

The Chemistry of Sites Binding Rubidium in *Chlorella*

DAN COHEN

From the Department of Agriculture, University of Oxford, England. Dr. Cohen's present address is the Department of Botany, The Hebrew University of Jerusalem, Israel

ABSTRACT The chemistry of sites that specifically bind Rb in *Chlorella pyrenoidosa* has been investigated by changing or modifying specific chemical groups or bonds in the cell and observing changes in binding capacity. Boiling the cells in water or in 70 per cent ethanol did not affect binding capacities of the sites. These results suggest that the integrity of the sites is independent of both hydrogen bonds and hydrophobic bonds, and that the sites, therefore, do not consist of a protein or protein-lipid complex. At 30°C, both 1 M HCL and 0.5 to 1 M NaOH rapidly inactivated 70 per cent of the sites, but over a range of pH 4.4 to 11.3, there was no effect. The sites are inactivated by strong chelating agents at 0.05 to 0.2 M and by reagents which reduce trivalent iron, and 4 to 10 atoms of iron per site are removed from the cells. Prolonged incubation in iron solutions, but not in solutions of Cu, Mn, or Mg, reversed to a considerable extent inactivation by EDTA. It is suggested that the sites probably bind trivalent iron tightly as chelation bridges which are essential to their structure. These structural bridges are broken when iron is removed by chelating agents or reduction, and are reformed in the presence of iron. Other experimental evidence indicates that amine, sulfhydryl, and carbonyl groups are not structural components of the sites.

INTRODUCTION

In a previous paper (Cohen, 1962), it was reported that there was a specific binding of Rb in the cells of *Chlorella* and the affinities of various ions for these binding sites were investigated. It was further established that the order of the affinity was not changed when the cells were frozen or boiled in water or 70 per cent ethanol or acetone. The chemistry of these sites is of interest, as compounds forming stable complexes with Rb or K in aqueous solutions are unknown. This paper is concerned with obtaining further information on the chemistry of the sites, which could be of assistance in elucidating the mechanism of binding and the possible factors which maintain the structure of the sites.

The approach has been to treat the cells in ways known to break or modify

specific chemical groups or bonds. A reduction in the binding capacity following a particular treatment would indicate that the groups known to be affected are important in the structure of the sites. On the other hand, if a treatment does not reduce the binding capacity, it would follow that the groups affected are not important.

EXPERIMENTAL METHODS

A thermophilic strain of *Chlorella pyrenoidosa*, 7-11-05, was used in this investigation, and the methods of growing and harvesting the cells have been described in the previous paper. In the present investigation, most of the experiments were undertaken with cells killed in boiling water for 30 minutes or in 70 per cent ethanol for an hour.

The method of measuring the specific binding of Rb is the same as that described in the previous paper. The principle of this method is as follows. The cells are first incubated in a standard medium containing high concentrations of ions that are non-competitive for the Rb sites, and a low concentration of Rb labeled with Rb⁸⁶. The quantity of Rb bound by the cells is then assessed by measuring the increase in concentration of Rb in the external medium following displacement from the sites by the addition of an excess of K. The concentration of the standard medium is as follows: MgSO₄, tris-HCL, and tris-phosphate, each at 10 mM, together with 0.01 per cent of the non-ionic detergent tween 80, with a pH of 6.2–6.6.

In the present investigation, after the cells had been subjected to the various chemical treatments, the cells were centrifuged, resuspended, washed in water, and the procedure repeated using the standard medium. Binding of Rb was then measured under standard conditions. For each experiment, the decrease in binding relative to that of the control cells was taken as a measure of the inactivation of the sites.

Solutions in which the cells had been treated were occasionally analyzed to find out which substances were released from the cells during treatment; such substances could indicate the nature of the reaction leading to inactivation of the sites. Prior extraction of the cells with boiling 70 per cent ethanol had the advantage that it reduced the release of substances not connected with inactivation during the subsequent treatment.

Samples of solutions to be analyzed for their metal content were wet ashed, if necessary, in a mixture of sulfuric and perchloric acids. The metals were assayed colorimetrically using a Hilger "uvispec" spectrophotometer. Iron was determined with 8-hydroxyquinoline 5-sulfonic acid (HQSA) at 570 m μ , or with *o*-phenanthroline at 505 m μ (Vogel, 1951). Copper was assayed with diethyldithiocarbamate (DIECA) at 440 m μ (Vogel, 1951). In solutions in which no copper could be detected, manganese was measured in the same way at 500 m μ . Hydroxamic acids were estimated by a modification of Schweet's method (1955).

EXPERIMENTAL RESULTS

The following treatments were found to have no effect on the binding sites: (a) boiling in water for 30 minutes, (b) boiling for more than an hour

in 70 per cent ethanol or acetone, (c) freezing and thawing three times, (d) treating with saturated *n*-butanol for 30 minutes at room temperature. The mean binding capacity after these treatments was 97.8 ± 4.5 per cent of that of the control cells.

Effects of Acid, Alkali, and Chelating Agents

Inactivation of the sites by acid and alkali was investigated at 30°C using heat-killed cells. In less than 5 minutes, a proportion of the sites was inactivated with 1 M HCL or 0.5 to 1 M NaOH, the remainder being stable for up to 60 to 80 minutes. The resistant fraction varied between experiments. The mean of the acid treatments was 28.8 per cent of the control, and that of the alkali treatments, 30.2 per cent. The difference between these treatments is not significant, as the standard error of the mean for the two treatments combined was 2.8 per cent of the control. When cells were treated for 60 minutes with solutions adjusted to a wide range of pH, there was no inactivation between pH 6.7 and 11.3, but reductions of 45.5 and 80.7 per cent at pH 2.5 and 1.7 respectively, suggested that some inactivation had already taken place at pH. 4.

Ethanol-extracted cells were employed when they were given more specific chemical treatments, at a temperature of 30°C. The solutions in which the cells were treated were analyzed for iron and other metals when it was thought that the treatments removed metals from the cells. In these instances, the ratio of the quantity of metal removed to the number of sites inactivated was calculated and will be termed the metal/site ratio.

Ninety-three to ninety-five per cent of the sites were inactivated after treatments for 2 to 3 hours with the sodium salt of the chelating agent ethylenediaminetetraacetic acid (EDTA) at concentrations of 0.1 to 0.15 M and at pH 9–10.2. The iron/site ratio was calculated for one such treatment and it was 15. Similar quantities of Mn and Fe were removed from the cells, but no Cu could be detected in the solutions. The sodium salt of 8-hydroxyquinoline 5-sulfonic acid (HQSA), another chelating agent, was used to study the effect of both concentration and length of treatment on the degree of inactivation. In Table I it is seen that inactivation takes place slowly and that a relatively high concentration of the reagent is required. The iron/site ratio is always of the same order, indicating that inactivation is approximately proportional to the removal of iron, four to ten atoms of iron being removed for every site inactivated.

A brief survey was made of other chelating and complexing reagents. Following treatment for 140 minutes with 0.2 M of the sodium salt of the chelating agent 1,2-dihydroxybenzene-3,5-disulfonic acid (tiron) at pH 10.0, 78.7 per cent of the sites were inactivated: the iron/site ratio was 4.8.

Treatment with 0.1 M NaCN at pH 9.4–9.7 for 4 hours caused no inactivation and removed very little iron. A 75 per cent saturated solution of *o*-phenanthroline (*circa* 5 mM) at pH 7.8 failed to bring about any inactivation after 4 hours and extracted little iron.

In contrast with other chelating agents, the affinity of phenanthroline for divalent Fe is much greater than that for trivalent Fe. The divergence suggested that Fe in the sites is trivalent and can be removed by reduction. Treatment of cells for 4 hours with 10 mM Na dithionite, a powerful reducing agent for trivalent Fe, inactivated 91.2 per cent of the sites, the pH falling from 7.3 to 5.6 during the treatment: the iron/site ratio was 9.4. Thioglycolate is both a chelating agent for ferrous iron and a strong reducing agent for ferric

TABLE I
THE EFFECTS OF CONCENTRATION AND DURATION OF TREATMENT
WITH 8-HYDROXYQUINOLINE-5-SULFONIC ACID (HQSA) ON THE
INACTIVATION OF SITES AND REMOVAL OF IRON

Experiment conducted with ethanol-extracted cells at 30°C and a pH of 9.2–9.3. Iron/site ratio expressed as atoms of Fe removed over number of sites inactivated.

Concentration of HQSA	Duration of treatment	Binding capacity as per cent of the control	Iron/site ratio
<i>mM</i>	<i>min.</i>		
0.5	163	87.2	6.7
4.0	39	91.1	4.5
4.0	163	83.2	5.7
20	38	85.3	4.3
20	162	60.6	4.3
80	150	10.5	10

iron, and 0.1 M of the sodium salt at pH 10.1 inactivated 46.1 per cent of the sites after 4 hours, the iron/site ratio being 6.1.

A prolonged posttreatment of the cells with a high concentration of iron was effective in reversing the inactivation caused by EDTA. The iron could be maintained in solution at pH 5–6, the pH of the “reversing” solutions, by using 10 to 18 mM succinate-imidazole as a buffer and by adding a low concentration of citrate to the medium. Results for a typical experiment in which reversal by Mn and Cu was also investigated are given in Table II. It is clear that only iron can reverse the inactivation. It should be added that before measuring their binding capacity the cells were left overnight in the standard medium which contains 10 mM Mg; Mg did not reverse the inactivation.

The inactivation by acid and alkali was further investigated to discover whether it involved removal of iron from the sites. If so, then the amount of iron removed should be at least equivalent to the degree of inactivation.

Also, the inactivation should be reversible by treatment in an iron solution. It is clear from the results of such an experiment (Table III) that the amount of iron removed from the cells was not enough to account for inactivation by acid, but was just sufficient to allow for inactivation by alkali.

TABLE II
THE REVERSAL OF EDTA INACTIVATION OF RB BINDING SITES
BY SUBSEQUENT TREATMENT WITH Fe, Cu, OR Mn

Experiment carried out with ethanol-extracted cells: Cu applied as 10 mM CuSO_4 , Mn as 10 mM MnCl_2 , Fe as a mixture of 30 mM FeCl_2 , 10 mM FeSO_4 , and 1 mM Fe citrate. Solutions buffered with 10 mM succinate-imidazole buffer: period of treatment 18.5 hours at 30°C. The pH of the individual solutions causing a reversal is indicated in the table.

Treatment with EDTA	Reversing metal	pH	Binding capacity as per cent of the control
—	—	7	100.0
+	—	7	6.1
+	Fe	5.85	39.2
+	Cu	5.8	6.7
+	Mn	6.7	8.9

TABLE III
THE REVERSAL OF THE INACTIVATION CAUSED BY ACID AND
ALKALI FOLLOWING A SUBSEQUENT TREATMENT WITH IRON

Ethanol-extracted cells were first treated at pH 1 for 90 minutes or at *circa* pH 12.5 for 85 minutes at 30°C; in the alkali the concentration of carbonate was high and the estimate of the pH inaccurate. The cells were then washed and subjected to 4 mM FeCl_2 and 0.7 M Fe citrate in 18 mM succinate-imidazole buffer at pH 6.5 and 30°C for 16.5 hours.

Treatment	Binding capacity as per cent of the control	Iron/site ratio
Acid	27.4	0.063
Acid, followed by iron	32.6	
Alkali	20.9	1.0
Alkali, followed by iron	44.9	

Further, inactivation by alkali was reversible by iron to a much greater extent than was inactivation by acid. Inactivation by alkali may therefore be similar to that caused by other iron-removing agents, but a different mechanism must be postulated to explain the effects of strong acids.

Tests for Specific Chemical Groups

As stated earlier, a series of experiments was concerned with examining the effects of compounds known to attack specific chemical groups. Sodium nitrite

attacks amine groups and it was observed that after treatment for 3.5 hours with a concentration of 0.6 M at pH 4.2 the level of inactivation was less than that produced by a pH of 3.8–4.0 alone. It was concluded therefore that amine groups are not essential for the binding of Rb by the sites.

Bromine water is a powerful oxidizing reagent for SH groups and olefinic bonds, and will introduce Br into phenolic aromatic compounds. When an excess of this reagent was used at pH 7.8 only 38.8 per cent of the sites were inactivated after 1.5 hours. Thus, sulfhydryl groups do not play an essential part in binding. Partial inactivation may have been caused either by a slow reaction of the reagents with part of the site, or by a completed reaction which caused only a partial loss of affinity.

Treatment at pH 7.3 with 1 M semicarbazide, which reacts with carbonyl groups, inactivated 24.4 per cent of the sites after 3 hours. Hydroxylamine, another reagent for carbonyl groups, at 0.1 M and pH 9.9–10.2, caused little inactivation of the sites after 2–3 hours; it did, however, react vigorously with carbonyl groups, as shown by the formation of hydroxamic acids. Therefore, carbonyl groups are seemingly not essential for binding.

It was observed that when either carbonate or bicarbonate was present, hydroxylamine did bring about a considerable degree of inactivation. This interaction was not investigated further, but it was noticed that during inactivation in the presence of carbonate, an orange substance with an absorption maximum at 430 $m\mu$ appeared in the solutions. The effects of varying the pH and the addition of iron on the absorption pattern led to the conclusion that this substance was a complex of iron with hydroxamic acids. It can therefore be suggested that inactivation of the binding sites by hydroxylamine in the presence of carbonate may be due to the removal of iron from the sites.

DISCUSSION

It has been established that the binding sites studied in this investigation are resistant to boiling both in water and in 70 per cent ethanol. The integrity of the sites thus does not depend on hydrogen bonds and hydrophobic bonds which are known to be readily broken down by boiling water and by hot hydrous organic solvents (Kautzman, 1959). Hydrogen and hydrophobic bonds are very important in maintaining the molecular structure of proteins, nucleic acids, and lipid-protein complexes and it would appear therefore that these substances do not play an important part in the structure of the binding sites. The sites are probably smaller than these macromolecules, and are stabilized by other chemical bonds. However, it is possible that some large heat-labile structure may have component sites which determine the

specificity of its reactions with ions in much the same way as a large labile enzyme molecule has a heat-stable coenzyme component.

The results for inactivation by the acid and alkali treatments pointed to the existence of two groups of sites which differ markedly in their stability. Heterogeneity of the sites, and in particular division into two groups of approximately 75 and 25 per cent, were also indicated by the findings of the previous paper (Cohen, 1962). Following a different approach, the sites were found to be divisible into two groups according to their pK_a and their affinity for Rb.

It has been shown that the sites were stable to strong acid and alkali to about the same extent, but the mechanism of inactivation appeared to differ in the two cases. During inactivation by alkali, as against that caused by acid, iron was removed during inactivation, which was partly reversible by incubation in an iron solution. This suggests that inactivation by alkali is at least in part due to removal of iron from the sites. The inactivation by acid and some part of the inactivation by alkali may best be explained on the basis of the breaking of a labile bond in the structure of the sites.

The stability of the larger fraction of the sites towards acid and alkali is similar to that of creatine phosphate, and is much less than that of many other phosphate esters (Ord and Stocken, 1959). However, no definite conclusions can be reached about the chemical nature of this bond. It is tempting to suggest that this bond is also metabolically labile, its making and breaking bringing about the large change in affinity necessary for the functioning of carriers in transport (Glynn, 1959).

It seems that trivalent iron stabilizes the structure of the sites. This view is supported by the following observations: (a) inactivation by EDTA is specifically reversed by iron, (b) the sites are also inactivated by reduction with dithionite, (c) the ratio of the number of atoms of iron removed from the cells to the number of sites inactivated is approximately constant for all the chelating and reducing reagents studied, (d) iron is released into those solutions of hydroxylamine which are effective in causing inactivation. It is suggested that trivalent iron, which has strong coordinating powers, forms chelation bridges which stabilize the structure of the sites. Removal of the iron breaks these bonds and inactivation ensues. These iron bridges are probably reformed when iron is resupplied, thus reversing the inactivation.

The evidence is insufficient for definite conclusions to be reached regarding the mode of binding of the iron in the sites. The iron must be tightly bound, as it is only removed when the concentrations of chelating agents are high and the period of treatment long. The slow rate of removal also suggests that binding of iron is partly covalent, or that removal is sterically hindered (Taube, 1952). A porphyrin linkage is unlikely, because in porphyrins, iron

is bound very firmly and is not released by treatments which removed iron from the sites in the present investigation.

Inactivation of the sites by dithionite is probably the result of the reduction of the iron to the divalent state, thereby lowering the affinity of the iron for the site, since most ligands bind trivalent iron more strongly than divalent iron. As a consequence, the removal of the iron from the sites would bring about their inactivation. The role of iron in the structure of the sites may be to hold negatively charged groups in the rigid configuration necessary for Rb binding. This would be analogous to the role of Zr in Zr-phosphate gels (Amphlett, McDonald, and Redman, 1958). Here, the specific binding of ions of the alkali metals by these gels is supposedly brought about by Zr ions linking the negatively charged phosphate groups in a rigid configuration favoring the binding of these ions. Iron and other heavy metals are known to stabilize the structure of various proteins (Gurd and Wilcox, 1956), intercellular cement (Ginzburg, 1958), and other biologically important structures by forming inter- and intramolecular chelation bridges.

Conway and Duggan (1958) suggested that iron is a structural component of K carriers in yeasts. They found that specific binding of K increases under anaerobic conditions, and suggested that alternating oxidation and reduction of the iron by respiratory enzymes causes the cyclical changes in the affinity of the carriers for K, which are considered necessary for their operation in transport. In the killed cells of *Chlorella*, it has been shown first that a reduction by dithionite leads to removal of iron from the sites. Second, that both this removal and its reversal are slow processes. It seems therefore unlikely that in living cells the postulated rapid metabolic oxidation and reduction of iron in the sites is the mechanism responsible for the changes in affinity for Rb.

The picture of the binding sites that emerges from this and the previous investigation is one of a rigid structure in which binding groups are so arranged that the space between them accurately corresponds to the size of an unhydrated Rb ion. The structure is maintained by chemical bonds which are stable both to hot water and to hot organic solvents. One or more of the bonds is broken at pH's below 3 and above 12. Iron constitutes an essential part of the structure, probably in the trivalent state as a chelation bridge.

This work was carried out while under tenure of the Reginald Graham Scholarship at Pembroke College, Oxford, for which I am grateful to the College and to the Friends of the Hebrew University of Jerusalem in Great Britain. I am greatly indebted to Professor G. E. Blackman, F.R.S., who supervised this work, for his continuous help and encouragement. I wish also to thank Miss M. A. Pratt, Dr. L. C. Reinhold, and Dr. J. Dainty for their help in preparing the manuscript, and Dr. B. Z. Ginzburg for stimulating discussions.

This work forms part of a Doctor of Philosophy thesis submitted to the University of Oxford.

Received for publication, December 18, 1961.

REFERENCES

- AMPHLETT, C. B., McDONALD, L. A., and REDMAN, M. J., *J. Inorgan. and Nuclear Chem.*, 1958, **6**, 220.
- COHEN, D., *J. Gen. Physiol.*, 1962, **45**, 959.
- CONWAY, E. J., and DUGGAN, F., *Biochem. J.*, 1958, **69**, 265.
- GINZBURG, B. Z., Ph.D. thesis, Hebrew University, Jerusalem, 1958.
- GLYNN, I. M., The sodium potassium exchange pump, Colloque de Biologie de Saclay, Pergamon Press, Paris, 1959, **1**, 46.
- GURD, F. R. N., and WILCOX, P. E., *Advances Protein Chem.*, 1956, **11**, 311.
- KAUTZMAN, W., *Advances Protein Chem.*, 1959, **14**, 1.
- ORD, M. G., and STOCKEN, L. A., in *Data for Biochemical Research*, (Dawson *et al.*, editors), Oxford University Press, 1959, 44.
- SCHWEET, R. S., *Biochim. et Biophysica Acta*, 1955, **18**, 566.
- TAUBE, H., *Chem. Rev.*, 1952, **50**, 69.
- VOGEL, A. I., *Quantitative Chemistry*, London, Longmans Green and Co., 2nd edition, 1951, p. 666 for Cu, pp. 646-647 for Fe.