac{M}{25000}$) Cu(NC ₃) ₂ | : of Ca(NC ₃) ₂ for | : | : | : |
| without Ca(NC ₃) ₂ | : each molecule of | : | : | : |
| | : Cu(NC ₃) ₂ | : 6.25 | : 2.50 | : 0.63 |
| Concentration of | : according to | : | : | : |
| dissociated Cu(NC ₃) ₂ | : isohydric | : | : | : |
| present | : principle | : 0.0061 | : 0.0004 | : 0.0001 |
| in | : from potentiometer: | : | : | : |
| mixture | : measurement | : 0.0058 | : 0.0004 | : 0.0001 |
| Concentration of dis- | : from | : | : | : |
| sociated Cu(NC ₃) ₂ | : conductivity | : | : | : |
| in physiologically | : measurements | : | : | : |
| equivalent solution | : (Jones' tables) | : 0.00004 | : 0.00004 | : 0.00004 |
| of this salt alone | : molecular - | : | : | : |

From these data the question arises, whether the influence of calcium nitrate in reducing the toxicity of the copper salt may be due to a direct effect produced in the solution or to some change brought about in the spore itself. Krönig and Paul (l. c.), in considering an effect similar to this , in which the toxicity of HgCl_2 upon Bacillus anthracis was decreased by the addition of NaCl , concluded that the change thus brought about is due to repression of the ionization of the salt of the heavy metal. That alteration in the ionization of $\text{Cu}(\text{NC}_3)_2$ cannot be the cause of the diminution of its toxicity as here observed is clearly shown by simple calculation from what is known as the isohydric principle. The percentage of dissociation of $\text{Cu}(\text{NC}_3)_2$ in the mixtures here ^{used} /should be determined by the concentration of the NC_3 radical. Following this generalization, the data given in Table I as "concentration of dissociated $\text{Cu}(\text{NC}_3)_2$, etc.," have been obtained. Seventy six per cent. of the copper salt of the first combination is seen to be dissociated. Now if only one per cent. of the copper salt here actually present in the mixture were ionized, the concentration of the Cu ions would correspond to that in a 0.00008m solution of $\text{Cu}(\text{NC}_3)_2$ alone, which, as has been shown, is stronger than is necessary to inhibit spore germination. It is therefore apparent that the decrease in the toxicity of the copper salt shown in these combinations with $\text{Ca}(\text{NC}_3)_2$ are not to be related to a decreased ionization of $\text{Cu}(\text{NC}_3)_2$.

Another theory to account for a similarly decreased toxicity of HgCl_2 in the presence of NaCl was advanced by Clark (l. c., page), who considered that a double salt was formed, such as Na_2HgCl_4 , only slightly dissociated in the presence of an excess of NaCl . This writer also worked with various copper salts in the presence of KNC_3 , K_2SC_4 , NH_2SC_4 etc. and suggested that the decreased toxicity of the copper salts which was uniformly observed in such combinations was likewise due to the formation of double salts.

In view of the work of Jones and Hosford¹³, - on the dissociation of double salts in dilute solution, it seems very improbable that the lowering of the toxicity of $\text{Cu}(\text{NO}_3)_2$ brought about by $\text{Ca}(\text{NO}_3)_2$, as shown in the present study, is due to increased ionization of copper owing to double salt formation. It is, however, possible to obtain direct evidence on this question by comparing the concentrations of ionic copper in the various solutions, with and without the calcium salt. The percentage of dissociation occurring in simple solutions of $\text{Cu}(\text{NO}_3)_2$ has been determined experimentally for many concentrations and these data are available in published tables¹⁴. From such data may be

13. Hosford, H. H., and Jones, Harry C., Conductivity, temperature coefficients of conductivity and dissociation of certain electrolytes. Am. Chem. Journ., 46:241-278. 1911.

14. Jones, Harry C., Electrical conductivity etc., of certain salts and organic acids. Pub. 170 Carnegie Institution of Washington.

derived the concentration of copper ions present in any solution containing only $\text{Cu}(\text{NO}_3)_2$, but no published data are yet available from which might be ascertained the concentrations of copper ions in the binary mixtures here dealt with.

The determinations here required of the relative concentrations of copper ions in solutions of $\text{Cu}(\text{NO}_3)_2$ with and without addition of the calcium salt, may be made by means of the potentiometer ¹⁵. With this instrument the solutions involved in the table given on page 26 were tested and the differences in electrical potential between the combination solutions of $\text{Cu}(\text{NO}_3)_2$ and $\text{Ca}(\text{NO}_3)_2$ and the corresponding simple solutions of $\text{Cu}(\text{NO}_3)_2$ were measured. The two solutions to be tested

15. Loomis, N. E., and Acree, S. F., A study of the hydrogen electrode, of the calomel electrode, and of contact potential. Amer. Chem. Journ. 46:585-620.

16. It is a pleasure to acknowledge here, that the suggestion of this method as applicable to the problem in hand is due to Professor S. F. Acree, of the Chemistry Department of this University, and that much valuable help in making the determinations was received from him and from Dr. C. N. Myers. This part of the work was carried out at the Chemistry Laboratory.

(for example, the simple solution containing 0.008m $\text{Cu}(\text{NO}_3)_2$ and the combination solution containing 0.008m $\text{Cu}(\text{NO}_3)_2$ and 0.05m $\text{Ca}(\text{NO}_3)_2$) were placed in the two arms of a U-tube separated below by a saturated solution of ammonium nitrate. Into each arm of the U-tube was introduced a copper electrode and these were connected with the potentiometer. The potentiometer was so calibrated that the difference in voltage between the two copper solutions might be read directly on the instrument. The copper electrodes were freshly plated and calibrated, for subsequent correction of the readings, with reference to any difference in potential which might exist between them when both are placed in the simple $\text{Cu}(\text{NO}_3)_2$ solution.

The difference in concentration of copper ions in the two solutions thus compared was calculated from the observed difference in electrical potential by the following formula:

$$V = 0.0591 \log_{10} \frac{C}{C_1}$$

in which V is the observed E. M. F. in volts, C and C_1 are the concentrations, respectively, of the copper ions in the two solutions and the quantity 0.0591 is a constant.

In the tests with which we are at present concerned

three entirely separate determinations were made, for each of which the reading was several times repeated. The results were in excellent agreement. It was found that the difference in electrical potential between the 0.008m solution of $\text{Cu}(\text{NO}_3)_2$ and the same solution containing also a 0.05m concentration of $\text{Ca}(\text{NO}_3)_2$, was 6mv., the simple solution having the higher potential. From this observation it appears, by substitution in the formula given above, that

$$\frac{C}{C_1} = 1.26 ,$$

or that the concentration of copper ions in the combination solution is $\frac{1}{1.26}$, or 0.794 as great as that in the simple one. Now, from Jones' conductivity tables (l.c. page 57) it is found by interpolation that the $\text{Cu}(\text{NO}_3)_2$ in our simple solution (0.008m) is about 90.6 per cent. dissociated at 25°C. It thus appears that the $\text{Cu}(\text{NO}_3)_2$ in the combination solution here considered must be dissociated to an extent equal to 0.794 x 90.6, or 71.9 per cent.

It has been shown earlier in this paper (page 32) that the $\text{Ca}(\text{NO}_3)_2$ in this particular combination should decrease the dissociation of the copper salt, on account of the common ion, to 76 percent. There is thus a difference of about 4 between the percentage of dissociation of the $\text{Cu}(\text{NO}_3)_2$ in this mixture, calculated from the concentration of the NO_3 ion, and that derived by the use of the electrical potential. This difference may, of course, be due to the formation of a double salt, following the hypothesis of Clark above mentioned, but it makes no difference in the present discussion whether it be 76 or 72 per cent. of the $\text{Cu}(\text{NO}_3)_2$

that is here dissociated; as has already been mentioned, the solution would still be toxic enough to inhibit spore germination in *Gloesporium* if only one per cent. of the total $\text{Cu}(\text{NO}_3)_2$ were dissociated.

The remaining two concentrations of $\text{Cu}(\text{NO}_3)_2$ (0.0004m and 0.001m, see the table already given), with and without additional $\text{Ca}(\text{NO}_3)_2$, were also subjected to potentiometer determinations of the copper ions present therein. In neither case was there any difference in electrical potential between the two corresponding solutions. It is therefore clear that at least 99 per cent. of the $\text{Cu}(\text{NO}_3)_2$ is to be considered as dissociated in these solutions, whether the calcium salt is present or not.

From the foregoing considerations, it seems quite clear that the influence of $\text{Ca}(\text{NO}_3)_2$ in reducing the toxic effect of $\text{Cu}(\text{NO}_3)_2$ on the germination of the spores here employed is not at all to be related to any changes brought about in the solution itself by the addition of the calcium salt. It appears that this antitoxic or antagonistic influence must be effective upon the spores, so altering them that they thus become capable of germination in solutions whose concentration of free copper ions would inhibit this process were it not for the presence of the calcium salt.

Whether the copper enters the spores and exerts its toxic action directly through some alteration in the protoplasm, or whether this toxic influence is exerted primarily

upon the spore walls, thus creating some disturbance in physico-chemical equilibrium which is subsequently propagated inward, is a question for the answering of which no evidence is yet at hand. Nevertheless, the present studies have clearly demonstrated that the presence of $\text{Cu}(\text{NO}_3)_2$ alone in the medium inhibits germination if the concentration be above a very low limit, produces markedly altered forms of renewed activity if the concentration is somewhat lower, and allows normal germination only when the solution is exceedingly dilute. While these facts must be interpreted to mean that the copper salt tends to upset the protoplasmic system in some way and that it is possible for that system to be so disturbed as either to inhibit germination absolutely or to allow the process to proceed in modified form, yet much more direct evidence of protoplasmic disturbance was frequently met with in the progress of this work. Spores which had been prevented from any germinational activity by the presence of $\text{Cu}(\text{NO}_3)_2$ frequently possessed a characteristic coarsely granular appearance, as though a precipitate or coagulum had been formed within the protoplasm. Furthermore, this same appearance was often encountered in spores which exhibited modified germination under the influence of copper. From this it appears that the granular appearance of the protoplasm does not necessarily denote death, but to settle this point conclusively and to determine whether the production of granulation

might not be a post mortem effect, the following experiment was performed.

Spores which had been for 18 hours in cultures with $\text{Cu}(\text{NO}_3)_2$ alone, at a concentration of 0.00003m, were employed. In this solution no germination has ever been found in the more than twenty separate cultures which have been observed, and the protoplasm usually appeared markedly granular. The spores were transferred from the $\text{Cu}(\text{NO}_3)_2$ solution to a new drop culture with distilled water and were returned to the thermostat. At the end of 24 hours observation showed that many of the granular spores had germinated normally, although the granular appearance was still very noticeable. Further proof that the granulated appearance with which we have to deal is not a post mortem effect of copper poisoning may be derived from the fact that the germinal tubes of spores which had germinated in a combination solution of 0.0001m $\text{Cu}(\text{NO}_3)_2$ and 0.00006m $\text{Ca}(\text{NO}_3)_2$, elongated considerably when the cultures were allowed to remain in the thermostat for a second day, although the spores showed the characteristic granulation.

It seems probable from these and other similar lines of evidence that the copper of the solution penetrates the spore wall and gives rise to the precipitation or coagulation effect just described.

4.

The influence of calcium upon the toxicity of copper is of interest on the problem of fungicides and fungicidal action. From the results obtained in the experiments described above the conclusion seems warranted that only a small portion of the soluble copper in Bordeaux mixture is effective as a fungicide. Yet it is a well known fact that a long list of fungous diseases including apple bitter-rot can be controlled by the proper applications of Bordeaux mixture to the host plants. The use of KCH instead of $\text{Ca}(\text{OH})_2$ in preparing the fungicide should result in a much more toxic mixture, for, as brought out in the preceding experiments, potassium is only about one fourth as effective as calcium in decreasing the toxicity of copper. From the standpoint of the host, however, it is quite probable that this anti-toxic effect of the calcium is important in preventing "spray-injury" to the foliage and fruit while the small amount of soluble copper present is, in most cases, sufficient to prevent fungous growth.

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Lead. In comparison with $\text{Cu}(\text{NO}_3)_2$ the nitrate of lead is considerably less toxic toward spores of *Gloeosporium*. The concentration inhibiting germination after 18 hours was found to be about 0.004m. With a concentration 0.002m some of the spores showed slight local swellings on the sides or ends. At this concentration spores were frequently found in which one half was brown or blackened while the other seemed devoid of protoplasm, appearing as if the protoplasmic contents had all collected in one end and had then taken on a thicker wall. No increase in size or local swelling was apparent in such cases. In a concentration of 0.001m the blackened bodies just mentioned were found, as well as swellings and also definite outgrowths from one or both ends of the spore. The latter (shown in Fig. 3) are of particular interest and require some attention here. They are spherical in shape, dark brown or black in color and appear in all respects similar to the chlamydo spores which have been frequently described as resulting from the germination, under certain special conditions, of spores of various *Gloeosporium* forms.

Appressoria have been described by Hasselbring¹⁷ as forming when nutrient materials are absent and when germinating spores or germ tubes come in contact with such hard surfaces as are furnished by the cover glass in a

Hasselbring, H., The appressoria of Anthracosas. Bot. Gaz. 42:135-142. 1906.

drop culture or by the epidermis of various fruits. This writer considers that they function as holdfasts and that they result from a contact stimulus acting upon spores or tubes which are not well nourished. That such bodies are frequently formed when germ tubes come in contact with the cover glass of a hanging drop culture, has often been demonstrated in the present studies. They are especially characteristic of cultures with certain salt solutions at concentrations somewhat below that at which germination is entirely suppressed but above that at which normal development occurs. Here then formation does not appear to be related to any contact stimulus, however. In some cases swollen bodies are produced which have the form of appressoria but which are hyaline like the usual spores and germ-tubes of this fungus, thus apparently differing from the appressoria only in not being brown or black in color. In the descriptions which follow the term appressoria^{will be used}/to denote the dark colored, appressorium-like bodies and the swellings of similar form but without dark appearance will be termed hyaline appressoria. These terms are applied merely in a descriptive way, without intended implication that the bodies thus designated may not be physiologically or otherwise different from the appressoria of the mycologists.

In concentrations of $Pb(NO_3)_2$ from 0.03Clm to 0.00005m most of the germination observed took the form of

appressoria. Some of the similar hyaline swellings also occurred in these cultures. When cultures where appressoria were common were allowed to remain in the thermostat for several days and were examined from time to time, these swellings continued to develop successively from the same spore, until sometimes as many as four appeared together at one end (see fig. 4). In the formation of chains of these bodies it appeared from the observation of different stages that a second or later swelling may be brought about either by the germination of one previously formed or by enlargement of the constricted portion of the tube between one previously formed and the spore itself, in the latter case possibly by new growth from the spore itself. In many cases a light colored area quite like that considered by Hasselbring to be a pore, could be distinguished and it may have been present in all cases.

Spores which had germinated in drop cultures by forming appressoria were transferred to new cultures in distilled water and returned to the thermostat. Nearly all the appressoria had thrust out normal germ tubes at the end of 24 hours.

An occasional spore germinated normally in a concentration of 0.0005m $Pb(NO_3)_2$, and a few normal tubes were found in 0.0004m concentration, but it was only in the 0.0002 concentration that no effect of the lead nitrate upon the germination of the spores was noticeable. In this

concentration the spores germinated with long tubes as in the simultaneous controls in distilled water.

Series including combinations of $\text{Pb}(\text{NO}_3)_2$ with $\text{Ca}(\text{NO}_3)_2$ were carried out at three different concentrations of the lead salt, 0.00016m, 0.00033m, and 0.00066m. With a 0.00066m concentration of $\text{Pb}(\text{NO}_3)_2$ no concentration of the calcium salt was found which entirely suppressed the formation of appressoria, though with a concentration of 0.05m $\text{Ca}(\text{NO}_3)_2$ only an occasional appressorium was found. This combination gave practically the same results as were found with a 0.00004m concentration of $\text{Pb}(\text{NO}_3)_2$ alone, i.e. somewhat over 50 per cent of the germination was normal, though a considerable number of swellings and appressoria were formed. In the combination of the same concentration of $\text{Pb}(\text{NO}_3)_2$ (0.00066m) with 0.004m of the calcium salt the effect produced was similar to that found in cultures with 0.00005m solution of $\text{Pb}(\text{NO}_3)_2$; only an occasional normal tube was found, these probably constituting less than 1 per cent of the total germination. With concentrations of 0.001m, 0.002m and 0.003m $\text{Ca}(\text{NO}_3)_2$ combined with a 0.00066m solution of the lead salt, practically no normal tubes were produced, germination taking the form of appressorium like bodies and swellings. These concentrations correspond to solutions of $\text{Pb}(\text{NO}_3)_2$ alone varying in concentration from 0.001m to 0.0001m.

In the second series of combinations of $\text{Pb}(\text{NO}_3)_2$

with $\text{Ca}(\text{NO}_3)_2$, the concentration of the first salt was 0.00033m and the same concentrations of $\text{Ca}(\text{NO}_3)_2$ were employed as in the series just described. The results in these experiments were similar to those of the previous series, with some differences due to the lower concentration of the lead salt. Practically no appressoria were formed with the combination containing a 0.05m concentration of $\text{Ca}(\text{NO}_3)_2$, germination being here about the same as that which occurred with a 0.0002m concentration of the lead salt alone. (see Fig. 5.) With a concentration of 0.01m $\text{Ca}(\text{NO}_3)_2$ the same effect was obtained as with one of 0.0004m $\text{Pb}(\text{NO}_3)_2$ alone, and with a 0.002m concentration of the $\text{Ca}(\text{NO}_3)_2$ about the same effect was evident as with a concentration of 0.0005m $\text{Pb}(\text{NO}_3)_2$ alone. In the combinations with 0.001m and 0.0005m $\text{Ca}(\text{NO}_3)_2$ the results were practically the same as with a concentration of 0.0001m $\text{Pb}(\text{NO}_3)_2$ alone; most of the germination here took the form of appressoria.

With a 0.00016m concentration of $\text{Pb}(\text{NO}_3)_2$ in combination with the same concentrations of $\text{Ca}(\text{NO}_3)_2$ as were employed in the two series above described, the concentration of the calcium salt with the majority of the germinating spores formed normal tubes (instead of swellings and appressoria), and the concentration which just allowed the formation of normal tubes was somewhat lower than in the previous series, as was to be expected. The combination containing a 0.002m concentration of $\text{Ca}(\text{NO}_3)_2$ gave practically the same effect as did a concentration of 0.0004m of the lead salt alone, while that containing a 0.001m concentration

of $\text{Ca}(\text{NO}_3)_2$ gave results similar to those obtained with a 0.00002m concentration of the lead salt alone.

Combinations containing $\text{Ca}(\text{NO}_3)_2$ in concentrations of 0.03333m, 0.04m and 0.05m respectively, produced the same sort of germination as was obtained with a concentration of 0.00033m $\text{Pb}(\text{NO}_3)_2$ combined with a 0.05m concentration of the calcium salt; germination was practically all normal, only an occasional appressorium or swelling being found.

From the above data the conclusion seems warranted that the same molecular ratio of the calcium salt to that of lead produces practically identical effects with the three concentrations of $\text{Pb}(\text{NO}_3)_2$ here used. Concentrations of 0.01m, 0.02m and 0.06m $\text{Ca}(\text{NO}_3)_2$ combined, respectively, with concentrations of 0.00016m, 0.00033m and 0.00066m $\text{Pb}(\text{NO}_3)_2$ produce practically the same effect on spore germination as 0.00005m concentration of the lead salt alone. It thus appears that for all three very different concentrations of $\text{Pb}(\text{NO}_3)_2$, the addition of $\text{Ca}(\text{NO}_3)_2$ in the proportion of six molecules of the calcium salt to one molecule of $\text{Pb}(\text{NO}_3)_2$ reduces the toxicity of the latter salt to a point where it almost, but not quite, inhibits normal germination and nearly restricts germinal activity to the formation of appressoria and other swellings. To obtain this same condition of germination a simple solution of $\text{Pb}(\text{NO}_3)_2$ must have a concentration of 0.00005m.

A similar series of equivalent effects upon spore

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germination, with different concentrations of the lead salt, is shown by taking as the index of physiological activity the prevalence of normal germ tubes in the cultures, as contrasted with swellings and aplanospores. This condition of affairs is attained in combination solutions containing, respectively, 0.02m $\text{Ca}(\text{NO}_3)_2$ combined with 0.00066m $\text{Pb}(\text{NO}_3)_2$, 0.01m $\text{Ca}(\text{NO}_3)_2$ combined with 0.00033m $\text{Pb}(\text{NO}_3)_2$, and 0.005m $\text{Ca}(\text{NO}_3)_2$ combined with 0.00016m $\text{Pb}(\text{NO}_3)_2$. These three combinations of the two salts all produce practically the same effect on germination as does a concentration of 0.00004m $\text{Pb}(\text{NO}_3)_2$ alone. Here the addition of about 30 molecules of $\text{Ca}(\text{NO}_3)_2$ to 1 (30:1) of $\text{Pb}(\text{NO}_3)_2$ reduces the toxicity of the lead salt until it is physiologically equivalent to that of a 0.00004m solution of the lead salt alone.

Combination of $\text{Pb}(\text{NO}_3)_2$ with $\text{Mg}(\text{NO}_3)_2$ shows a similar influence of the alkaline earth upon the toxicity of the heavy metal though with somewhat different concentrations. Equivalent physiological effects were produced by 0.00066m $\text{Pb}(\text{NO}_3)_2$ in combination with 0.02m $\text{Mg}(\text{NO}_3)_2$, by 0.00033m $\text{Pb}(\text{NO}_3)_2$ in combination with 0.01m $\text{Mg}(\text{NO}_3)_2$ and by 0.00005m $\text{Pb}(\text{NO}_3)_2$ alone. Here the addition of about 30 molecules of $\text{Mg}(\text{NO}_3)_2$ for each molecule of $\text{Pb}(\text{NO}_3)_2$ in the solution reduced the toxicity of the latter salt so that it became equivalent, in both combinations, to a simple solution containing 0.00005m $\text{Pb}(\text{NO}_3)_2$. Under these conditions most of the germinal activity took the form of the produc-

tion of appressoria and other swellings, but a small amount of normal germination was observed in all cases. Thus, $\text{Hg}(\text{NO}_3)_2$ appears to be only about one fifth as effective in reducing the toxicity of the lead salt as is $\text{Ca}(\text{NO}_3)_2$.

Magnesium nitrate was not employed in concentration sufficiently high to inhibit all lead effects. The nearest approach to normal germination was obtained in a combination solution containing 0.05m $\text{Mg}(\text{NO}_3)_2$ and 0.00033m $\text{Pb}(\text{NO}_3)_2$, in which about half of the germination was normal.

From the results obtained with the different combinations of calcium and magnesium nitrates with $\text{Pb}(\text{NO}_3)_2$, it is evident that the decrease in toxicity of the $\text{Pb}(\text{NO}_3)_2$ due to the other salt cannot be caused by a depression of the ionization on account of the common anion. For in the combination containing 0.004m $\text{Ca}(\text{NO}_3)_2$ with 0.0066m $\text{Pb}(\text{NO}_3)_2$, about 90 per cent of the lead salt was calculated to be in the dissociated condition. In the combination containing 0.02m $\text{Ca}(\text{NO}_3)_2$ or $\text{Mg}(\text{NO}_3)_2$ together with a 0.00066m concentration of the lead salt the latter should be about 40 per cent. dissociated. The presence of either of these concentrations of dissociated lead salt alone in a culture solution would either prevent germination entirely or cause abnormal growth. That the decrease in toxicity may have been due to the formation of a double salt remains possible; at least no direct evidence to the contrary was obtained.

With the concentrations of the $\text{Pb}(\text{NO}_3)_2$ that

inhibited germination no granular appearance of the protoplasm such as was found in physiologically similar solutions of $\text{Cu}(\text{NO}_3)_2$ was evident. Yet it is clear that the lead salt either directly or indirectly affects the protoplasm through the spore wall, as is shown by the formation of the dark bodies occupying one half of the spore in many cases. This response was observed, as has been mentioned, in the higher concentrations of the lead salt. A discussion of the different forms of germination will be taken up below, after the effects of the combinations of calcium and magnesium nitrates with the other salts have been discussed.

Zinc. Zinc nitrate alone and in combination with the nitrates of calcium and magnesium was studied in much the same way as were the nitrates of copper and lead. Zinc nitrate inhibited germination in 0.25m concentration. Many local swellings of the spore walls were found in concentrations of 0.08m and 0.04m, while local swellings and short tubes were present in concentrations of 0.008m and 0.004m. Normal germination occurred in a concentration of 0.002m. Combinations of $Zn(NO_3)_2$ with $Ca(NO_3)_2$ and $Mg(NO_3)_2$ were tested using a concentration of 0.04m $Zn(NO_3)_2$. In these combinations the calcium salt was employed in concentrations ranging from 0.000125m to 0.02m. Some normal germination occurred with all concentrations of $Ca(NO_3)_2$ below and including that of 0.0005m, but none was observed in the mixture containing a 0.000125m concentration of this salt. It thus appears that normal germination of these spores in a 0.04 $Zn(NO_3)_2$ solution may be brought about by the addition of $Ca(NO_3)_2$ in the proportion of one molecule of this to every 80 molecules of $Zn(NO_3)_2$ present in the solution.

The concentrations of $Mg(NO_3)_2$ which were employed in these combinations with the 0.04m solution of $Zn(NO_3)_2$ ranged from 0.00025m to 0.025m. Here it was found that no normal germination occurred until the magnesium salt reached a concentration in the mixture of 0.0025m. This means that to produce any normal germination in the $Zn(NO_3)_2$ solution here used, by addition thereto of $Mg(NO_3)_2$, it is necessary to add one molecule of the

latter salt for every 16 molecules of $Zn(NO_3)_2$ already present. It therefore requires 5 times as much of the magnesium salt to counteract the toxic influence of $Zn(NO_3)_2$ in the concentration here used as is required of the calcium salt to produce the same effect.

The relatively small amounts of the calcium and magnesium salts which are required to inhibit the toxic effect of $Zn(NO_3)_2$ in 0.04m concentration preclude any possibility that antagonistic influence of either of the former salts might be related to decreased dissociation of the zinc salt brought about by their addition, so that this consideration needs no attention in this case.

It is interesting to recall here that the effectiveness of $Mg(NO_3)_2$ in counteracting the toxicity, in the two concentrations studied, of $Pb(NO_3)_2$, so as to allow some normal germination of the spores, was also found to be only about one fifth as great as that of $Ca(NO_3)_2$. The relative effectiveness of the nitrates of calcium and magnesium is thus seen to be the same whether they are employed to counteract the toxicity of $Pb(NO_3)_2$ or that of $Zn(NO_3)_2$.

Zinc nitrate did not seem to stimulate the spores to form appressoria, though hyaline swellings were common in the more concentrated solutions and no direct evidence was obtained as to whether the zinc nitrate enters the spores.

Aluminum. Aluminum nitrate was used at certain concentrations, both alone and in combination with magnesium and calcium nitrates. Series were carried out, and several times repeated, including the combinations and concentrations which are tabulated below.

Table II.

| Concentration of $Al(NO_3)_3$, : | | Concentration of $Mg(NO_3)_2$ | |
|-----------------------------------|-----------|-------------------------------|----------|
| molecular | | or of $Ca(NO_3)_2$, | |
| molecular | | molecular | |
| Common | Decimal | Common | Decimal |
| fraction | fraction | fraction | fraction |
| 9/2000 | : 0.0045 | : 1/100 | : 0.01 |
| 8/2000 | : 0.004 | : 2/100 | : 0.02 |
| 6/2000 | : 0.003 | : 4/100 | : 0.04 |
| 4/2000 | : 0.002 | : 6/100 | : 0.06 |
| 3/2000 | : 0.0015 | : 7/100 | : 0.07 |
| 2/2000 | : 0.001 | : 8/100 | : 0.08 |
| 1/2000 | : 0.0005 | : 9/100 | : 0.09 |
| 1/4000 | : 0.00025 | : 95/1000 | : 0.095 |

(2)

In none of the cultures with $\text{Al}(\text{NO}_3)_3$ did the addition of either $\text{Mg}(\text{NO}_3)_2$ or $\text{Ca}(\text{NO}_3)_2$ bring about any alteration at all in the behavior of the spores. Although many other proportions of these pairs of salts might have been tried, it appears probable that, if either the calcium or magnesium salt, at any concentration, exerts any influence upon the toxicity of $\text{Al}(\text{NO}_3)_3$, in any concentration, some evidence in this direction would have been obtained from the experiments which were carried out. This seems still more probable from the fact that different concentrations of the aluminum salt alone produced markedly different effects upon the activities of the spores, a matter which will now receive attention.

In the highest concentration of $\text{Al}(\text{NO}_3)_3$ used, 0.0045m, only a few local swellings of the spores were observed. A blackening of half of the spore was frequently found here, samples of spores so modified by the aluminum salt being shown in Fig. 6. No normal germination occurred in this solution. In the two lowest concentrations of $\text{Al}(\text{NO}_3)_3$, however, much of the germination was normal. Aluminum nitrate appears to influence the form of germination occurring in these spores with much the same results as have been described for cultures poisoned with $\text{Pb}(\text{NO}_3)_2$. The types of renewed activity which occur with $\text{Al}(\text{NO}_3)_3$ are: (1) the formation of a dark body occupying about half of the spore, (2) the growth of a small local swelling at one end

of the spore, and (3) the development of one or more appressoria, the latter sometimes having their characteristic dark color and sometimes being hyaline. Unless the formation of the internal dark bodies, which seem to arise directly through protoplasmic activity, may be taken as evidence in favor of a penetration of the toxic salt, no information as to whether $\text{Al}(\text{NO}_3)_3$ enters the spores was obtained.

21

Nickel. Nickel nitrate proved to be very slightly toxic to these fungus spores, its effect on germination being manifest only in very high concentrations. On this account no study was made of the possible influence of calcium and magnesium nitrates upon this toxicity.

The toxic effects of $\text{Ni}(\text{NO}_3)_2$ alone may be added here. A concentration of 0.5m inhibited germination. In a concentration of 0.25m, numerous local swellings of the spores were observed. Both local swellings and normal germination were present in a 0.125m solution of this salt, and germination was perfectly normal, as in the distilled water control, where a concentration of 0.05m or lower was employed.

Germinating spores in 0.25m concentration of $\text{Ni}(\text{NO}_3)_2$ frequently exhibited a granular appearance such as has been described for spores in solutions of $\text{Cu}(\text{NO}_3)_2$ which were not quite concentrated enough to inhibit all activity.

TOXICITY OF SINGLE SALTS

The present section will be devoted to the relative degrees of toxicity exhibited by the various substances heretofore dealt with, together with certain other considerations bearing upon the problem of chemical stimulation as evidenced in the simple solutions employed in these studies. Besides the substances already mentioned, nitric acid entered into the experimentation, and the information gained regarding the toxicity of this substance will also find place here.

From what has preceded it appears possible to group the effects of the different treatments into four distinct kinds of physiological response on the part of the fungus spores here employed. (1) A given treatment may be without effect; under such treatments the spores germinate normally within 18 hours, just as they do in the controls with distilled water. (2) Germinal activity may be manifest, but in other ways than that which is here considered as normal. Under treatments which produce this sort of response, the activities of the spores results (a) sometimes in an actual decrease in the exposed surface as well as in the volume of the organism, and (b) sometimes in an increase in both volume and surface, the latter, however, increasing to a much lower degree - as related to the volume - than is the case with normal germination. In the first category (a, above) belong the phenomena involved in the production of the dark bodies which have been described as

forming within the spore wall and occupying only a portion of its volume. These appear to have essentially the characteristics of chlamydospores formed within the organism. All other renewed activities of the spores which have been described belong to the second category (b), and embrace those growth processes which result in more or less restricted swellings, especially at the ends of the spores, in apressoria, with either dark or hyaline wall, and in short germ tubes somewhat resembling the normal but of much greater diameter. (3) No renewed activity may occur at all during a period of from 18 to 20 hours under the given treatment, although capacity to germinate if transferred from this treatment to another (as to distilled water) may still be retained. Here the treatment prevents germinal activity but does not destroy viability, at least within the given time limits. (4) Viability, or power to germinate in water, may be destroyed within a period of from 18 to 24 hours; the organism is killed outright.

It has already become clear in this work that most of the substances dealt with produce death within the assumed time limits, if applied at a sufficiently high concentration (4). With a somewhat lower concentration of the injurious material germination is inhibited but viability is retained throughout the given period (3). When the concentration is still lower germinal activity becomes manifest but takes other forms than those recognized as normal (2).

Finally, when the concentration of the toxic agent is still further decreased, the stimulation threshold in the present sense is passed and normal germination becomes the rule.

Livingston¹⁸ working with a green alga has presented a somewhat similar series of responses to chemical stimuli. This writer studied the effect of a large number of nitrates and sulphates on a form of Stigeoclonium, adding different concentrations of the salts to a nutrient medium in which the alga was grown. With a dilute nutrient solution, in which the alga had a characteristic filamentous form, its response to stimulation might be considered as of three types. (1) death, (2) change in phenomena of growth, and (3) change in phenomena of reproduction. The response to a high concentration was usually death, while addition of a toxic salt somewhat below the fatal concentration stimulated the production of the palmella form, with spherical cells and division occurring in all directions. It is interesting that with relatively similar concentrations of the salts, (that is at concentrations somewhat lower than those required to inhibit germination) the spores dealt with in the present paper exhibited responses similar to those which Livingston found with his alga; the production of appressoria and swellings in this fungus appears physiologically similar to the production of the palmella form

 18. Livingston, B. E., Chemical stimulation of a green alga. Bull. Torr. Bot. Club 32:1-54, 1905.

in Stigeoclonium. The third type of response discussed by Livingston, change in phenomena of reproduction, of course finds no parallel in this investigation.

For convenience of comparison, the limits of the various responses of the fungus spores here dealt with, to the different treatments employed, have been presented in the form of a table and are given below. The only nitrate occurring in the table that has not already received attention is the acid. In a series of cultures with HNO_3 the germination at a 0.005m concentration was practically all normal while at 0.01m and all higher concentrations no germination took place in the 18 hour period. The acid prevented germination for the period of 18 hours at a 0.02m concentration and killed the spores at 0.5m. No concentration was found, therefore, at which the germination took the form of any of the various abnormal growths found at some concentration with all the other substances used, nor was there any apparent coagulation of the protoplasm at concentrations below that which killed the spore. From these considerations it would seem then that the various abnormal growths and effects on the protoplasm of the spore cannot be due to the acid present in the solution as a result of hydrolysis of the salts, but must be related either directly or indirectly to the metals themselves.

Turning now to the tables, the different substances are there arranged in the order of the concentration which

just allowed normal germination in a period of 18 hours. In the first column are listed the substances dealt with. The second column presents the concentrations in which the spores are killed in 18 hours and fail to germinate later, after transfer to distilled water. Here, as in many other instances, the critical concentrations must not be regarded as definite in the sense of the more exact physical sciences; the concentrations employed in the experimental series were frequently rather widely separated, and were this not the case, the variability of the organism in its resistance to toxic substances would render quite useless any attempt to define such critical points with very great accuracy. In all such work as the present, dealing with large numbers of organisms, the internal conditions of the cells must be as important in determining reactions as are the external ones, and we are as yet unable either to control or measure the former excepting in a very general way.

In the third column are found the concentrations at which the different salts inhibit germination for at least 18 hours. When spores from these cultures were removed from the toxic solution and placed in water for a day they germinated. The fourth column gives the maximum concentration in which germination occurred. In all cases this growth was abnormal, showing swellings, swollen tubes and other unusual structures, such as are illustrated in figs. 1-6.

In the fifth column the concentrations given are the maxima in which any normal germination was observed. The sixth column presents the highest concentrations in which no toxic influence was manifest.

FOLD OUT

From the table it will be seen that the copper salts are by far the most toxic of all the salts here studied. These two salts are very closely similar in their effect on the germination of the spores, which is in accord with the results of Clark (loc. cit.) and points almost conclusively to the commonly accepted idea that the toxicity of such copper salts is mainly or entirely due to the copper ion. The last mentioned writer found that the concentration of copper salts which inhibited germination were higher than those producing the same effect in this investigation, a fact that may probably be due to differences in the organism he worked with; he found that his five fungi varied markedly in their response to the same stimulus. Also, Clark usually made use of nutrient media while the present studies were carried out without its employment. It is quite possible that the presence of nutrient substances might have modified the effect of the poison. Duggar¹⁹ found the nitrate of copper considerably more toxic than the sulphate and also considerably more toxic than it is here shown to be. In the work of Stevens²⁰, the two salts affected germination similarly and the concentra-

19. Duggar, E. H., Physiological studies with reference to the germination of certain fungus spores. Bot. Gaz. 31:38-65 1901.

20. Stevens, F. L., The effect of aqueous solutions upon fungus spores. Bot. Gaz. 26:377-406. 1898.

tions required to prevent germination varied, with the different fungi used, from m/3400 to m/200. Many other investigations of the effect of copper on the germination of fungus spores have been carried out, with varying results. The effect of this poison on algae has been studied by Livingston, and Kahlenberg and True²¹, Heald²², Jensen²³, Szűcs¹³, and others, have investigated its toxicity toward higher plants. The results obtained indicate that fungus spores are considerably more resistant toward the toxic effects of copper than are either the algae or higher plants.

In the order of their toxicity, $Pb(NO_3)_2$ follows the copper salts, though not closely, with $Al(NO_3)_3$ next. The effects of the last two salts on plants seems to have been studied but little. Livingston employed them, however, and found the same relation to hold true as is here brought out. Also, Jensen finds that $Pb(NO_3)_2$ is somewhat less toxic toward wheat seedlings than is $Cu(NO_3)_2$. The nitrates of zinc and nickel, which are next in the order of diminishing toxicity, are not exceedingly toxic toward the spores of Gloeosporium. It is interesting to note that

 21. Kahlenberg, Louis, and True, Rodney H., On the toxic action of dissolved salts and their electrolytic dissociation. Bot. Gaz. 22:81-124. 1896.

22. Heald, F. D., the toxic effect of dilute solutions of acids and salts upon plants. Bot. Gaz. 22:125-153. 1896.

23. Jensen, G. H., Toxic limits and stimulation effects of some salts and poisons on wheat. Bot. Gaz. 43:11-44. 1907.

Clark, working with fungi, and Livingston, with an alga, found nickel salts to be more strongly toxic than those of zinc, a result which is opposite to that obtained in this investigation.

Magnesium, calcium, and potassium nitrates and cane sugar end the list, in the order named. It is noticeable that, with these last four compounds at concentrations just below that required to inhibit germination, growth takes the form of swollen bodies which tend to give a lower value to the ratio of surface to volume of the organism than that resulting from normal germination. This reaction to toxic stimuli, at concentrations just below those required to inhibit growth, occurs commonly with all the salts here used. That it is found with cane sugar, which is usually regarded as non-toxic (see True²⁴) suggests that such reactions may be brought about by high osmotic pressure as well as by chemical stimulation in the true sense of this term. This suggestion is in accord with the conclusions of Livingston (l c. 18) who has shown that his form of Stigeoclonium will assume the palmella form in response either to a toxic stimulus or to one of relatively high osmotic pressure. Whether this effect of sugar, and similar effects produced by high concentrations of calcium, magnesium and potassium nitrates is really due, in the present instances, to osmotic

24. True, Rodney H., The physiological effects of certain plasmolyzing agents. Bot. Gaz. 26:407-416. 1898.

pressure relations was not determined experimentally; the minuteness of the spores must render experiments involving plasmolysis exceedingly difficult with such organisms as those here employed. It is obvious, however, that the high concentrations of the calcium, magnesium and potassium salts must be capable of exerting an extremely high osmotic pressure if the ectoplasm of the spore is not readily permeable to them.

From the table of relative toxicities it may be seen that the different compounds arrange themselves in the same order, with the exception of HNO_3 , if other types of reaction to the chemical stimuli are taken as criteria. This suggests that the physiological response of death, on the one hand, and the various morphogenic responses considered in this paper, on the other, are ultimately related to the same properties or characteristics of the various stimulating substances which bring them about. Copper is the most toxic metal here dealt with, whether we consider its toxicity as bringing about changes that result in death or as bringing about the less final changes that lead to abnormal growth. In the same way, magnesium, calcium and potassium are the least toxic metals of the list, on whatever physiological criterion we may base our judgement.

GENERAL DISCUSSION

The results of the experiments described in the foregoing pages show that, in certain cases at least, the effect of a toxic salt on the germination of the conidia of Glomerella cingulata may be influenced by the addition of calcium, magnesium or potassium nitrates. That this effect is not due to depression of ionization of the toxic salt has been demonstrated. That it cannot be due to the formation of undissociated double salts has been shown for the combination of $\text{Cu}(\text{NO}_3)_2$ with $\text{Ca}(\text{NO}_3)_2$ and of $\text{Zn}(\text{NO}_3)_2$ with calcium or magnesium nitrates. The influence of calcium upon the toxicity of the salts of the heavy metals here employed must then be related to an effect of the $\text{Ca}(\text{NO}_3)_2$ on the spore or to an effect on the contained protoplasm is made probable from the fact that copper, lead, aluminum and nickel nitrates sensibly affect the protoplasm in various ways without producing any apparent changes in the spore wall. If now this antagonistic action of the two salts is to be related to the protoplasm of the spore, the question naturally arises, whether the effect of the salt of the lighter metal, in decreasing the toxicity of the other salt toward the protoplasm may not be related to some specific effect or effects of the salts.

Loeb²⁵ has advanced the theory of ion-proteid

25. Loeb, J., On ion-proteid compounds and their role in the mechanics of life phenomena. I., The poisonous character of a pure NaCl solution. Am. Journ. of Physiol. 7:327-338.

3

formation to explain this sort of action, suggesting that salts combine with the proteids of living protoplasm to form ion-proteid compounds. He considers it possible that one or both of the ions of the anti-toxic salt may thus combine with the proteid molecule. In his work with NaCl he found that a solution of NaCl was poisonous unless a little calcium and potassium were present, and considers that the ions of the last mentioned salts may be substituted in the ion-proteid molecule of living matter. He suggests that the organism cannot live without some of these proteid compounds containing calcium and potassium as well as those containing sodium.

The work of Osterhout²⁶ on "balanced solutions" also supports this theory of Loeb's.

Ostwald²⁷ in his work on Gammarus concludes that the toxicity of a solution is related to its power of being adsorbed, and "dass eine Lösung um so giftiger ist, je stärker sie adsorbiert wird." Morowitz²⁸ considers that

26. Osterhout, W. J. V., On the importance of physiologically balanced solutions for plants. I. Marine plants, l.c. II. Fresh water and terrestrial plants. Bot. Gaz. 44:259-272-1907.

27. Ostwald, Wo., Ueber die Beziehungen zwischen Adsorption und Giftigkeit von Salzlösungen für Susswassertiere (Gammarus) Pflugers Archivs 120:19-30.1907.

28. Morowitz, Hugo, Ueber Adsorption und Kolloidfällung Kolloid Chemische Beihefte 1:301-331. 1909-10.

when a salt comes in contact with an organism it is adsorbed by the surface layer and diffuses thence into the interior. The amount thus entering in a given time is related to the amount adsorbed. If an otherwise indifferent but strongly adsorbed substance be present the amount of adsorbed poison should be less.

True and Gies²⁹ also relate the antagonistic effect here considered to the protoplasm and seem to consider that it is due to an accelerating action of calcium acting against a retarding influence exerted by copper. Szűcs, as mentioned earlier in this paper, likewise/considers the influence of one salt upon the toxicity of another to be due to a mutual effect of the two salts upon the protoplasm. Loeb³⁰ later advanced the theory that the influence of one salt on the toxic action exerted by another upon an organism was due to an effect upon the outer colloidal membrane of the organism whereby it was made impermeable to the toxic salt.

Osterhout³¹ working with cut disks of Laminaria, comes to the conclusion that " The antagonistic action of salts is largely or entirely due to the fact that they hinder or prevent one another from entering the protoplasm."

29. True and Gies. Loc. Cit.

30. Loeb, J., The mechanistic conception of life. Page 173, University of Chicago Press, 1912.

31. Osterhout, W. J. V., The permeability of protoplasm to ions and the theory of antagonism. Science N. S. 35:112-115. 1912.

The antagonistic action of salts upon the germination of fungus spores as here recorded may be, in some cases, explained by considering that both the salts are taken up by the protoplasm but that the adsorption of the toxic salt was so retarded by the other salt that the amount taken up was below that necessary to inhibit the vital activities. In the case of $\text{Cu}(\text{NO}_3)_2$ it was found that the amount of $\text{Ca}(\text{NO}_3)_2$ necessary to inhibit the toxicity of the copper salt varied with its concentration. This variation ranged from a molecular proportion of 5Ca to 8 Cu with a low concentration of $\text{Cu}(\text{NO}_3)_2$ to 6 Ca to 1 Cu at a relatively high concentration. If it is assumed that the salts in the solution are first adsorbed by the plasma membrane or form ion-proteid compounds therein and diffuse thence into the interior, and also that the amount of salt adsorbed is directly proportional to the concentration of its solution and to the time during which it acts, then it is quite conceivable that a higher proportion of the calcium salt would be required to inhibit the toxic effect of a higher concentration of $\text{Cu}(\text{NO}_3)_2$. For if the proportion of the slightly toxic calcium salt to that of copper was greater the ratio of the calcium to the copper adsorbed by the spore would be greater and the total quantity of the copper salt adsorbed in a given time might be no more than at one of the dilute concentrations of copper salt alone in which the spores germinated. With the combination of $\text{Pb}(\text{NO}_3)_2$ and $\text{Ca}(\text{NO}_3)_2$ this reasoning could not hold as the same molecular ratio of

calcium to lead at the three concentrations of $Pb(NO_3)_2$ employed produced the same effect on spore germination. With $Zn(NO_3)_2$ 1 molecule of calcium to 80 molecules of zinc was found to reduce the toxicity of the latter to one twentieth. It is difficult to believe the rate of adsorption of this relatively high concentration of zinc salt can be so greatly reduced by the addition of such a small amount of calcium.

In combinations of $Al(NO_3)_3$ with $Ca(NO_3)_2$ or $Mg(NO_3)_2$ the toxicity of the aluminum salt is not reduced at all. It was to be expected, from the results obtained by Loeb³² in his experiments upon the influence of NaCl upon the toxicity of $MgSO_4$, that a higher molecular proportion of the bivalent metal would be needed to inhibit the toxicity of the trivalent aluminum than was required in the case of bivalent poisons. The work of Szűcs (l.c. page) on the relative efficiency of certain metals of different valencies in inhibiting the poisonous action of quinine hydrochloride and the comparative proportions of KNO_3 and $Ca(NO_3)_2$ required to noticeably influence the toxicity of copper in the present work are in accord with the results of Loeb just cited. If now the toxicity of a salt is dependent upon the amount absorbed, as Ostwald has

32. Loeb, J., The dynamics of living matter. New York, 1906. Page 49.

suggested, and if when two salts are present the ratio of absorption of both will be decreased as would seem to be the case from Morowitz' hypothesis, then it would seem that a proportion of 80 molecules of calcium to 1 of aluminum should decrease the toxic effect of $\text{Al}(\text{NO}_3)_3$ at least to a limited extent.

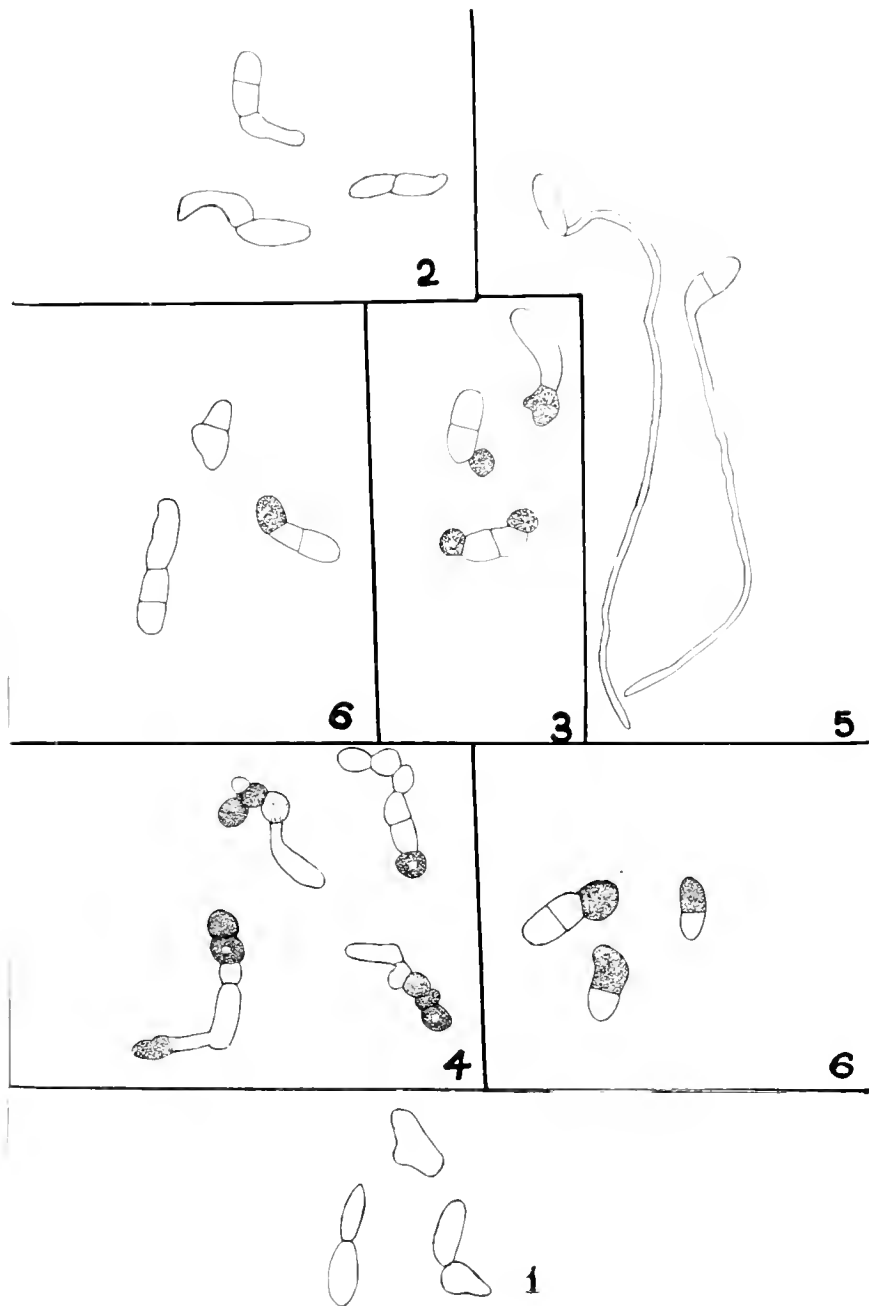
The whole matter might be explained by assuming that the protoplasm is made impermeable to the toxic salt by the presence of the other salt in the medium, as suggested by Loeb in his later theory and by Osterhout from his work on *Laminaria*. Yet if this were the case it seems probable that a ^{constant} quantity of the salt of the lighter metal would be required irrespective of the concentration of the toxic substance. This, however, is obviously not the case in the experiments described above. In the combination of $\text{Pb}(\text{NO}_3)_2$ with $\text{Ca}(\text{NO}_3)_2$ the amount of the calcium salt required to bring about a given response in the presence of the lead salt varies with the concentration of the latter. In this case the concentrations of the two salts are always in constant molecular ratio to each other. A similar state of ^{obtains} affairs when the nitrate of magnesium is used instead of that of calcium, although the molecular ratio is here somewhat different. Again in the combinations of $\text{Cu}(\text{NO}_3)_2$ with $\text{Ca}(\text{NO}_3)_2$ here employed the amount of the calcium needed for a given response varies with the concentration of the copper salt. This combination differs from that with lead nitrate however for here the molecular ratio of

one salt to the other varies with the concentrations of the toxic salt.

A consideration of the toxic stimulation of the single salts as brought out in this investigation suggests that the influence of the salts upon the protoplasm of the spore is specific, at least in certain cases. The nitrates of lead and aluminum, in concentrations somewhat below those which inhibit germination, frequently cause (either directly or indirectly) in the spore a dark chlamyospore-like body. The presence of copper at a similar concentration as regards toxicity causes a granular appearance of the protoplasm. Much the same effect was observed in concentrations of nickel nitrate not quite strong enough to inhibit germination. Toxic concentrations of $Zn(NO_3)_2$, however, fail to produce any visible alteration in the protoplasm. If then the effects of the different salts on the spores are not the same it would seem reasonable to consider that the requisite antidote might not be identical in all cases; a substance which inhibited the poisonous effects of one toxic salt might have no influence upon the toxicity of another. This may be the reason that neither calcium or magnesium nitrate has any apparent influence upon the toxic effect of aluminum nitrate.

It is useless, however, to attempt to draw conclusions as to the dynamics of these antagonistic salt actions.

It is of course logically possible that they may be explained in some simple manner as has been suggested by some of the various writers on this subject, yet the exceeding complexity of the system within the cell must make possible a great variety of explanations.



- Fig. 1. Some types of germination found in 1.6m sucrose.
- Fig. 2. Germinating spores from the combination solution of 0.00006m $\text{Ca}(\text{NO}_3)_2$ and 0.0001m $\text{Cu}(\text{NO}_3)_2$.
- Fig. 3. Typical germination in 0.00033 m $\text{Pb}(\text{NO}_3)_2$ solution.
- Fig. 4. Growth from spores after four days in 0.001m $\text{Pb}(\text{NO}_3)_2$ solution.
- Fig. 5. Typical germination in 0.00033 m $\text{Pb}(\text{NO}_3)_2$ in combination with 0.005m $\text{Ca}(\text{NO}_3)_2$.
- Fig. 6. Typical germination from 0.005m $\text{Al}(\text{NO}_3)_3$ solution.

VITA

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