CHANGES IN THE PERMEABILITY OF ASCOSPORES OF NEUROSPORA TETRASPERMA DURING GERMINATION*

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Ascospores of *Neurospora* afford a unique opportunity for the study of different physiological states within the same organism. These spores remain dormant and metabolize at a relatively low rate until they are subjected to heat (Goddard, 1939) or to chemical activators (Emerson, 1948; Sussman, 1953). As a result of such treatment, a dramatic rise in metabolic rate ensues accompanied by other changes which culminate in the germination of the ascospores. Since the duration of the dormant stage is at the control of the investigator, and since the transition to the activated condition is easily recognized by physiological markers, such as oxygen uptake, comparative studies of these different stages can be undertaken.

The experiments reported in this paper deal with the changes in permeability which accompany germination, as exemplified by the response to the chelating agent ethylenediamine tetraacetic acid (EDTA) and to certain cationic exchange resins.

Materials and Methods

Ascospores were obtained in the manner outlined by Goddard (1935). Strains 374 and 377 of *N. tetrasperma* were crossed and grown at 25°C. Storage of ascospores was carried out at approximately 80 per cent relative humidity at a temperature of 4°C. Under these conditions only a slow decline in the ability to germinate occurs. Activation was accomplished by heating the spores at 58°C. for 10 to 20 minutes in a constant temperature water bath. Conidia were used immediately after being harvested from Westergaard's agar (Ryan, 1950).

The concentration of spores was estimated from density readings taken in a Klett colorimeter with a blue filter (Klett No. 42). In general, and except where otherwise stated, a final concentration of 1 mg. dry weight per ml. of spores, corresponding to a Klett reading of 180, was used in the experiments to be reported. Experiments were usually run in 25 ml. Erlenmeyer flasks to which were added 2 ml. of the spore suspension. The activated ascospores were shaken for 5 hours at a rate of 80 oscillations per minute with a throw of 10 cm. at a temperature of 28°. Five drops of formaldehyde were added at this time in order to kill the spores, after which counts were

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performed. This was accomplished by a modification of the method described by Davies, Duckworth, and Harris (1948) and adapted as described previously (Sussman, 1953).

Ordinary distilled water as well as such water passed through a mixed-bed resin (amberlite MB-3, analytical grade) was used and no differences could be observed in the results of experiments wherein comparisons were made. Therefore, these were used interchangeably in the experiments to be reported.

Ethylene diaminetetraacetic acid (EDTA) was obtained from the Bersworth Chemical Corporation, Framingham, and from the Alrose Chemical Company, Providence. The analytical grade of the disodium salt was used and the pH was adjusted to pH 7.1 by the addition of either NaOH or HCl. Unless otherwise mentioned, EDTA was always used at this pH.

EDTA was analyzed spectrophotometrically using the absorption of its copper complex at 280 and 340 mμ (Plumb, Martell, and Bersworth 1950; Technical Bulletin No. 2, Bersworth Chemical Company). All samples were filtered through sintered glass funnels (F grade) before their absorption was read.

Calcium was determined[1] by means of the Beckman flame photometer attachment and the model DU spectrophotometer at a wave length of 553 mμ. By this means amounts as low as 1 p.p.m. could be detected. Sodium, potassium, and magnesium concentrations were read at 588, 766, and 383 mμ respectively.

RESULTS

Effect of EDTA upon Dormant and Activated Ascospores.—Since ascospores can germinate in distilled water it might be assumed that they contain a sufficient endogenous supply of minerals. As a preliminary to studying their mineral requirements the depletion of these endogenous reserves was attempted by means of EDTA. With this in mind, dormant ascospores were incubated in 0.028 M EDTA, at 28°C. on a shaking machine for varying periods of time. Aliquots were withdrawn, washed by centrifugation in 5 changes of distilled water, activated, and reincubated in distilled water. There was no significant change in the germinability of dormant ascospores incubated in EDTA for as long as 5 months.

Activated ascospores were then suspended in different concentrations of EDTA, and incubation and counting were accomplished as before. In contrast to the previous results, those given in Fig. 1 show that the germination of activated spores, as determined after 5 hours of incubation, is almost completely inhibited by very low concentrations of EDTA. The K^+ salt of EDTA gave results comparable to those obtained with the Na^+ salt. That the respiratory increase associated with activation is also inhibited by EDTA is demonstrated by the data in Fig. 2. In this experiment ascospores were suspended in 0.028 M EDTA and heat-activated. They were then pipetted into Warburg vessels

[1] I am indebted to Professor A. G. Norman who performed the mineral analyses reported in this paper and to Mr. Leo Vander Beek who performed most of the germination counts.

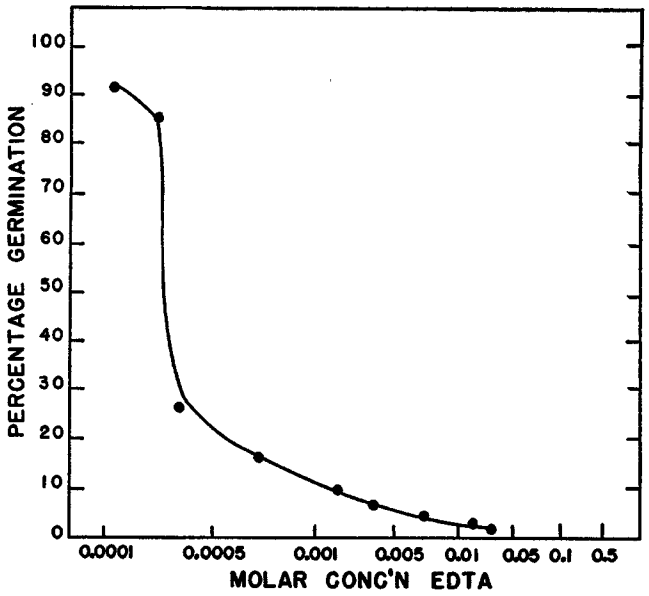

FIG. 1. Effect of different concentrations of EDTA upon the germination of ascospores of *N. tetrasperma*. The control amount of germination in distilled H_2O was 93 per cent.

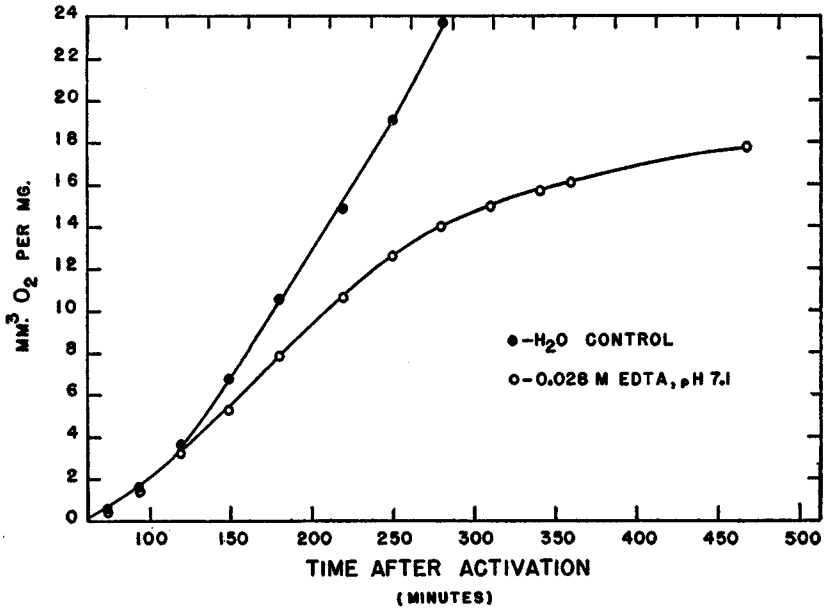

FIG. 2. Effect of EDTA upon the respiration of activated ascospores of *N. tetrasperma*. EDTA was added at 0 time. Respiratory data computed on the basis of dry weight of ascospores.

of 7 ml. capacity and the oxygen uptake measured in the usual way at 26°C. and at a shaking rate of 120 oscillations per minute. In order to obtain measurable O_2 uptake under these conditions, about four times the concentration of spores used in the germination experiments was used in these experiments. The results outlined in Fig. 1 are, therefore, not strictly comparable to these.

Conidia in a concentration of 20,000 per ml. were also treated with several concentrations of EDTA, and 1×10^{-2} M was found to be completely toxic to the germination process.

Because EDTA was used as the sodium or potassium salt, the effect of these cations upon activated ascospores was studied. Cells were heat-activated after suspension in distilled water and were dispensed into Warburg vessels into which relatively high concentrations of Na^+, K^+, or their combination were tipped. Table I furnishes the data on the total oxygen uptake and the per-

TABLE I

The Effect of Na^+ and K^+ upon the Respiration and Germination of Activated Ascospores of N. tetrasperma

Treatment	O_2 consumed after 405 min.	Germination after 405 min.
	*mm.*³	*per cent*
0.2 M NaCl	237	90
0.2 M KCl	205	92
0.1 M NaCl + 0.1 M KCl	259	93
H_2O control	384	93

centage germination of ascospores 405 minutes after activation. It is apparent that ascospore germination is not affected by these concentrations of Na^+ and K^+. On the other hand, their respiration was reduced by 47 per cent in the presence of K^+ and by 39 per cent in the presence of Na^+. In any case, the effects of EDTA cannot be explained as being due to the presence of the alkali ions.

The Prevention of EDTA Poisoning.—EDTA was known to be a strong chelating agent so its effect was assumed to be due to the removal of essential ions from their site of action in the organism. The reversal of such poisoning was therefore attempted by the addition of a mineral which could be complexed by the chelator. To this end, ascospores were suspended in 0.028 M EDTA, and were activated after the addition of various concentrations of $CaCl_2$. Table II shows that Ca^{++} can prevent the toxic effects of EDTA upon both the germination and respiration of ascospores.

Whether the reversal of EDTA poisoning by Ca^{++} can be effected at any time after activation was then investigated. Ascospores were activated in 0.028 M EDTA and dual aliquots were withdrawn at intervals thereafter.

5×10^{-2} M Ca^{++} was added to one sample and the other was washed free of EDTA by centrifugation in 5 changes of water before being resuspended in distilled water and reincubated for 350 minutes at 31°C. Table III shows that only a third of the spores germinated if Ca^{++} was added 120 minutes after activation and that none germinated if the addition was made after 240 min-

TABLE II

The Effect of Calcium upon the Toxicity to Ascospores of 0.028 M EDTA at pH 7.1

Concentration Ca^{++}	Germination	Q_{O_2} after 270 min.
	per cent	
0	0	0.5
0.012 M	69	16.2
0.010 M	66	14.6
0.006 M	48	7.6
0.003 M	9	2.0
0.001 M	5	0.8
Control with zero EDTA	68	16.4

TABLE III

The Reversibility of the Toxicity of EDTA at Various Times after Activation of Ascospores of N. tetrasperma

Time after activation when Ca^{++} was added to EDTA-poisoned ascospores	Germination	
	Ca^{++} added	Washed cells
min.	*per cent*	*per cent*
0	87	92
30	86	—
60	89	91
90	91	—
120	30	—
150	11	—
170	—	6
180	11	—
240	5	8
300	0	—
360	0	—

utes. In general, cells from which the EDTA was washed responded in the same way.

Since rather large concentrations of Ca^{++} were added to EDTA-poisoned cells, the effect of this ion on untreated ascospores was next investigated. However, concentrations as large as 1×10^{-2} M were found to have only a small effect, or none at all upon ascospore germination or respiration.

The precipitous decline in the ability of Ca^{++} to prevent the toxicity of

EDTA beyond 120 minutes after activation suggested that a change in the spores occurred at that time. This possibility was investigated manometrically by adding 1×10^{-2} M EDTA at various times after activation. As can be seen in Fig. 3, if EDTA is added immediately upon activation, about 2 hours are required for its effect to become manifest. However, when EDTA is added 120 minutes after activation its effect is almost immediate. Surprisingly enough, spores which were incubated for 225 minutes after activation were less sensitive to EDTA since no respiratory effect became apparent until 125 minutes

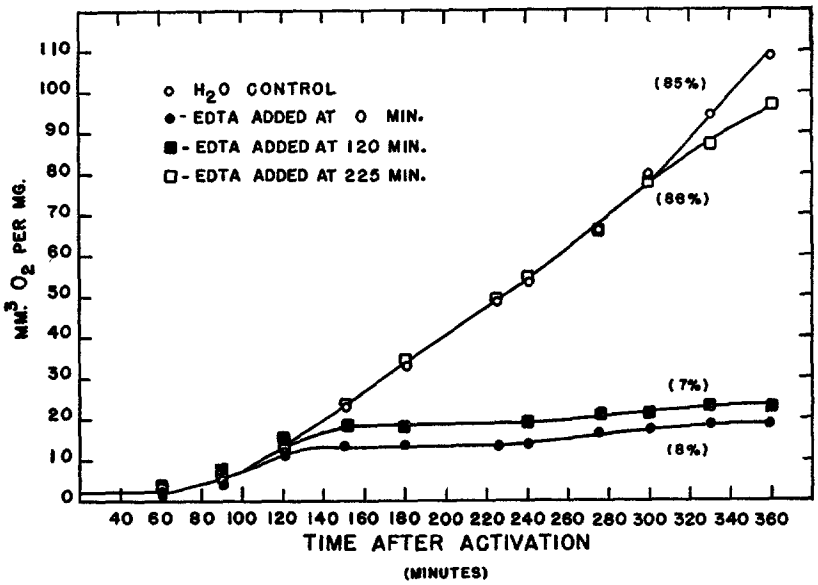

FIG. 3. Effect of addition of EDTA to ascospores of *N. tetrasperma* at various times after activation. The figures in parentheses refer to the amount of germination obtained with each treatment.

after addition. The respirometric data are supported by germination counts (Fig. 3) which further indicated the sensitivity of the spores at 120 minutes after activation.

The change in the response of ascospores to EDTA can be construed to be due either to an increased permeability to EDTA or to the diffusion of minerals out of the cell in response to the chelating agent. In order to distinguish between these possibilities the concentration of EDTA was determined at various times after its addition to activated spores.

To perform this experiment, a suspension of ascospores in 0.015 M EDTA was made up to a Klett reading of 360 (2 mg./ml.). 50 ml. of this suspension was heat-activated and incubated by shaking at 28°C. 5 ml. aliquots were

withdrawn before activation and at intervals thereafter and were treated as follows: 1 ml. samples were added to equal volumes of distilled water or 0.1 M $CaCl_2$ whereupon both samples were reincubated; the remaining 3 ml. was filtered through an F grade sintered glass funnel and was used for the analysis of residual EDTA as described in the section on Methods. The results are given in Table IV and suggest that little, if any, EDTA is absorbed by the ascospores. The amount of EDTA that would be expected to disappear by free diffusion in this system is approximately 0.5 per cent of the total. This calculation was made upon the basis of a volume of spores equivalent to 1/100 that of the suspension as determined by centrifugation in a hematocrit tube. No allowance for interstitial water was made and it was assumed that the

TABLE IV

EDTA Uptake and Germination of Ascospores at Various Times after Activation

Time after activation when sample was withdrawn	Residual EDTA	Germination:	
		In H_2O	In Ca^{++}
min.		*per cent*	*per cent*
Dormant ascospores	0.0150 M	0	0
0	0.0150 M	0	91
60	0.0144 M	0	90
120	0.0143 M	0	27
160	0.0150 M	0	11
240	0.0150 M	0	3
300	0.0148 M	0	0

ascospores contained 50 per cent H_2O. The sensitivity of the method is such, however, that the penetration of such an amount might not be detectable.

The above data suggested that the EDTA acts by withdrawing a necessary ion, or ions, from activated ascospores, and that this effect becomes possible only after 120 minutes. To test this possibility, eight 250 mg. samples of ascospores which were harvested 1 month previously were washed repeatedly with deionized water. Six of the eight samples were heat-activated while the remaining two served as dormant controls. After activation, two of the samples (0 hour samples) were suspended in 10 ml. of water and centrifuged. The supernatant was collected by filtration through an F grade sintered glass filter and stored in the cold. The dormant controls and the remaining four activated samples were suspended in 5 ml. of deionized water in 250 ml. Erlenmeyer flasks and shaken at 31°C. The supernatants from two of the activated samples were collected after 1 hour and those from the remaining samples after 2 ½ hours of shaking. All samples were made up to 10 ml. with deionized water. Analyses of the Ca^{++}, Mg^{++}, Na^{+}, and K^{+} content of the supernatants were then performed and the results tabulated in Table V. Clearly, only Na^{+} and

Ca^{++} are lost in significant quantities from ascospores suspended in distilled water. Moreover, these ions are released only after 150 minutes of incubation; that is, at a time corresponding with the onset of germination.

It was now apparent that significant amounts of chelatable cations were released from ascospores upon germination in water. It remained to be seen whether the inhibitory effect of EDTA could be traced to the binding of Ca^{++} or other cations. Therefore, six 250 mg. aliquots of ascospores were shaken in 10 ml. of 0.028 M EDTA at 31°C. overnight. Since the strong emission of Na^{+}

TABLE V

Concentration of Cations in the Supernatant Fluid Recovered from Dormant, Activated, and Germinating Ascospores of N. tetrasperma Which Were Incubated in Deionized Water

Time after activation	Concentration of cations in supernatant, *mg./gm. ascospores*			
	Ca^{++}	Mg^{++}	Na^{+}	K^{+}
min.				
Dormant controls	0	0	0	0
0	0	—	—	—
60	0.06	—	—	—
150	0.56	0	0.6	0

TABLE VI

Concentration of Cations in the Supernatant Recovered from Dormant, Activated, and Germinating Ascospores of N. tetrasperma Which Were Incubated in 0.028 M EDTA

Time after activation	Concentration of cations in supernatant, *mg./gm. spores*			
	Ca^{++}	Mg^{++}	Na^{+}	K^{+}
min.				
Dormant controls	0	0	0	0
0	0	0	0	0
150	0.6	0	1.16	11.2

in the flame photometer interfered with the readings of the other cations, the NH_4^{+} salt of EDTA was used. It had previously been determined that this salt of EDTA was almost equally as toxic as the Na^{+} salt. The EDTA-treated dormant ascospores were washed with five changes of EDTA and made up to 10 ml. Four of the samples were heat-activated and the remainder served as non-activated controls. The supernatants of two of the activated sets of ascospores were immediately recovered by filtration as in the previous experiment, while the other flasks were shaken at 31°C. for 150 minutes. After this time the supernatants of all the samples were collected and analyzed for their content of Na^{+}, K^{+}, Ca^{++}, and Mg^{++}. The results are given in Table VI and show a striking difference between ascospores incubated in water and in

EDTA. This lies in the fact that at least 11.2 mg. K^+ were released from 1 gm. of spores in the presence of EDTA whereas those incubated in water lost none. On the other hand, about 0.6 mg. of Na^+ and Ca^{++} per gm. of spores was lost under the latter set of conditions. Again, as in the case of ascospores germinated in water, the minerals were released only by ascospores which had been incubated at least 150 minutes after activation.

In order to determine the proportion of the cell's content that was lost by these treatments, the mineral content of the ascospores was analyzed. To accomplish this, two 500 mg. aliquots of ascospores were added to 125 ml. Erlenmeyer flasks. They were then suspended in 10 ml. of 0.028 M NH_4 versenate and shaken 24 hours at 30°C. After being washed free of the EDTA by centrifugation in five changes of deionized water the ascospores were poured into crucibles which were then placed in a muffle furnace. Ashing was performed at 525°C. for approximately 22 hours. Thereupon the ash was dissolved in 2 ml. of 5 N HCl and diluted to 10 ml. with water. The solution was clarified by

TABLE VII

The Ash Content of Ascospores of N. tetrasperma

mg./gm. ascospores			
Ca^{++}	Mg^{++}	Na^+	K^+
0.54	12.0	7.2	15.6

centrifugation and its mineral content determined by means of the flame photometer. The results are given in Table VII. By comparison with the data in Table VI it is seen that about 72 per cent of the K^+ content of the cell is lost in the presence of EDTA. All the Ca^{++} of the cell is lost under these conditions as well as in water alone. On the other hand, insignificant amounts of Na^+ and Mg^{++} are lost in water or in EDTA.

The Non-Specificity of Ca^{++} in the Reversal of EDTA Poisoning.—It was to be expected that any cation which can be bound by EDTA would serve to reverse its toxic action. Therefore, ascospores were activated in 0.028 M EDTA, pH 7.1, and were subsequently treated with concentrations of cations ranging from 1×10^{-1} M through 1×10^{-3} M. The results given in Table VIII show that Ca^{++} and Mg^{++} detoxify EDTA completely and Cu^{++}, Mn^{++}, and Co^{++} do so to a lesser extent. NH_4^+ was ineffective in reversing EDTA toxicity and Na^+ had only a slight effect.

The fact that Co^{++} and Cu^{++} do not completely prevent EDTA toxicity even at concentrations in excess of that of EDTA suggested that a balance between the toxicity of the chelating agent and the ion must be reached before maximal reversal can be accomplished. This is confirmed by the data in Fig.

4 which show a sharp maximum for the Cu^{++} concentration which is most effective in detoxifying EDTA.

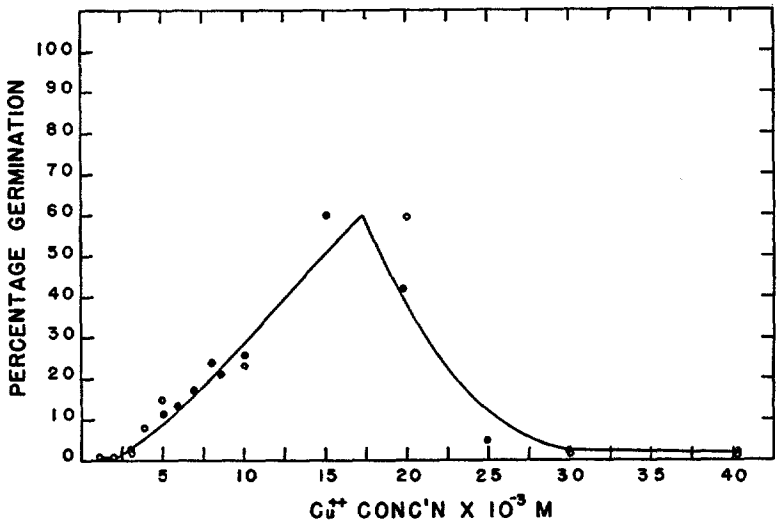

FIG. 4. Reversal of EDTA toxicity by Cu^{++}. Spores were treated with 0.02 M EDTA at pH 7.1. The percentage of germination of controls in H_2O was 94 per cent; that of spores treated with EDTA but with 0.05 M Ca^{++} was the same as for the H_2O control.

TABLE VIII

The Effect of Various Ions upon the Toxicity of EDTA to Ascospores of N. tetrasperma

Compound added	Concentration reversing most effectively	Germination
		per cent
$CuSO_4 \cdot 5H_2$	1.5×10^{-2} M	60
NaCl	5.0×10^{-2} M	7
$MgSO_4 \cdot 7H_2O$	5.0×10^{-2} M	92
$MnCl_2 \cdot 4H_2O$	1.0×10^{-2} M	53
$CoSO_4 \cdot 7H_2O$	1.0×10^{-2} M	59
NH_4Cl	None were effective	0
$CaCl_2 \cdot 2H_2O$	5.0×10^{-2} M	94
H_2O control	—	96

It was shown in Table III that neither Ca^{++} nor washing will reverse EDTA toxicity beyond about 150 minutes after activation. This suggested that a combination of minerals would be required to reverse the poisoning. Therefore ascospores were activated in 2.8×10^{-2} M EDTA and were incubated for 150 minutes at 31°C., at which time 1 ml. aliquots were removed and added to

solutions containing one cation or combinations of cations. These additions were also made to spores which were washed free of EDTA. The cations tested in this way were those listed in Table VIII, as well as Fe^{++}, and Zn^{++}. These were tested in concentrations ranging from 1×10^{-4} M to 1×10^{-2} M and in mixtures containing all the cations in a concentration of 1×10^{-4} M. In addition, the major elements of the minimal *Neurospora* medium (Ryan, 1951) were used in single strength and double strength as well as in the following fractions of the single strength mixture: 1–2, 1–10, 1–100, and 1–1000. Also the trace elements for the above medium were made up separately and were used double strength, single strength, and also in 1–2, 1–5, 1–10, and 1–100 strengths. Finally, combinations of the major and minor elements were used

TABLE IX

Protection from EDTA Toxicity by Pretreatment of Dormant Ascospores with Cu^{++} or Ca^{++}

Ascospores were incubated in 5×10^{-2} M Ca^{++} and 1×10^{-2} M Cu^{++} for 14 hours, washed free of the supernatant, and were activated and incubated in distilled H_2O.

Concentration of EDTA	Percentage germination spores treated with:	
	Ca^{++}	Cu^{++}
Water control	83	3
0.0035 M	—	36
0.0070 M	18.5	23.5
0.0140 M	—	42
0.0280 M	26.5	—
0.0350 M	33	21
0.0700 M	7	—
0.1400 M	0	—

but none of the many individual cations used, or combinations thereof, could reverse EDTA toxicity after ascospores had been incubated in the EDTA for longer than 150 minutes after activation.

The previous experiments have shown that chelatable minerals in solution can prevent the toxicity of EDTA. The next experiments were designed to explore the possibility that protection from EDTA toxicity could be afforded ascospores by prior treatment with such minerals. Therefore, two aliquots of dormant ascospores were made up to a Klett reading of 180 in distilled water. These were added to equal volumes of 1×10^{-1} M $CaCl_2 \cdot 2H_2O$ and 2×10^{-2} M $CuSO_4 \cdot 5H_2O$ respectively and shaken 14 hours at 28°C. At this point, spores were washed five times by centrifugation in distilled water and were heat-activated. 1 ml. samples of both the Ca^{++}- and Cu^{++}-treated spores were then added to 1 ml. of each of several concentrations of EDTA and the resulting suspensions were incubated and formalinized after 5 hours. The data in Table IX show that pretreatment of dormant ascospores with relatively

high concentrations of either Cu^{++} or Ca^{++} will, in large measure, prevent the toxic effects of EDTA. For example, Fig. 1 shows that any concentration of EDTA above 0.0035 M is almost completely toxic to activated ascospores. Yet, when spores were pretreated with Cu^{++} or Ca^{++}, appreciable germination occurred even at concentrations which were ten times higher.

Effect of pH upon EDTA Toxicity.—The dissociation constant of the EDTA–metal complex is markedly affected by pH in the case of some cations but not appreciably, in the physiological range, in the case of others (Martell and Calvin, 1952). This, then, affords a possible means for determining which

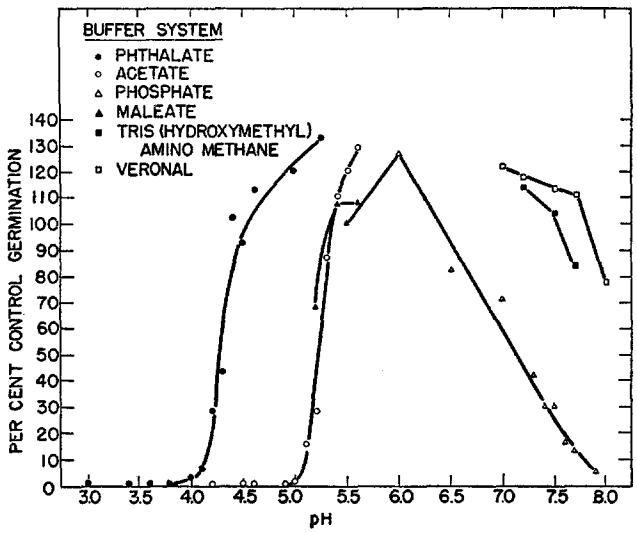

FIG. 5. Effect of pH and buffers upon the germination of ascospores of *N. tetrasperma*. All buffers were used at a concentration of 0.1 M.

essential cations are bound by EDTA. Therefore, the effect of pH upon ascospores was first investigated in order to learn within what range it was possible to work.

With this in mind, a series of buffers was used to cover the pH range from 3.0 to 8.0. Ascospores were heat-activated and aliquots were added to enough buffer to provide a final concentration of 0.1 M. As Fig. 5 shows, there was no germination below pH 4.0 in any of the buffers used. However, phthalate permitted germination to occur as low as pH 4.5 whereas acetate did not permit germination until a pH of 5.5 was reached. In the alkaline range, phosphate buffer greatly hindered germination at pH 7.9. On the other hand, the organic buffers used in the same range did not inhibit germination markedly at this pH. That this effect was probably due to the precipitation of $Ca_3(PO_4)_2$ was shown by experiments in which added Ca^{++} prevented the toxicity of

phosphate buffers even at pH 7.9. The effect of the concentration of phosphate buffer was also investigated and it was found that lower concentrations of buffer will not depress the percentage of germination at pH 7.9. For example, 0.02 M phosphate buffer at this pH had no effect at all upon germination.

Enough information was now at hand so that the effect of pH upon EDTA toxicity could be ascertained. To accomplish this a series of parallel EDTA concentrations was prepared at pH 5.6 and 7.1. Ascospores were heat-activated in distilled water and 1 ml. aliquots were added to an equal volume of each of the EDTA solutions. The results given in Table X show that the toxicity of the chelating agent to ascospores is markedly reduced by lowering the pH. Experiments were also carried out at pH 4.5 with concentrations as high as

TABLE X

pH and Its Effect upon the Toxicity of EDTA to Ascospores of N. tetrasperma

EDTA concentration	Germination	
	pH 5.6	pH 7.1
	per cent	*per cent*
0.00035 M	85	14
0.00070 M	72	10
0.00140 M	57	5
0.0035 M	29	6
0.0070 M	18	2
0.0140 M	10	2
H_2O control	90	

0.01 M but germination was found to be inhibited only about 27 per cent even though one-thirtieth this concentration poisons completely at pH 7.1.

Effect of Ion-Exchange Resins.—The effectiveness of EDTA in inhibiting germination suggested that ion-exchange resins might also be toxic. Therefore, two cationic exchange resins were used: amberlite IR-120, a sulfonic acid resin, and amberlite IRC-50, a carboxylic acid resin. All the resins used were products of the Rohm and Haas Co., Philadelphia. The different cationic forms of the IR-120 resin were made by treatment of the analytical reagent grade (H^+ form) with the appropriate chloride. Such treatment was continued until the pH of the effluent was neutral. The resins were air-dried after being washed until no trace of chloride was detectable by the formation of a precipitate with $AgNO_3$. The approximate exchange capacity of these resins was computed from the data furnished by the manufacturer. The commercial grade of the IRC-50 (Na^+ form) resin was converted into its various forms by the use of the chloride salts as above. The concentration of cations in this resin was determined, after elution of the cation, by the use of the flame photometer as described previously.

Experiments were performed by adding 2 ml. of heat-activated ascospores (Klett reading of 90) to flasks containing weighed amounts of the resins. Incubation and counting were performed as usual and the results summarized in Table XI. These data show that the H^+ form of IR-120 entirely prevents germination in amounts furnishing as little as 0.0125 m.eq. None of the other

TABLE XI

Effect of Resins upon the Germination of Ascospores of N. tetrasperma

Resin	Type	Form	Exchange capacity	Germination	pH
			m.eq./2 ml.	*per cent*	
IR-120	Sulfonic acid (strong acid)	H^+	0.0125 0.010 0.005	0 34 88	3.7
"	" "	Na^+	5.0	8	—
"	" "	K^+	5.0	86	6.3
"	" "	Mg^{++}	5.0	83	6.1
"	" "	Ca^{++}	2.5 0.5	65 91	5.7
IRC-50	Carboxylic acid (weak acid)	H^+	10.0 1.0	0 83	4.2
"	" "	Na^+	.5 0.05	0 26	8.8
"	" "	K^+	0.215 0.0215	0 62	6.3
"	" "	Mg^{++}	0.7	78	6.1
"	" "	Ca^{++}	1.1	77	5.7

forms of the resin which were tried were toxic in amounts much higher than those which proved toxic when the H^+ form was used. On the other hand, the Na^+ and K^+ forms of IRC-50 proved to be the most toxic forms of this type of resin.

The next experiment sought to determine whether actual contact of the ascospores with the resin was necessary for the toxic effects to occur. With this in mind, 4 gm. of IR-120, containing about 20 m.eq. of H^+ was suspended in 5 ml. water and placed in a dialysis bag. 30 ml. of a suspension of activated ascospores (Klett reading of 90) was added to 250 ml. of deionized water in a

300 ml. beaker and the filled dialysis bag was attached to a stirring motor. Stirring was performed at 23°C. and was continued for 18 hours. No germination occurred despite the fact that controls without resin germinated normally. On the other hand, when the experiment was repeated using an amount of IRC-50 in the Na^+ form equivalent to that used above, no inhibition of germination could be detected.

That diffusible impurities are not responsible for the toxic effects of these resins was demonstrated by the following experiment. Three gm. of IR-120 (H^+) and of IRC-50 (Na^+) were shaken in 10 ml. of deionized H_2O overnight and the supernatants collected. Equal volumes of the supernatant and of a suspension of activated ascospores were mixed and incubated as usual but germination was normal in both cases.

To determine whether the pH change induced by the addition of the H^+ form of IR-120 could explain the toxic effect of the resin, the supernatant fluid was collected by filtration after spores had been incubated with the resin. Although ascospores which were in contact with the resin were completely prevented from germinating, those exposed only to the supernatant germinated normally.

Dormant ascospores were shaken overnight with enough IR-120 (H^+) to provide 20 m.eq. of H^+ and with approximately 40 m.eq. Na^+ of the IRC-50 resin. The spores were separated from the resins and activated, incubated, and counted as before. Germination was equal to that of the untreated controls in both cases so that the dormant ascospores proved to be completely insensitive to high concentrations of the resins.

DISCUSSION AND CONCLUSIONS

Whereas dormant and newly activated ascospores of *Neurospora tetrasperma* are insensitive to relatively high concentrations of ethylene diaminetetraacetic acid, germinating spores are inhibited by concentrations as low as 0.0035 M. That the change in sensitivity to the chelating agent occurs at about 120 to 150 minutes after activation (the start of germination) is suggested by the following: (1) Respiratory inhibition does not occur until this time (Fig. 1). (2) EDTA can be washed free of the spores until this time without any ill effects on the cells (Table III); and chelatable ions can prevent the inhibition until this time. (3) If EDTA is added at about 120 minutes after activation, very little lag occurs before its effect upon respiration becomes manifest (Fig. 3).

In an attempt to explain the differences in the response of the ascospore at different stages to the chelating agent, residual EDTA was determined. Since no measurable uptake occurred at any time after activation, changes in permeability to EDTA probably cannot be invoked as an explanation of these differences. The failure of this compound to penetrate, at least the dormant

ascospores, corroborates the evidence obtained by others with bacteria and algae (Hutner *et al.*, 1950) and with fungi (Reischer, 1951).

These results also parallel the observations of Keilin and Hartree (1947) who worked with *Bacillus subtilis* and found that 8-hydroxyquinoline was toxic to both germinating and vegetative cells in low concentrations. On the other hand, resting spores were quite resistant to the chelating agent.

The effect of the resins in these experiments confirms the observation that non-penetrating cation-binding substances can inhibit the germination of ascospores. As in the case of EDTA, resins do not affect dormant ascospores, even in relatively high concentrations. The fact that the strong acid resin (IR-120) in the H^+ form is the most toxic of the preparations used was not surprising since this form is almost completely dissociated. Such an effect could have been due either to a deleterious change in pH or to an exchange of H^+ ions for required cations. However, the fact that the supernatant from spore suspensions poisoned by this resin was not toxic when taken alone argues against an effect of pH. Moreover, the failure of the H^+ form of IRC-50 to prevent germination, despite the low pH of its supernatant fluid, suggests that pH alone cannot explain the toxicity of resins. The toxicity of the Na^+ and K^+ forms of the weak acid resin (IRC-50) when compared with the innocuous nature of the strong acid resin forms of these alkali ions is more difficult to explain. A possible explanation lies in the data of Kunin and Barry (1949) which showed that the exchangeable Na^+ ions of sulfonic acid resins are more difficult to replace by Ca^{++} or Mg^{++} ions than are the Na^+ ions of carboxylic resins. It is possible that the K^+ form acts in the same way. Therefore, in the case of the Na^+ and K^+ forms of the weak acid resin, at least, their toxicity may be a function of the ease of replaceability of their exchangeable ions by divalent cations.

Therefore the toxic effects of EDTA and resins might be attributed to two properties which they have in common: first to a supply of an exchangeable ion, and second, to cation-binding sites. Inhibition is probably initiated by the replacement of necessary cations within, or on, the cell by those furnished by the EDTA or resin. That the supply of an exchangeable cation alone is not sufficient was shown when high concentrations of NaCl or KCl failed to inhibit germination (Table I). Furthermore, binding sites alone are not sufficient because the most toxic forms of resins were those which had readily dissociable ions. This last point was also confirmed by experiments wherein activated ascospores were placed in a dialysis bag and irrigated with distilled H_2O. No effect upon germination was apparent, the assumption being that an exchangeable cation was lacking to replace the necessary ones in the cell. On the other hand, when activated ascospores were placed in a dialysis bag and irrigated with several changes of 0.1 M KCl, germination was entirely prevented. There-

fore, the dual requirement of an exchangeable cation and of binding sites for the removal of the cations lost by the cell seems to be supported.

The nature of the essential cation, or cations, and their location in the cell are not known. Because the experiments attempting to reverse EDTA toxicity with the usual cations used in growth media were negative, it can be argued that essential minerals might have been omitted, or that the wrong combinations were used, but this seems unlikely in view of the many combinations of different ions that were used. If the toxic effect is due to the removal of an essential mineral, or minerals, the failure to reverse by addition is more likely due to the disturbance of a mineral complex the disruption of which is irreversible. In the case of resins, at least, the loss of cationic organic growth factors must also be considered as a possibility.

The data in Table X suggest that the minerals being interfered with are less tightly chelated at a low pH. This is not the result to be expected if trace cations such as Fe^{++}, Cu^{++}, Co^{++}, Ni^{++}, Zn^{++}, or Cr^{++} were being lost from the cell (Schwarzenbach, 1949). On the other hand, the alkaline earth metals are chelated less strongly at a low pH. Of this group, Mg^{++} does not appear to be involved since little or none diffuses out of the cell upon germination. Ca^{++} is, however, released from the cell with the inception of germination but because its addition will not reverse the toxic effect, it is not likely that its loss alone is responsible. Moreover, despite the fact that K^+ is lost in relatively large amounts as a result of EDTA toxicity it is unlikely that its loss alone can explain this effect. The toxicity of the K^+ salt of EDTA and the failure of K^+ alone or in combination with other cations to reverse this, are strong evidence in support of this contention. Since the time when K^+ is lost coincides with that when respiratory inhibition by EDTA becomes manifest, it is possible that this cation is lost as a result of the lessened respiration. This would be in line with the suggestion of others that K^+ accumulation is directly related to carbohydrate metabolism (Scott *et al.*, 1951, and others).

Some interesting aspects of the physiology of spore germination arise from this work. For example, the start of germination signals an important change in the permeability of the ascospores. It is only at this time that Ca^{++} and Na^+ are released from the cell. Moreover, it has also been shown that toxic ions such as Cu^{++} can enter the cell only after germination commences (unpublished results of the author). Therefore, the rate of both the entrance and exit of cations is altered as a result of germination. In this connection it is of interest to note the results of Powell (1953) who found that large amounts of the Ca^{++} salt of pyridine dicarboxylic acid were released from bacterial spores upon germination. These results and many others have suggested a role for Ca^{++} ions in the maintenance of the heat resistance of bacterial spores. The fact that Ca^{++} is released during the germination of *Neurospora* ascospores suggests that their heat resistance may be due to similar reasons.

SUMMARY

The respiration and germination of activated ascospores of *Neurospora tetrasperma* have been shown to be almost completely inhibited by concentrations of ethylene diaminetetraacetic acid (EDTA) as low as 0.0035 M. In contrast, however, dormant ascospores are insensitive to this chelating agent. At any time up to about 150 minutes after activation Ca^{++} or Mg^{++} can completely reverse this toxicity but Cu^{++}, Co^{++}, and Mn^{++} only partially reverse it. After this time, the minerals of the *Neurospora* "minimal" medium taken singly, or in various combinations cannot reverse this effect. Adding EDTA at 120 minutes after activation eliminates the lag period associated with its effect upon respiration. Inhibition occurs even though the cells seem to be impermeable to EDTA. Cationic exchange resins, as another example of a non-penetrating metal-binding agent, gave effects similar to those noted with EDTA. Of the resins used the H^+ form of IR-120 and the Na^+ and K^+ forms of amberlite IRC-50 were the most toxic to activated ascospores. On the other hand, dormant ascospores were entirely unaffected by the resins. The release of Ca^{++} from activated ascospores coincided with the period of maximum sensitivity to EDTA. More than 60 per cent of the cell's content of K^+ is released by EDTA-inhibited ascospores. A low pH decreased the effectiveness of EDTA as a poison. The data are consistent with the possibility that non-penetrating metal-binding agents are toxic because of the irreversible removal of essential cations from the cell. The kinetic data for the inhibitory effects, and for the release of Ca^{++} establish that the permeability of germinating ascospores to minerals changes drastically as a result of activation.

BIBLIOGRAPHY

Davies, O. L., Duckworth, R. B., and Harris, G. M. C., 1948, Method for estimating percentage germination of fungal spores, *Nature*, **161,** 642.

Emerson, M. R., 1948, Chemical activation of ascospore germination in *Neurospora crassa*, *J. Bact.*, **55,** 327.

Goddard, D. R., 1935, The reversible heat activation inducing germination and increased respiration in the ascospores of *Neurospora tetrasperma*, *J. Gen. Physiol.*, **19,** 45.

Goddard, D. R., 1939, The reversible heat activation of respiration in *Neurospora*, *Cold Spring Harbor Symp. Quant. Biol.*, **7,** 362.

Hutner, S. H., Provasoli, L., Schatz, A., and Haskins, C. P., 1950, Some approaches to the study of the role of metals in the metabolism of microorganisms, *Proc. Am. Phil. Soc.*, **94,** 152.

Keilin, D., and Hartree, E. F., 1947, Comparative study of spores and vegetative forms of *Bacillus subtilis*, *Antonie van Leeuwenhoek. J. Microbiol. and Serol.*, **12,** 115.

Kunin, R., and Barry, R. E., 1949, Carboxylic, weak acid type, cation exchange resin, *Ind. and Eng. Chem.*, **41,** 1269.

Martell, A. E., and Calvin, M., 1952, Chemistry of the Metal Chelate Compounds, New York, Prentice-Hall, Inc.

Plumb, R. C., Martell, A. E., and Bersworth, F. C., 1950, Spectrophotometric determination of displacement series of metal complexes of the sodium salts of ethylenediaminetetraacetic acid, *J. Physic. and Colloid Chem.*, **54,** 1208.

Powell, J., 1953, Biochemical changes occurring during the germination of bacterial spores, *Biochem. J.*, **54,** 205.

Reischer, H. S., 1951, Growth of Saprolegniaceae in synthetic media. I. Inorganic nutrition, *Mycologia*, **43,** 142.

Ryan, F. J., 1950, Selected methods of *Neurospora* genetics, *in* Methods in Medical Research, (R. W. Gerard, editor), Chicago, Year Book Publishers, Inc., **3,** 51.

Schwarzenbach, G., 1949, Chelate complexes of cobalt with and without other ligands, *Helv. Chim. Acta*, **32,** 839.

Scott, G. T., Jacobson, M. A., and Rice, M. E., 1951, The influence of glycolytic factors on the potassium and sodium content of *Saccharomyces cerevisiae*, *Arch. Biochem.*, **30,** 282.

Sussman, A. S., 1953, The effect of heterocyclics and other compounds upon the germination of ascospores of *Neurospora tetrasperma*, *J. Gen. Microbiol.*, **8,** 211.

The Versenes *Techn. Bull. No. 2*, Bersworth Chemical Co., Framingham, Massachusetts, 2nd edition, 1951.