PHYSIOLOGY OF THE CELL SURFACE OF NEUROSPORA ASCOSPORES.
IV. THE FUNCTIONS OF SURFACE BINDING SITES1,2

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The previous paper in this series (6) has disclosed that the surface of Neurospora ascospores can serve as a reservoir of cations which can enter the cell after adsorption. For example, Ag+, UO2++, Cu++, and other cations are removed from solution by dormant live ascospores and by killed ones as well. Furthermore, the kinetics of uptake and the fact that these materials can be eluted readily suggest that they are localized on the cell surface. If the dormancy of such "coated" ascospores is broken they fail to germinate due to the presence of the toxic cations on their surface. This effect has been demonstrated in the case of organic bases like Polymyxin-B (5) as well as for the substances mentioned above. These observations suggest, therefore, that the cell surface is the source of cations which are excluded from vital centers in the spore until germination is induced.

However, the fact that such absorbed ions can penetrate does not establish that this route is obligatory. It is also possible that the cations must be eluted before they enter and that the adsorption sites merely concentrate them; or, alternate means of penetration may exist which are independent of preliminary surface localization.

That surface adsorption of cations is widespread is demonstrated by its occurrence in bacteria (7, 9), erythrocytes (1), fungi (10, 13) and higher plants (8, 17). Therefore, an investigation of the relation of adsorption to penetration was undertaken in order to define the role of this ubiquitous phenomenon.

MATERIALS AND METHODS

Ascospores of Neurospora tetrasperma were obtained, stored and prepared for use by the methods described previously (12). The latter paper also describes the techniques used in the heat-activation as well as the germination of the spores. The concentration of these cells was determined by the use of a Klett colorimeter with a blue filter (Klett # 42) whereby a reading of 180 corresponded to a concentration of 1 mg (dry wt) per ml of spore suspension.

"Coated" ascospores were prepared by mixing equal volumes of suspensions containing 1 mg per ml of ascospores and 2 x 10^-2 M of the unbuffered "coating" material. This mixture was incubated at 20°C on a reciprocal shaking machine for 24 hours whereupon the supernatant fluid was decanted after centrifugation and the ascospores washed in 4 changes of water. The "coated" ascospores were resuspended in water and stored at 4°C until used.

The measurement of oxygen uptake was performed by the standard manometric procedures outlined in Umbreit et al (15). Unless otherwise noted, the Warburg vessels contained 0.5 ml of the spore suspension, 0.5 ml of a solution of the cation to be tested, or an equivalent volume of water, and 0.2 ml of KOH in the inset as an absorbent for carbon dioxide. All respirometric experiments were carried out at 25°C at a shaking rate of 120 oscillations per minute. The dry weight of the spore suspensions used was obtained by keeping aliquots in an oven at 105°C overnight.

Ag110 was determined by means of an end-window Geiger tube and scaler. As before, aluminum pans containing 0.2 ml of the solution were used and enough counts were recorded to assure less than 2% counting error.

Hexol nitrate (see list of abbreviations) was prepared according to the method of Werner (16), as modified by Sutherland (14). The cobalt hexamine salts were generously provided by Prof. Robert W. Parry of the Department of Chemistry, University of Michigan. List of abbreviations:

1. PCB: phenylmercuribenzoic acid.
2. Hexol nitrate: hexa-ethylendiamino-hexol tetraacetic acid whose chemical formula is

![Chemical Structure](attachment:chemical_structure.png)

This and the subsequent description of cobalt coordination compounds is used as provided in Sidgwick (11).

3. Cobalt hexamine: hexamine cobalt (III) chloride whose formula is Co(NH3)6Cl3.
RESULTS

As a result of the disruption of dormancy in ascospores of Neurospora a 20-fold enhancement of oxygen uptake is observed (4). Such metabolic activity is a sensitive indicator of the effect of toxic materials upon the cells (12) and serves to indicate the point where such inhibition is first manifest. With these possibilities in mind, the effect of adsorbed cations upon the oxygen uptake of ascospores was studied.

To accomplish this, a suspension of ascospores that had been "coated" with UO$_2^{++}$ was activated and its oxygen uptake compared with that of a similar but untreated suspension of spores that served as a control. In addition, the oxygen uptakes of untreated spores suspended in $1 \times 10^{-8}$ M UO$_2$(NO$_3$)$_2$ immediately and 105 minutes after activation were also studied. The results provided in figure 1 demonstrate that no effect of the UO$_2^{++}$ is obvious until 100 minutes of incubation after activation. At this time the oxygen uptake of both "coated" spores and those suspended in a UO$^{+}$ solution after activation is inhibited although the effect of the latter treatment is much greater. However, in no case did germination occur.

It is worth mention that the effect of the UO$_2^{++}$ that was tipped at 105 minutes was almost immediate although the amount of inhibition was somewhat lower than in the case where the cation was included in the spore suspension from the start.

This experiment was repeated with spores that were treated with Ag$^+$ in the same manner as above except that the effect of tipping at 105 minutes was not studied. As in the case of spores treated with UO$_2^{++}$ those treated with Ag$^+$ were affected only after 100 minutes.

In order to test the effect of an organic cation upon this system, phenylmercuribenzoic acid (PCB) was used in the same way as were the inorganic substances. Again, as in the latter instances no effect of PCB was demonstrable until incubation had proceeded beyond 90 to 100 minutes after activation (fig 2). However, in this case, the "coated" cells were affected at least 20 minutes after those that had been incubated continuously in the poison although the former cells still did not germinate. An experiment was also performed in which the oxygen uptake of dormant PCB-"coated" cells was compared with that of untreated ones and it was found in both cases that the $Q_{O_2}$ ($\mu$l oxygen per mg dry weight per 60 minutes) was about 0.5.

It had been reported previously (13), that certain strongly basic substances like Polymyxin-B, protamine and complex salts of cobalt served to prevent the adsorption of methylene blue on ascospores of N. tetrasperma. This observation provided a means whereby the role of adsorbing sites in the penetration of cations might be tested. For example, if adsorption of cations on surface sites is a necessary prelude to their penetration, then the blocking of such sites by the materials listed above should prevent this process. Alternatively, if adsorption is not required, then the entrance of cations should proceed unimpeded by such treatment.

Accordingly, experiments were designed to test whether cobalt hexammine chloride and hexol nitrate prevented the surface-binding of Ag$^+$, as well as that...
TABLE I
UPTAKE OF Ag\textsuperscript{110} by Ascospores of N. tetrasperma after Treatment for 24 Hours with Complex Cobalt Salts at a Concentration of 1 x 10\textsuperscript{-4} M

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CPM after 30 min</th>
<th>UPTAKE in CPM</th>
<th>PERCENTAGE REMOVAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexol nitrate</td>
<td>81</td>
<td>108</td>
<td>57</td>
</tr>
<tr>
<td>Cobalt hexamine</td>
<td>76</td>
<td>113</td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td>23</td>
<td>166</td>
<td>88</td>
</tr>
<tr>
<td>Starting solution</td>
<td>189</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Final concentration of ascospores was 1.0 mg per ml and that of Ag\textsuperscript{110} was 2 \( \mu \)g per ml. Incubation in the mineral was continued for 30 minutes. The figures provided are the average of triplicate counts.

of methylene blue, to ascospores. Dormant cells were “coated” with each of these substances and, after being washed free of the complex salts, aliquots containing 1 mg per ml of the spore suspensions were mixed with an equal volume of a solution containing 4 \( \mu \)g per ml (3.7 x 10\textsuperscript{-8} M) of Ag\textsuperscript{110} and incubated on a shaking machine at 20\°C for 5 hours. At this time the cells were precipitated by centrifugation and aliquots of the supernatant fluid were pipetted onto planchets and their radioactivity determined as described before. Exactly similar treatment was accorded a set of untreated ascospores which served as the control in this experiment. The results provided in table I show that the adsorption of Ag\textsuperscript{+} by dormant ascospores is markedly interfered with by previous treatment with cobalt hexamine chloride and hexol nitrate.

It was now possible to examine the effect of blocking adsorbing sites upon the uptake of Ag\textsuperscript{+} by ascospores after activation. With this in mind, aliquots of Ag\textsuperscript{+}, hexol nitrate-, and cobalt hexamine-coated ascospores were made up to 16 mg per ml and activated as was a suspension of untreated spores at the same concentration. Thereupon, aliquots of these spore suspensions were diluted to 8.0 and 4.0 mg per ml and all were incubated at 30\°C for 1 hour after activation. At this time, an equivalent volume of a solution containing 4 \( \mu \)g per ml Ag\textsuperscript{110} was added to each of the flasks containing the spore suspensions described above and incubation was resumed for a total of 2.5 hours after activation. The ascospores were then removed by centrifugation and aliquots of the supernatant were plated out for counting of residual Ag\textsuperscript{110}.

Since the time of incubation had been large enough to assure the protrusion of a germ tube, the percentage germination was determined after the spores had been killed by the addition of formaldehyde. The results provided in table II lead to the conclusion that Ag\textsuperscript{110} is not excluded from the cell by the presence of any of the surface “coatings” used; in fact, the amount taken up is greater under these circumstances. Moreover, although germination was complete in all the spore suspensions to which water had been added, none occurred in those which had been treated with Ag\textsuperscript{110}.

Another way of testing the effect of “coating” materials is to determine whether such treatment alters the rate at which Ag\textsuperscript{+} inhibits the oxygen uptake of activated ascospores. Accordingly, activated spore suspensions that had previously been coated with hexol nitrate were added to Warburg vessels as before and enough Ag\textsuperscript{+} tipped 105 minutes after activation to give a final concentration of 5 x 10\textsuperscript{-8} M. As can be seen in figure 3, the oxygen uptake of “coated” cells is inhibited as rapidly as that of controls so that the penetration of Ag\textsuperscript{+} does not seem to be affected by such treatment. Identical results were obtained when cobalt hexamine was used instead of hexol nitrate.

<table>
<thead>
<tr>
<th>Spore Conc ( \text{mg/ml} )</th>
<th>Controls</th>
<th>Hexol Nitrate</th>
<th>Cobalt Hexamine</th>
<th>Ag\textsuperscript{+}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Uptake of Ag\textsuperscript{110} in cpm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>34</td>
<td>30</td>
<td>29</td>
<td>87</td>
</tr>
<tr>
<td>4.0</td>
<td>54</td>
<td>84</td>
<td>77</td>
<td>134</td>
</tr>
<tr>
<td>8.0</td>
<td>82</td>
<td>117</td>
<td>128</td>
<td>140</td>
</tr>
</tbody>
</table>

Incubation of the spores in the metal was performed as described in the text. The final concentration of Ag\textsuperscript{110} was 2 \( \mu \)g per ml and the initial counts in this solution totaled 176 cpm.

**TABLE II**
UPTAKE OF Ag\textsuperscript{110} BY VARIOUS CONCENTRATIONS OF ACTIVATED ASCOSPORES TREATED WITH HEXOL NITRATE, COBALT HEXAMMINE, OR Ag\textsuperscript{+}

**Fig 3.** Effect of “coating” with hexol nitrate upon the time when Ag\textsuperscript{+} affects the oxygen uptake of activated ascospores of *N. tetrasperma*. Final concentration of Ag\textsuperscript{+} after tipping 105 minutes after activation was 1 x 10\textsuperscript{-4} M. Points represent the average of readings in duplicate vessels. Germination: control, “coated” with hexol nitrate—91%; tipped, “uncoated”—0%; tipped, “coated”—0%. 
DISCUSSION AND CONCLUSIONS

The above results suggested that prior adsorption to sites on the cell surface did not result in faster penetration to the interior of the cell or to the sites that are sensitive to heavy metals. In fact, in the data summarized in figures 1 and 2, the inhibition by Ag⁺, UO₂²⁻, and PCB of cells "coated" with these materials took longer to materialize than in the cases where the cells were incubated continuously in these substances. This was so despite the fact that the former cells had been exposed to the cations at least 24 hours previous to the latter. These data are supported by the results of the experiments which demonstrated that the blocking of adsorbing sites with hexol nitrate or with cobalt hexammine did not reduce the amount of Ag⁺ taken up by the cells after activation. Furthermore, the effect of these substances upon the oxygen uptake of germinating ascospores was not delayed by such blocking. Therefore, these results lead to the conclusion that surface binding of cations is not a prerequisite to penetration, at least in ascospores of N. tetrasperma.

Abundant confirmation of the fact that ascospores undergo a drastic change in permeability as a result of activation is furnished by these experiments. For example, figures 1 and 2 demonstrate that the activated cells become permeable to cations only after they have been incubated for 105 minutes under these conditions. This is also supported by previous data (5) showing that cells "coated" with Polymyxin-B, a basic polypeptide, has the same effect as do the cations studied above. It might be argued that these data could be accounted for by assuming that slow diffusion into the activated cells is possible throughout the course of incubation and that it takes about 100 minutes for toxic amounts to accumulate. That this is not very likely is suggested by the fact that the first indication of the effect of each of the cations tested occurs at the same time. Furthermore, when UO₂⁺ was tipped 105 minutes after activation, instead of being added to the spores from the start (fig 1), there was no lag in the expression of the poison's effect, suggesting again that a barrier to penetration is removed or modified to permit the entrance of cations at this time.

According to Rothstein and Hayes (10), the binding sites on the surface of the yeast cell are associated with less than 2 % of the total cellular cations. These sites must, therefore, involve only a small fraction of the cellular structure. This calculation was made for Neurospora ascospores on the basis of previous work (12) in which was reported the amount of K⁺, Na⁺, Mg²⁺ and Ca²⁺ to be found in these cells. Because only a negligible amount of cations other than those are present the total concentration is approximately 0.785 micromoles per mg dry weight of ascospores. It should be noted that this figure is several-fold higher than in the case of yeast but this is understandable, perhaps, in view of the fact that the ascospores are a type of resting cell and store relatively large amounts of food materials. Using the data provided in the previous paper (6) surface bound Ag⁺, UO₂²⁻, Cu⁺⁺, Th⁴⁺ and Al³⁺ account for 9.8, 11.3, 5.5, 2.7 or 3.8 %, respectively, of the total cations in the cell. In all the cases studied, therefore, a larger proportion of cations is bound to the surface of Neurospora than to that of yeast.

Whether this is the case for other resting cells is still not known but the concentration of trace elements on the cell surface from the external environment would provide such cells with a ready supply of such materials when needed. From the practical standpoint, the presence of relatively small amounts of a toxic cation could result in the inhibition of germination. The data in tables I and II suggest that "coating" the cell results in the enhancement of uptake during germination so that it is possible to consider such treatment as acting as an adjuvant would in the application of fungicides. It is possible, then, that one way in which cobalt hexammine and hexol nitrate act is by neutralizing charges on the surface of the cell.

If the assumption is made that the cations bound to the surface of the dormant ascospore are arranged in a single layer then the proportion of the surface that is occupied can be calculated. However, before this could be done it was necessary to calculate the surface area of the ascospore and, to this end, 100 measurements of its axes were made with the results averaging 31.7 and 14.9 μ. Examination shows that the ascospores have approximately the shape of prolate spheroids, that is, of ellipsoids which are generated by the rotation of ellipses

\[ x = a \cos \theta; \quad y = b \sin \theta \quad (0 < b \leq a) \]

about the x-axis. By somewhat extended but elementary computations, it can be shown that the area A(a, b) of such an ellipsoid is given by the formula

\[ (1) \quad A(a, b) = 4ab \int_0^1 \sqrt{1 - c^2u^2} \, du \quad (Edwards, 1932), \]

where \( c^2 = 1 - \frac{b^2}{a^2}. \) It is readily seen that the value of the integral in the last expression lies between \( \pi/4 \) and 1. In other words, \( A(a, b) = 4ab\pi \lambda; \) where \( \lambda \) depends on the ratio \( b/a, \) but lies in every case between 1 and \( \pi/4; \) and in any case \( A(a, b) \leq 4ab\pi. \) As judged from the ratio of the axes of the ascospores \( (a/b), \) the extreme values of the integral given above are 0.92 and 0.82, while the average is 0.85. Therefore, the average surface area of the ascospores was computed by substituting 0.85 for the integral in (1) and the absolute maximum was obtained by setting this equal to 1, with the following results:

- Maximum Area 3.0 × 10¹¹ sq. Å
- Average Area 2.5 × 10¹¹ sq. Å

By reference to the tables of ionic radii presented by Ephraim (3) it was now possible to compare the area available on the cell surface to that occupied by

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3 The authors are indebted to Prof. George Piranian of the Department of Mathematics, University of Michigan, for the derivation provided below.
a single layer of ions used in these experiments. For the purposes of this calculation, it is assumed that the ions are spherical. A consideration of table III reveals that many more molecules are absorbed to the surface than there is space to accommodate them, if the assumption is made that they occur in a single layer. The discrepancy is 4-fold in the case of Th+++ and 7-fold for Ag+, using the value obtained for the average area of the spore surface, suggesting that these ions must be arranged in a 3-dimensional pattern on the ascospore surface. Even if the figure for the maximum possible area is used there is still a large discrepancy between the amount of space available on the spore and that required for ions arranged in a single layer. The possibility that the adsorbed materials are arranged in depth is supported by recent work (Lowry and Sussman, unpublished) describing the lamellar structure of the cell wall of Neurospora ascospores.

To summarize it seems clear from the data presented in this paper and in the others in this series that cations adsorbed to the cell surface can enter when germination commences. Therefore, the adsorbing sites might serve as a source of trace minerals for the cell, especially since these are usually multivalent and are strongly bound by the surface. This is in agreement with the idea of some workers (8) that it is more reasonable to consider the concentration of adsorbed minerals on the root surface to be the amount available to the plant rather than that of a dilute nutrient solution.

**Summary**

The prior adsorption of Ag+, UC4++, and phenylmercuribenzoate (PCB) to sites on the cell surface did not result in faster penetration to the interior, as judged by their effect upon the respiration of germinating ascospores of *Neurospora tetrasperma*. Moreover, the blocking of adsorbing sites by treatment with hexol nitrate or cobalt hexamine chloride did not result in decreased uptake of Ag+ or in a delay in the time when the respiratory effects of Ag+, UC4++, and PCB were manifested. These results lead to the conclusion that adsorption to the surface of the cell is not a necessary prelude to penetration. Cations bound to the surface account for about 3 to 11% of the total cations of the cell. Calculations of the area of the cell disclosed that adsorbed cations are probably arranged in depth on the cell surface rather than in a single layer.

**Literature Cited**


