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THE STACKING OF THE THYLAKOIDS OF TWO LEGUMINOSAE

DIFFERENTIAL RESPONSES TO H^+ AND DIVALENT CATIONS

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The stacking of the thylakoids of lupine and horse bean has been studied by the digitonin method in relation to the concentration of H^+, Ca^{2+} or Mg^{2+}. (1) The isolectric point appears to be 4.7 for the two species. At this pH, Ca^{2+} has no effect on the stacking of the lupine thylakoids but it lowers the stacking of those of horse bean. (2) At pH 7.6, for any given Ca^{2+} concentration, the horse bean thylakoids fix a lesser amount of Ca^{2+} than those of lupine but they are more stacked. (3) The surface potential has been estimated by the use of the Gouy-Chapman model, modified to take account of H^+ and Ca^{2+} binding. Simulation of the experiments indicates that the results may be explained by supposing that (i) the anionic groups are less numerous on the horse bean thylakoids than on those of lupine, (ii) they are arranged such that the affinity for the binding of Ca^{2+} or Mg^{2+} is higher for horse bean and virtually nil for lupine, and (iii) the divalent cation binding per se promotes the stacking when the coulombic repulsion is sufficiently weakened by screening and binding.

Introduction

A comparative study of Ca^{2+} fixation to isolated chloroplasts from lupine (*Lupinus luteus* L., calcifuge) and horse bean (*Vicia faba* L., var *minor*, calcicole) was carried out previously [1–3]. When chloroplasts from the two species were extracted with a chloroform/methanol mixture following $^{45}Ca^{2+}$ labelling, the label preferentially appeared in the protein fraction [1]. Fixation of Ca^{2+} to the thylakoid membranes was completely abolished when a water-soluble carbodiimide was used to modify the carboxyl groups of the membranes [2]. These results indicated that carboxyl groups act as major Ca^{2+}-receptor sites. A direct correlation between the amount of calcium fixation and the carbodiimide-mediated incorporation of a [^{14}C]glycine ethyl ester was observed: chloroplasts from lupine fixed more calcium than those of horse bean because they contain a greater amount of carboxyl groups. The results on the whole strongly suggest that the interaction of Ca^{2+} with the thylakoid membrane surface is associated with a diffuse electrical layer [3].

Barber et al. [4] pointed out the importance of such electrostatic interactions in various reactions in photosynthesis: they have proposed that metal cations induce membrane stacking by neutralizing charges on the membrane surfaces. Such interactions may be treated semiquantitatively in terms of the Gouy-Chapman diffuse double layer theory.

In the present study we show that Ca^{2+} does not affect the stacking of the thylakoids from lupine and horse bean in the same way. Analysis of our results with the help of the Gouy-Chapman modified model to include the binding of H^+ and Ca^{2+} reveals that the

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; LHCP, light-harvesting chlorophyll *a*/*b*-protein complex; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

different stacking responses to pH may be explained by different affinities for divalent cation binding. Furthermore, we are led to suggest that Ca^{2+} or Mg^{2+} binding becomes per se a factor of the stacking when the electrostatic repulsion between the membranes is sufficiently weakened by screening and binding.

Materials and Methods

Chloroplasts were isolated from lupine or horse bean leaves using the procedure reported in Ref. 1.

The chloroplast pellets were resuspended in 330 mM sorbitol and 5 mM Hepes adjusted to pH 7.6 with Tris. The outer envelopes of the chloroplasts were ruptured by resuspending the pellets in 5 mM Hepes-Tris (pH 7.6) for 5 min. The osmotically ruptured chloroplasts were then collected by centrifugation at $10000 \times g$ for 10 min and resuspended in 330 mM sorbitol and 5 mM Hepes-Tris (pH 7.6) for experiments on calcium fixation or in 100 mM sorbitol adjusted to pH 7.6 with Tris for experiments on stacking.

Calcium fixation. Calcium-fixation measurements were made, according to Ref. 1, by using thylakoid suspensions consisting of various concentrations of $^{45}CaCl_2$ added to the basic medium and containing 100 μg chlorophyll/ml. For experiments on calcium fixation in the protein and lipoprotein fractions, the pellets obtained after Ca^{2+} fixation were treated with a chloroform/methanol mixture according to Ref. 1.

Stacking of thylakoids. The degree of stacking was monitored by the effectiveness of digitonin in disrupting the membrane system [5–7]. Thylakoid membranes were suspended in 100 mM sorbitol-Tris (pH 7.6) and the indicated salt concentrations for 5 min at 25°C and then incubated with 0.5% digitonin according to the procedure of Anderson and Boardman [8]. The mixture was then centrifuged according to the method of Barber et al. [9].

The percentage of chlorophyll in the pellet was determined following the method of Whatley and Arnon [10] and gave a relative measure of the stacking. In experiments where the pH was varied, the medium also included 100 mM sorbitol, 1 mM citric acid and 1.5 mM K_2HPO_4. The pH was adjusted, if necessary, with Tris or HCl.

Electrophoresis. Electrophoresis was performed on an FF5 Hobein and Bender apparatus [11]. The electrode buffer was 15 mM Hepes-Tris (pH 7.6). The chamber buffer was 5 mM Hepes-Tris (pH 7.6) and was collected in 90 tubes after a 5 min flow period. The electric field was 100 V/cm (current 35 mA). The temperature was 4.5°C.

Proteins were measured by the method of Lowry et al. [12]. Digitonin (analytical reagent grade) was purchased from Calbiochem, ^{45}Ca from CEA France and other reagents were grade A.

Results

Calcium fixation

Fig. 1 shows the amount of Ca^{2+} which co-sediment with the thylakoids; part of this calcium is bound, and part is free in the diffuse layer. The thylakoids from lupine retain more Ca^{2+} than those from horse bean. The same difference is observed for fixation of Ca^{2+} on protein and lipoprotein fractions. The fixation curves reach a plateau. The apparent affinities are about 250 μM Ca^{2+} for both species. It has been shown that the thylakoids from lupine bear 50% more carboxylic acid groups than those of horse bean, and that the neutralization of these groups by EDC virtually suppresses the fixation of Ca^{2+} [2]. This may explain the difference between the curves for both species in Fig. 1.

Stacking of thylakoids

Effect of divalent cations. Ca^{2+} and Mg^{2+} enhance the stacking with equal effectiveness (Fig. 2). Their effect is more pronounced on the thylakoids from horse bean than on those from lupine. At 100 μM Ca^{2+} or Mg^{2+}, the former are 50% stacked, whereas the latter need more than 15 mM Ca^{2+} or Mg^{2+} to attain the same stacking level. Comparison of the results of Figs. 1 and 2 shows that in the two species the stacking of the thylakoids is not associated with the same amount of fixed Ca^{2+}: the thylakoids from lupine are more effective in fixing Ca^{2+}, but the fixation seems to have little effect on their stacking. The reverse is true for horse bean (Fig. 2, upper).

Effect of pH. Fig. 3 shows that the maximum stacking level is obtained at about pH 4.7 for both species. This value has been reported to be the isoelectric point of the thylakoids from spinach [13]. At this pH, the thylakoids from lupine are almost totally stacked with or without Ca^{2+}. For horse bean,

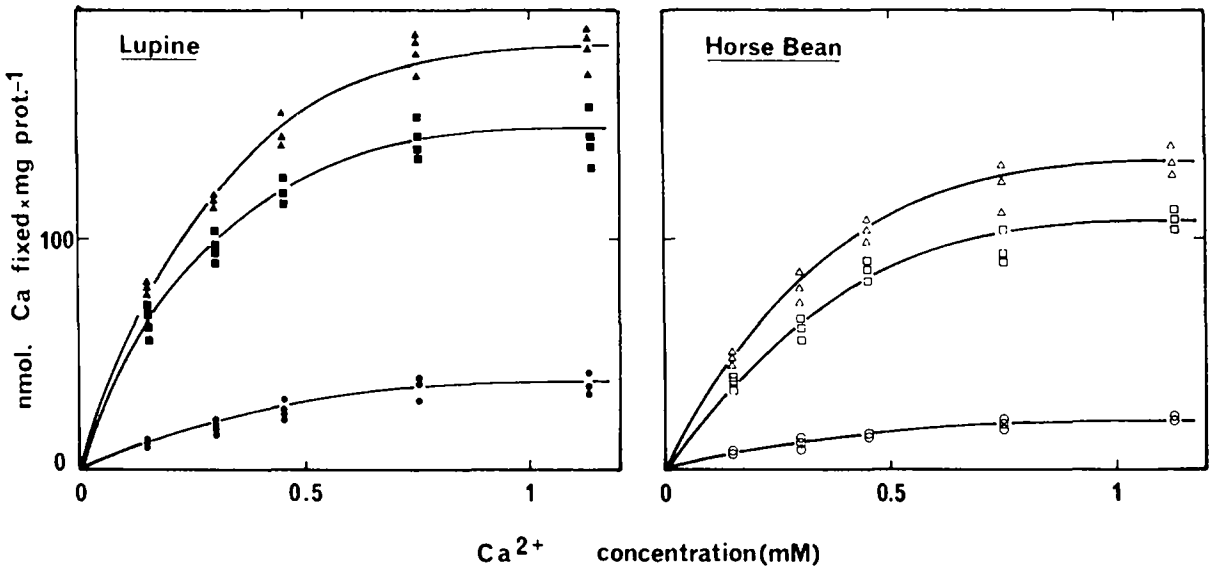

Fig. 1. Concentration dependence of $^{45}Ca^{2+}$ fixation to thylakoids of lupine and horse bean at pH 7.6. The medium contained 5 mM Hepes, 330 mM sorbitol and $CaCl_2$ as indicated. After 3 min incubation, the thylakoids were pelleted, briefly rinsed with Ca^{2+}-free solution and aliquots were treated with a chloroform/methanol mixture. (▲,△) Thylakoids, (■,□) chloroform/methanol-insoluble fraction, (●,○) chloroform/methanol-soluble fraction.

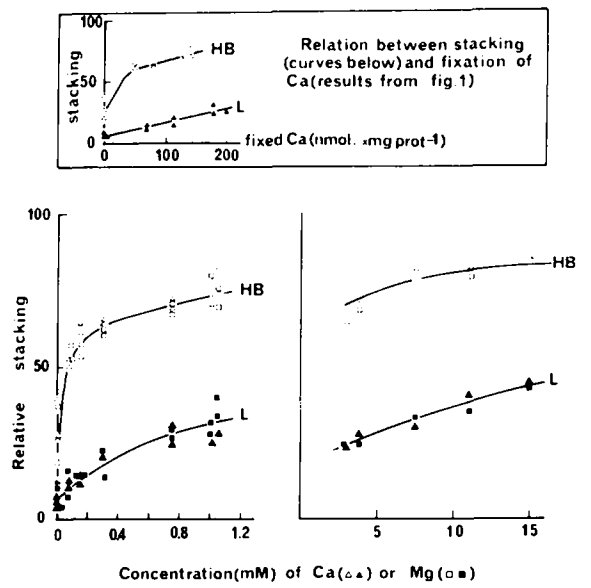

Fig. 2. Effect of divalent cations on the percentage of chlorophyll in the 10 000 × *g* pellet (stacking) of thylakoids after incubation with 0.5% digitonin. The experimental details are described in Materials and Methods (HB, horse bean; L, lupine).

the presence of 6 mM Ca^{2+} lowers the maximum level of stacking. This further suggests that Ca^{2+} does not affect the stacking of the thylakoids from the two species by the same mechanism.

Free flow electrophoresis

The horse bean thylakoids were injected at a position located at the vertical of the 60^{th} fraction collecting tube. The anode corresponds to tube 0 and the cathode to tube 90. When the injection buffer (pH 7.6) contained no calcium, the thylakoids were collected in the 22^{nd}–24^{th} tubes; the anionic marker bromophenol blue migrated to the same tubes. When the thylakoids, pretreated with 12 mM Ca^{2+}, were injected in a buffer containing the same Ca^{2+} concentration, they migrated to the 60^{th}–65^{th} tubes. In some runs, the thylakoids were free of Ca^{2+} before being injected in the 12 mM Ca^{2+} buffer; they migrated towards the anode in the first centimetre, then progressively returned towards the same tubes as did the Ca^{2+}-pretreated thylakoids. In other runs, Ca^{2+}-pretreated thylakoids were injected in a Ca^{2+}-free buffer; they first migrated towards the 60^{th}–65^{th} tubes, and after a few centimetres, were deviated in the direction of the 22^{nd}–24^{th} tubes.

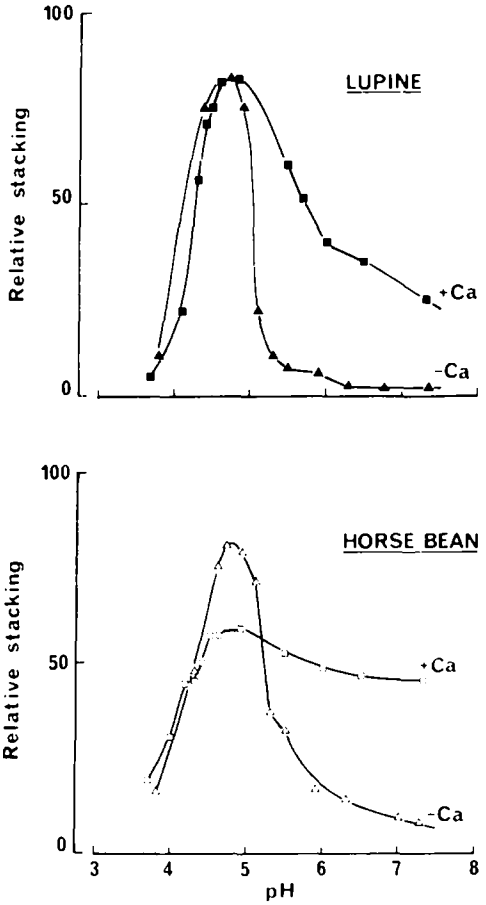

Fig. 3. Effect of pH on stacking. The thylakoids were suspended in media containing 1 mM citric acid, 1.5 mM KH_2PO_4, 100 mM sorbitol (▲,△) and eventually 6 mM $CaCl_2$ (■,□). The pH values were adjusted with HCl or Tris.

Theory

The electrical interactions between an infinitely flat, uniformly charged surface and an electrolytic solution may be described by the Gouy-Chapman model. Briefly, by using the Poisson law for describing the effect of ions on the potential and the Boltzmann law for describing the effect of the potential on ions, one obtains for the appropriate limiting conditions the so-called Grahame equation [4,14,15]

$$\Sigma C_i\left(1 - \exp\left(\frac{Z_i F\psi_0}{RT}\right)\right) + \frac{2\pi N_A}{\epsilon RT}\cdot\sigma^2 = 0 \qquad (1)$$

where C_i is the bulk concentration of the ith ion, ψ_0 the surface potential, σ the surface charge density, N_A Avogadro's number and the other symbols have their usual meanings.

This relationship predicts that the ions exert a screening effect on ψ_0. The valency dependence of this effect may explain the marked influence of divalent cations on surface phenomena such as the voltage-conductance shifts on axons [16]. In other cases it appears that the surface charge is not only screened but also masked by the binding of ions. This is evident when ions with the same valency have different effects on surface phenomena [17]. The binding of the ith ion is taken into account by using the mass action law for correcting σ (e.g., see Ref. 18 for H^+, or Ref. 17 for Ca^{2+}). For instance, if one ion binds to one fixed charge:

$$\sigma = \sigma_{max} - \frac{\sigma_{max}\cdot C_{io}}{C_{io} + K_i} \qquad (2)$$

where K_i is the intrinsic dissociation constant, and C_{io} the local concentration given by the Boltzman law:

$$C_{io} = C_i \exp\left(-\frac{Z_i F\psi_0}{RT}\right) \qquad (3)$$

If j kinds of fixed ionic groups are present:

$$\sigma = \sum_j \sigma_{max\,j} \sum_i \left(1 - \frac{C_{io}}{C_{io} + K_{ij}}\right) \qquad (4)$$

By combining Eqns. 1, 3 and 4 one obtains an implicit relationship from which ψ_0 may be computed by iteration.

Discussion

When Ca^{2+} is the major cation in the medium, and the sole divalent cation as is the case for Fig. 1, the amount fixed (bound plus free in the diffuse layer) approximately measures the number of fixed negative groups. This may be easily shown by using the theoretical model described above. Experimental evidence for this relationship in the case of carboxyl groups has been published [24–26].

From the values of the plateaus of the Ca^{2+}-fixation curves (Fig. 1), one may assume that the

thylakoids of lupine and horse bean, respectively, bear about 400 and 300 nequiv./mg protein of dissociable anionic groups. It is clear from Fig. 1 that these surface groups belong to the proteins, and it has been shown that they essentially are carboxylic acids [2]. The pK of such a function is near 4.5 [18]. The computations indicate that when the values of the number of anionic groups and of their pK values are fixed, the value of the isoelectric point is only determinated by the numbers of basic groups and by their pK values, irrespective of the area of the membrane. It seems likely that most of the basic groups consist of arginine and perhaps lysine [9]. The pK values of the guanidine and ϵ-amino groups, respectively, are 12 and 9. When the pH remains less than 8, as in our work, it is not essential to distinguish the two pK values. Under these conditions, it appears that the observed pI value (4.7) is obtained by using contents in basic groups equal to 60% of those of anionic groups. This value is somewhat higher that (47%) derived from the amino acid composition of LHCP from spinach [19].

At pH $<$ 4.7, the stacking is not affected by Ca^{2+}. This is consistent with the hypothesis of a positive net surface charge in this pH range. When the pH increases above 4.7, the effect of Ca^{2+} on the lupine thylakoids is still in accordance with the building up of a negative net surface charge, the repulsive field of which is attenuated by the divalent cation. The stacking response to Ca^{2+} of the horse bean thylakoids is markedly different from that of the lupine thylakoids. For these latter, Ca^{2+} has no effect at pH = pI (as expected), and its efficiency in promoting the stacking diminishes when the net negative surface charge is increased along with the pH. For horse bean thylakoids, Ca^{2+} is effective in decreasing the stacking at pH = pI, and this effect remains approximately the same when the pH is increased to 7.5.

If the stacking level is taken as reflecting the coulombic repulsion, then it must be concluded that when the pH increases, Ca^{2+} prevents the net positive charge of the horse bean thylakoids from being reduced to zero at pH 4.7, and maintains it at a constant value at higher pH values. The electrophoretic behaviour of these thylakoids is consistent with a slight positive surface charge at pH 7.6 in the presence of Ca^{2+}, in contrast with a negative surface charge in Ca^{2+}-free medium. For this to occur, it is necessary that Ca^{2+} is able to compete with H^+ for interacting with the anionic groups at pH values equal to pI or even below, and that the resulting effect is the maintenance of a net positive charge on the membrane. These two conditions are fulfilled if Ca^{2+} specifically binds with the anionic groups. If the intrinsic affinity constant is sufficiently low, then the binding will be effective even at pH $<$ pI, in spite of a relative exclusion of Ca^{2+} of the diffuse layer. Moreover, the binding masks a fraction of the anionic groups and prevents the net charge from becoming negative when the pH increases above pI. The fact that small amounts of fixed Ca^{2+} are more effective in promoting the stacking of the horse bean thylakoids than that of the lupine thylakoids (Fig. 2, upper) strengthens the hypothesis of a direct association between the divalent cation and the anionic groups of the horse bean thylakoids. The similarity between the effects of Ca^{2+} and Mg^{2+} (Fig. 2) does not really weaken the hypothesis of specific binding because for most of the dicarboxyl groups, the affinity constants for the two cations are similar [20]. We have used the above theoretical model with H^+ and Ca^{2+} binding for computing the surface potential of the thylakoids. The binding of divalent cations to monovalent anionic groups is taken into account by postulating that these receptors are pre-existing pairs of carboxyl groups. The concentration of the receptors is simply half the concentration of the monovalent groups and the structure of Eqn. 2 is not affected. The specific spatial arrangements of the two carboxyl groups are supposed to confer different intrinsic affinities on Ca^{2+} for the $(\text{-}COO^-)_2$ sites.

The selected values of the parameters are listed in Table I. Fig. 4B shows the curves of the surface potential computed for the experimental conditions of Fig. 3. Clearly, the model correctly accounts for the qualitative characteristics of the experimental curves of stacking vs. pH insofar as the stacking is supposed to reflect the coulombic repulsive interactions. The charge balance is depicted in Fig. 4A. It can be seen that on the horse bean thylakoids, the high affinity for Ca^{2+} results in the masking of a large fraction of the negative charges so that the positives ones are in excess even at high pH values. The same situation appears in Fig. 5. The resulting positive surface potential expels the cations from the diffuse layer, such

TABLE I

SELECTED VALUES OF THE PARAMETERS USED IN THE THEORETICAL MODEL

R^- and R^+ are the acidic and basic groups. The formers' contents were estimated from the data of Fig. 1. The latters' contents were then chosen to obtain a p*I* value which coincides with the stacking maxima. The indicated values of affinity constants are those which give the best agreement between the calculated potential curves and stacking data of Figs. 4 and 5. The area *A* corresponds to negative surface charge densities in the 1–4 $\mu C/cm^2$ range for the different experimental conditions

	R^+ (nmol/mg protein)	pK^+	R^- (nmol/mg protein)	pK^-	$K_{Ca} = K_{Mg}$ (M)	A (m^2)
Horse bean	200	12	320	4.5	10^{-3}	1
Lupine	300	12	480	4.5	0.5	1

that the Ca^{2+} retained by the horse bean thylakoids (Fig. 1) is bound for the most part. In contrast, the lupine thylakoids bear a net negative surface charge at high pH values because they have a poor efficiency in Ca^{2+} binding. One can calculate, using Eqn. 3, that the local free Ca^{2+} concentration reaches 50 mM

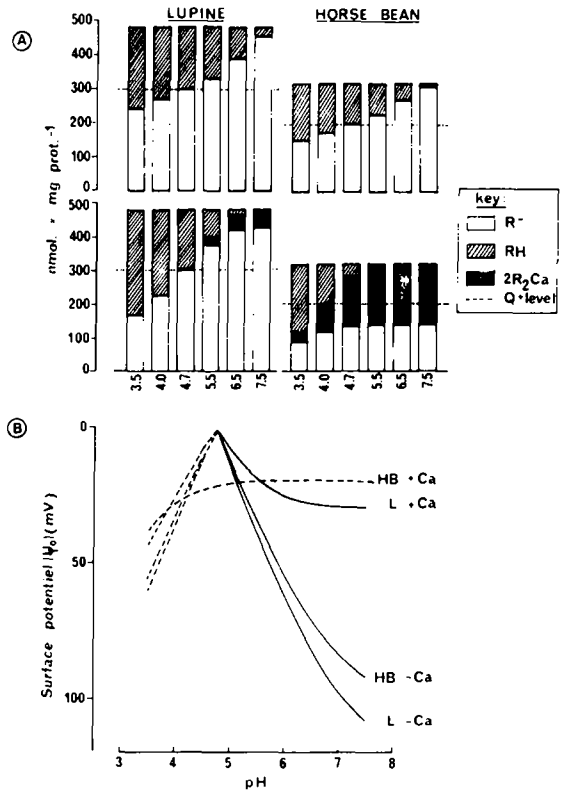

Fig. 4. Simulation of the experiment described in Fig. 3 by the Gouy-Chapman model with H^+ and Ca^{2+} binding. The parameters are given in Table I. A, forms of the anionic groups R and charge balance; B, absolute values of the surface potential. (- - - - -) Positive values, (———) negative values. (HB, horse bean; L, lupine).

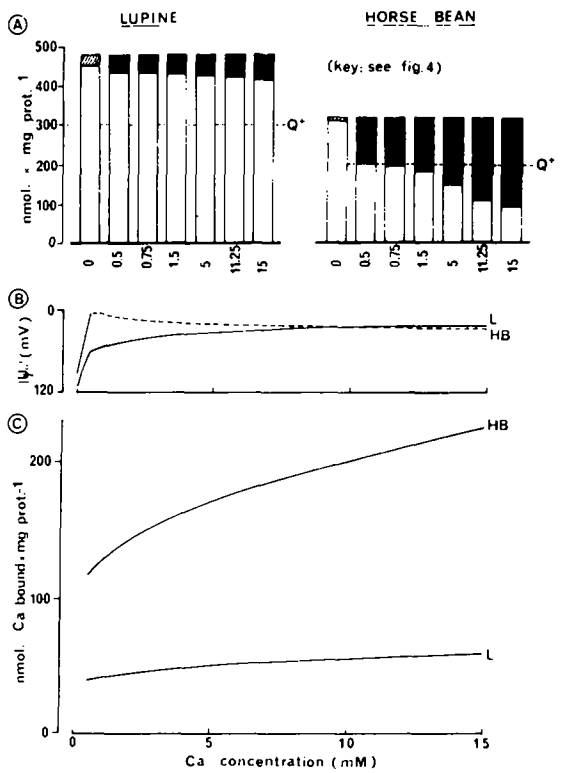

Fig. 5. Simulation of the experiment described in Fig. 2 by the Gouy-Chapman model with H^+ and Ca^{2+} binding. The parameters are given in Table I. A, forms of the anionic groups R and charge balance; B, absolute values of the surface potential. (- - - - -) Positive values, (———) negative values; C, content in bound Ca^{2+} (HB, horse bean; L, lupine).

when the medium contains 1 mM Ca^{2+}. This may explain why the lupine thylakoids co-sediment with more Ca^{2+} than those of horse bean (Fig. 1).

Fig. 5B shows the computed curves of surface potential vs. Ca^{2+} concentration under the experimental conditions of Fig. 2. There is no longer any agreement between the observed stacking and the calculated potential for horse bean: the stacking is steadily enhanced when the Ca^{2+} concentration is increased while the calculated repulsive strength increases. This discrepancy may be overcome if one supposes that the stacking has two major determinants with opposite effects, namely, the repulsive interactions and the amounts of bound divalent cations. It then appears that the experimental data of Figs. 2 and 3 may be accounted for by the theoretical results of Figs. 4 and 5, simply by assuming that the cation binding is the main determinant of the stacking when the repulsive strength is weak (approximately for $|\psi_0| < 25$ mV). This effect of cation binding may be due to the masking of discrete negative charges which prevented the achievement of stacking even after the long-range repulsive forces were weakened by net charge attenuation and by screening.

In summary, our experimental results show that the interactions between Ca^{2+}, H^+ and the membrane surface are different for the thylakoids of both species. The theoretical analysis reveals that the difference may be explained by assuming that (i) the stacking is prevented by high net surface charge, (ii) it necessitates the binding of divalent cations, and (iii) both species differ in the affinity constants of binding. Point i is generally accepted. The control of stacking by the local positive space charge density has been recognized [4,18,21–23] but it is considered to act via the modulation of surface potential by screening and binding. This does not contradict our hypothesis of a more direct involvement in the stacking (point ii) because the attenuation of electrostatic repulsion is a prerequisite for the stacking.

We do not know whether the differences between the two species reported here are related to the differences in their ecological status. Nevertheless, recent work in our laboratory (Cournier, S., unpublished data) has given analogous results with two other calcicole/calcifuge plants (*Vitis vinifera* L., *Vitis riparia* Michx).

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