BBA 75045

LIPID–PROTEIN INTERACTION AT THE AIR–WATER INTERFACE

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(Received September 27th, 1966)
(Revised manuscript received February 20th, 1967)

SUMMARY

1. The spreading of a protein solution with a glass rod into the plane of a cholesterol monolayer greatly reduced the time necessary to attain an equilibrium surface pressure in the mixed monolayer. The results obtained with this spreading method coincided with those obtained by injecting the protein solution into the bulk of the subphase supporting the lipid monolayer.

2. On a phosphate buffer solution (pH 7.2) there was a linear relationship between the total surface pressure of the mixed monolayer and the molar fraction of amino acid residues at the air–water interface; this suggests a non-specific dissolution process in which the hydrocarbon chains of the amino acid residues and the cholesterol molecules are the chemical species involved.

3. The free energy of mixing for this process, as well as for the surface dissolution of cetyl alcohol and cholesterol, practically coincided with that corresponding to an ideal system; this suggests that the driving forces for both processes could be of entropic origin and that no specific interactions occur between the components of the surface solution.

INTRODUCTION

The structure of biological membranes as bimolecular leaflets of lipids with adsorbed proteins[1] is now generally accepted. Such structure strongly suggests lipid–lipid and lipid–protein interactions which can explain the structural stability as well as the modifications of the functional properties of the membrane by processes in which small energetic transitions are involved[2].

Lipid–protein interactions can be studied at an air–water interface by injecting a protein solution in the aqueous subphase supporting the lipid monolayer[3–6]. The observed increase of surface pressure (at constant area) or of area (at constant surface pressure) with this method is the resultant of three successive steps: diffusion in the bulk subphase, adsorption, and interaction. The interpretation of these phenomena points to the interaction of the lipid with some specific group in the protein molecule.

TRURNIT's technique[7] for direct spreading of bovine albumin at an air–water interface[8] seems a suitable method for direct spreading of proteins into the plane of a lipid monolayer, thus avoiding the steps previous to the interaction; using this method the interaction of cholesterol with bovine albumin and other substances has been studied and through the determination of the excess free energy of mixing, the interaction between cholesterol and bovine albumin at the air–water interface has been evaluated.

MATERIALS AND METHODS

Instruments

Surface pressures were measured with an automatic recording surface balance constructed according to TRURNIT AND LAUER[9]. The trough (14 cm × 90 cm × 0.3 cm) and the mica float were Teflon coated. An electrically driven Gilmont micropipette allowed the delivery of solutions at the air–water interface (± 0.0001 ml). The balance could be operated at constant area (± 0.14 cm^2) or at constant surface pressure (± 0.1 dyne/cm); it was located in an air-conditioned room. All experiments were performed at 20° (± 1°).

Reagents

Bovine albumin was two times crystallized (Armour Laboratories); its homogeneity and purity was checked by free electrophoresis and analytical ultracentrifugation. Bovine growth hormone was prepared after SONNENBERG AND DELLACHA[10]. Insulin was obtained from Elanco, Argentina; [5-valine]-angiotensin from Ciba; sodium dodecylsulphate from Sigma and was recrystallized from ethanol; sodium hexadecylsulphate and cholesterol were obtained from Schuchardt and cetyl alcohol from Fluka; both lipids gave in preliminary experiments areas per molecule in accord with the accepted values.

Inorganic reagents were Analytical grade. Purified petroleum ether was used for the lipid solutions; 1 ml spread in 1000 cm^2 produced less than 0.5 dyne/cm surface pressure when the surface was reduced to 100 cm^2. Deionized water, freshly distilled from alkaline permanganate, was used throughout.

The subphase was always a phosphate buffer (pH 7.2, I 0.15, made up with NaCl); bovine albumin, [5-valine]-angiotensin, sodium dodecylsulphate and sodium hexadecylsulphate spreading solutions were prepared in this buffer. Insulin was dissolved in 0.01 M HCl. Bovine growth hormone was dissolved in glycine–HCl buffer (pH 3). When 2 ml of these solutions were added to 1000 ml of the phosphate buffer solution no change was observed in the pH value.

Methods

Constant area. The trough filled with the phosphate buffer solution was cleaned by sweeping the surface four times with Teflon barriers. Using a flat circular Teflon support, a glass rod (5 mm diameter and 75 mm long) with an hemispheric top was placed vertically in the trough, just beneath and in contact with the tip of the micropipette previously filled with the protein or the alkylsulphate spreading solution. The micropipette was secured vertically to the rack of the balance. The required amount of cholesterol was then spread with a pipette using the petroleum

ether solution. A compression curve was obtained to 35–40 dyne/cm, followed by a decompression to 10 dyne/cm. At this point a leak test was performed by connecting a constant-pressure device; no displacement of the mobile barrier in 30 min was taken as a criterion for the absence of leaks in the system in which case the constant-pressure device was then disconnected. All the experiments at constant area were performed with the cholesterol monolayer at 10 dyne/cm initial surface pressure. Turning on the switch of the electrically driven micropipette, the required amount of protein or alkylsulphate spreading solution was delivered through the glass rod, starting simultaneously a stopwatch. In all experiments the diameter and length of the glass rod, the speed of flow, and the concentration of the protein spreading solution were those recommended by TRURNIT for the spreading of bovine albumin at the air–water interface. No attempts have been made to adjust these parameters to reduce the equilibration time for sodium dodecylsulphate and sodium hexadecylsulphate. The increasing surface pressure was recorded until equilibrium was attained; all the values of surface pressure increment given correspond to the equilibrium value.

At the end of each experiment (2–4 h) a leak test was performed measuring the surface tension of the buffer solution behind the barrier and the float, to take account of any soluble molecules which had passed by diffusion under the barriers to the "clean" surface. Values obtained were always within 0.10–0.15 dyne/cm of the pure phosphate buffer solution. Also, sweeping the surface behind the float did not produce any surface pressure variation.

Compression curves. Mixed monolayers of cholesterol and cetyl alcohol were obtained by separate spreading at 0 dyne/cm of the respective petroleum ether solutions. Mixed monolayers of cholesterol and bovine albumin were obtained by spreading the bovine albumin solution by means of the glass rod into the cholesterol monolayer at 0 dyne/cm surface pressure. Compression curves were obtained at a rate of 4 cm/min in all experiments.

RESULTS

Constant area

Equilibrium surface pressure was rapidly attained with bovine albumin, bovine growth hormone, insulin and [5-valine]-angiotensin; after an initial period of 10 min, no further change in surface pressure was observed up to 2 h. With sodium dodecylsulphate and sodium hexadecylsulphate, equilibrium surface pressure was attained only after a period of 30 min. Fig. 1 presents a plot of the increment of surface pressure ($\Delta\pi$) against the number of bovine albumin molecules added to the cholesterol monolayer at 10 dyne/cm initial surface pressure. Similar curves were obtained for the experiments with bovine growth hormone, insulin, [5-valine]-angiotensin, sodium dodecylsulphate and sodium hexadecylsulphate. All the plots were always obtained with the average results of at least three separate experiments.

The molar fraction of amino acid residues (x_R) was calculated at the interface using the formula

$$x_R = \frac{n_p \cdot n_r}{n_p \cdot n_r + n_{ch}}$$

in which n_p is the number of protein molecules at the interface, n_r is the number of

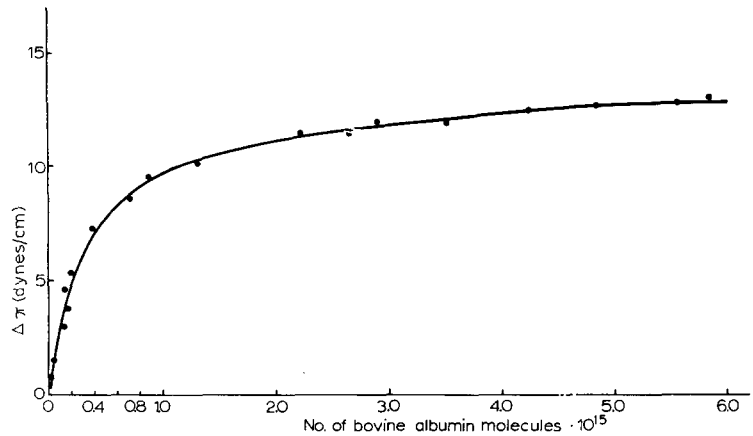

Fig. 1. Increment of surface pressure ($\Delta\pi$) in a cholesterol monolayer at 10 dyne/cm initial surface pressure with the number of bovine albumin molecules added at constant area.

amino acid residues per protein molecule and n_{ch} is the number of cholesterol molecules at the interface. Fig. 2 represents a plot of the total surface pressure against the molar fraction of amino acid residues of bovine albumin at the interface and Fig. 3 the same plot for the rest of the substances studied.

Compression curves

Fig. 4 shows compression curves for mixed monolayers of cholesterol and

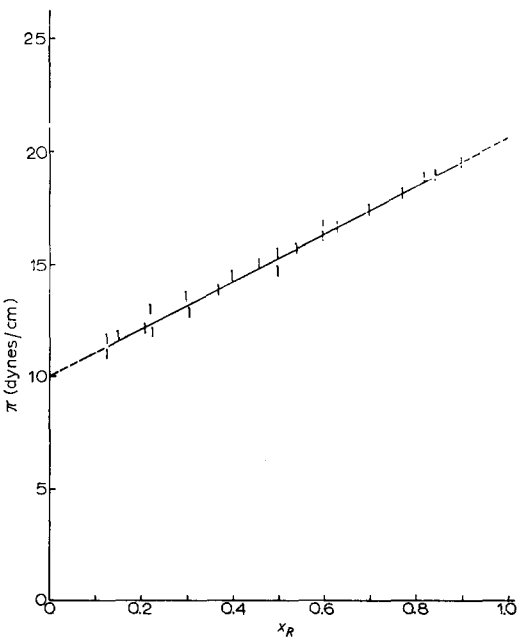

Fig. 2. Total surface pressure (π) as a function of molar fraction of amino acid residues of bovine albumin (x_R) at constant area. Initial surface pressure of cholesterol monolayer: 10 dyne/cm.

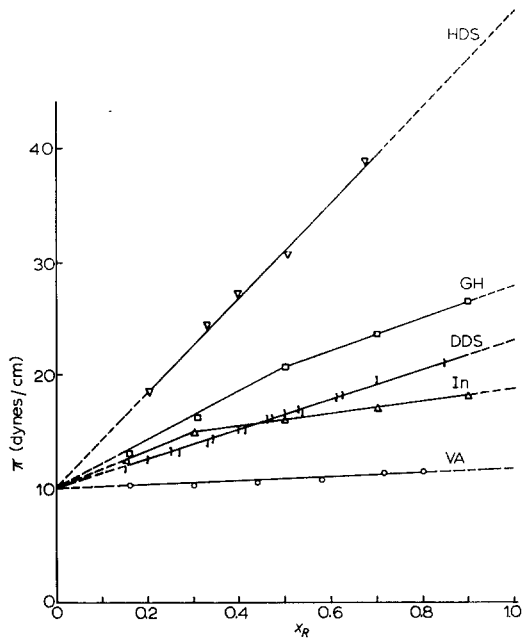

Fig. 3. Total surface pressure (π) as a function of molar fraction (x_R) for: sodium hexadecylsulphate (HDS); bovine growth hormone (GH) (amino acid residues); sodium dodecylsulphate (DDS); insulin (In) (amino acid residues); [5-valine]-angiotensin (VA) amino acid residues. Initial surface pressure of cholesterol monolayer: 10 dyne/cm.

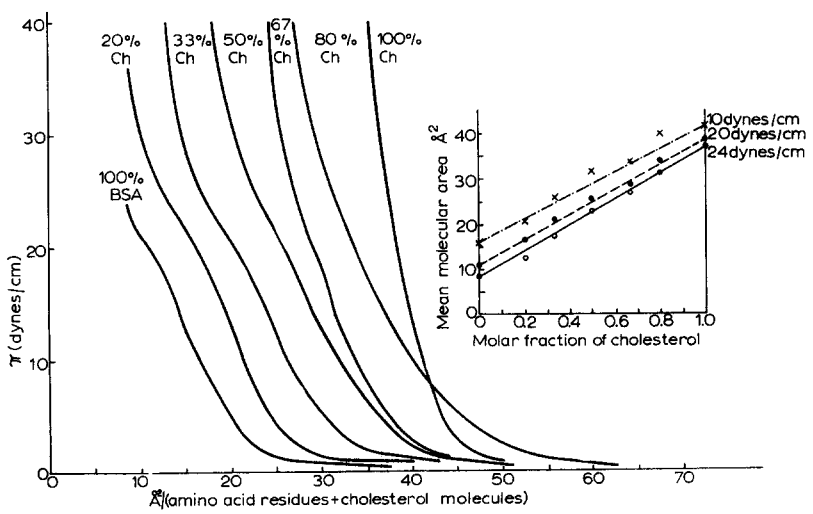

Fig. 4. Compression curves for mixed monolayers of bovine albumin (BSA) and cholesterol (Ch). Amino acid residues *plus* cholesterol molecules at the interface: $1 \cdot 10^{17}$.

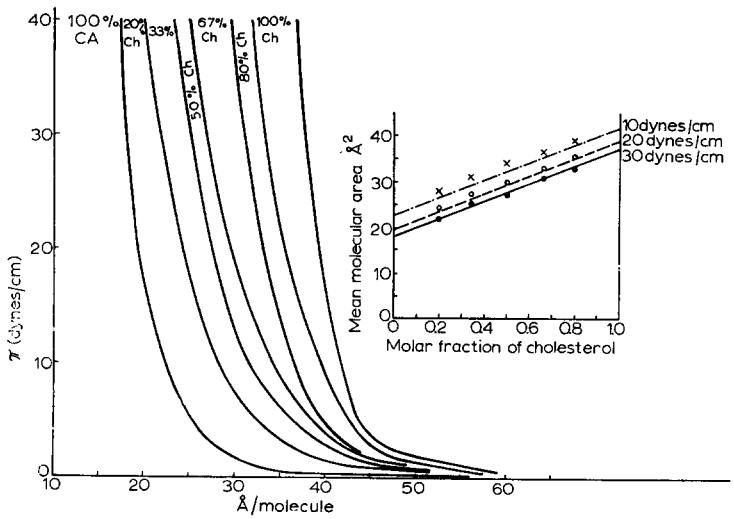

Fig. 5. Compression curves for mixed monolayers of cetyl alcohol (CA) and cholesterol (Ch). Total number of molecules at the interface: $1 \cdot 10^{17}$.

bovine albumin and the abscissa values give the mean molecular area *i.e.* the total area divided by the number of molecules of cholesterol *plus* the number of amino acid residues of bovine albumin at the interface; the inset presents the plot of the mean molecular area against the molar fraction of cholesterol. Fig. 5 shows compression curves for mixed monolayers of cholesterol and cetyl alcohol with the inset showing a plot of the mean molecular area against the molar fraction of cholesterol. All curves are the average of at least three separate experiments.

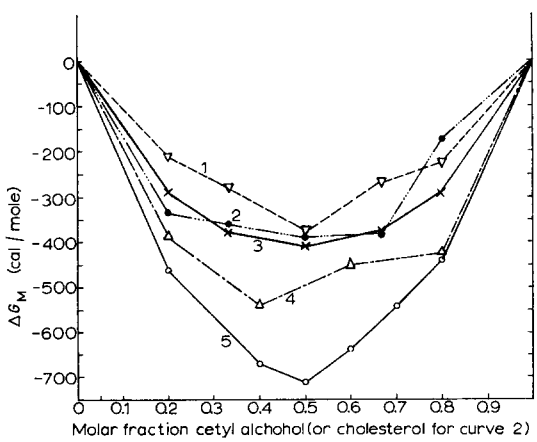

Fig. 6. Free energy of mixing (ΔG_M) as a function of molar fraction. Curve 1 (▽— — —▽), cetyl alcohol–cholesterol. Curve 2 (●—···—●), cholesterol–bovine albumin. Curve 3 (×—×), ideal system, calculated from $\Delta G_M^I = RTN_1 \ln N_1 + RTN_2 \ln N_2$. Curve 4 (△— - —△), cetyl alcohol–$C_{14}H_{29}CH(CH_3)OSO_3Na$, calculated from the data of GOODRICH[11]. Curve 5 (○—○), cetyl alcohol–sodium hexadecylsulphate, calculated from the data of GOODRICH[11].

From these compression curves the excess free energy of mixing[11] was calculated using the equation

$$\Delta G_M^E = \int_0^\pi (\delta_{12} - N_1\delta_1 - N_2\delta_2)d\pi$$

where δ_{12}, δ_1 and δ_2 represent, respectively, the area of the mixed monolayer and the areas of the pure monolayers of cholesterol and bovine albumin, and N_1 and N_2 represent the respective molar fractions. For bovine albumin the molar fraction is that corresponding to the molar fraction of its amino acid residues. The integrals

$$I_{12} = \int_0^\pi \delta_{12}d\pi, \quad I_1 = \int_0^\pi \delta_1 \, d\pi, \quad I_2 = \int_0^\pi \delta_2 \, d\pi$$

were separately evaluated from the corresponding compression curves. The free energy of mixing (ΔG_M) was calculated from the equation

$$\Delta G_M = \Delta G_M^E + \Delta G_M^I$$

in which ΔG_M is the excess free energy of mixing and ΔG_M^I is the free energy of mixing for an ideal system. Fig. 6 represents a plot of ΔG_M against the molar fraction.

DISCUSSION

In classical experiments on lipid–protein interactions at interfaces, injecting the protein solution into the aqueous subphase supporting the lipid monolayer, three processes must be considered: the carrying of protein molecules from the bulk to the surface, the unfolding of molecules, and their interaction with the lipid. The surface pressure, at constant area, after a relatively rapid initial rise, slowly increases until equilibrium surface pressure is attained after a period of not less than 1 h.

The results obtained in the experiments described here show that the cholesterol–protein system reached equilibrium surface pressure at constant area in about 10 min for bovine albumin, bovine growth hormone, insulin and [5-valine]-angiotensin. MURAMATSU AND SOBOTKA[8], using the glass-rod method for spreading bovine albumin at air–water interfaces confirmed the "instantaneous" unfolding of bovine albumin molecules, taking as criterion for the complete unfolding the independence with time of the ratio surface pressure:area. Having in mind the molecular rearrangements that must take place during the formation of the mixed lipid–protein monolayer, the reduction in time necessary to attain equilibrium surface pressure can be interpreted assuming the unfolding of the protein almost completed when the molecules reach the lipid monolayer through the glass rod; in this way the process has been practically reduced to the interaction of the unfolded protein with the lipid molecules at the monolayer.

The curve of Fig. 1, representing the equilibrium surface pressure increment ($\Delta\pi$) as a function of the number of bovine albumin molecules added, has the same general features as that obtained by ELEY AND HEDGE[6] for the same system, injecting the bovine albumin solution under the cholesterol monolayer at 10 dyne/cm initial surface pressure, with an equilibration time of at least 1 h; this confirms the equivalence of the final results in both methods of protein spreading.

The mechanism of penetration of bovine albumin or sodium dodecylsulphate[12]

in cholesterol monolayers has been interpreted[3–5] as due to the interaction of these molecules with cholesterol, forming a stoichiometric complex and to the subsequent dissolution of the molecules in this surface complex. In the experiments here described, the plot of the total surface pressure against the molar fraction of amino acid residues of bovine albumin at the surface (Fig. 2) gave a straight line, within the limits of the experimental error, which can be represented by the equation $\pi_t = \pi^0_{ch} + mx_R$. This equation, being of the same form as that representing the total vapour pressure of a tridimensional binary system as a function of the molar fraction, suggests that the process can be considered as a bidimensional dissolution, with the amino acid residues and the cholesterol molecules being the components of this binary surface solution.

It has been suggested[6] that the hydroxyl groups of the cholesterol monolayer interact with peptide bonds of the protein; since the amino acids in proteins are linked by peptide bonds, the same graphic representation should be obtained on substituting the molar fraction of peptide bonds at the interface for x_R.

Experiments performed with cholesterol and sodium dodecylsulphate or sodium hexadecylsulphate (Fig. 3) have the same linear dependence of the total surface pressure on the molar fraction of sodium dodecylsulphate or sodium hexadecylsulphate at the interface. No peptide bonds being present in these systems, these results seem to favour the hypothesis of a simple mechanism of dissolution between cholesterol and the hydrocarbon chains of amino acid residues in the case of proteins. It must be pointed out that with sodium dodecylsulphate, sodium hexadecylsulphate, bovine albumin and [5-valine]-angiotensin the extrapolated intercept (π^0_{ch}) coincides with the experimental value of the surface pressure for the pure cholesterol monolayer.

For bovine growth hormone and insulin a change in the slope can be clearly seen: having in mind the phenomena of association and dissociation described for these molecules in bulk solution[13] or at interfaces[14] we shall consider only the behaviour of bovine albumin.

A thermodynamic approach to this dissolution process can be performed in terms of the excess thermodynamic properties. GOODRICH[11] studying interactions in mixed monolayers of alkylsulphates and the homologous alcohols concluded, from an experimental evaluation of the excess free energy of mixing, that a release of energy (Van der Waals) occurs if the hydrophobic portions of the two components are sufficiently symmetrical to pack intimately together; the introduction of hindering groups along the paraffin chain greatly reduces the magnitude of the released energy.

Excess (Gibbs) free energy of mixing is defined[15] for a binary system at constant temperature and pressure as $\Delta G^E_M = \Delta G_M - \Delta G^I_M$, the superscript I denoting ideal. The free energy of mixing for an ideal binary system, being a function of the molar fraction, can be expressed by

$$\Delta G^I_M = RTN_1 \ln N_1 + RTN_2 \ln N_2$$

in which N_1 and N_2 are the molar fractions of the components. Substituting and rearranging we obtain

$$\Delta G_M = \Delta G^E_M + RTN_1 \ln N_1 + RTN_2 \ln N_2$$

The first term on the right can be evaluated experimentally; the other terms being independent of the specific chemical nature of the components, can be calculated.

The free energy of mixing for a given binary system is a measure of the tendency of the system to realize the process of dissolution; for an ideal mixture ($\Delta H_M^I = 0$) the "driving force" for the process is entirely of entropic origin

$$\Delta G_M^I = -T\Delta S_M^I = RTN_1 \ln N_1 + RTN_2 \ln N_2$$

The analysis of the curves of Fig. 6 permits some speculation about the spontaneous tendency of the dissolution process; it can be seen that the most negative values for the free energy of mixing are those of the mixed monolayer of cetyl alcohol and sodium hexadecylsulphate, *i.e.* the most symmetrical hydrocarbon chains. When a hindering group is located in the hydrocarbon chain (mixed monolayers of cetyl alcohol and $C_{14}H_{29}CH(CH_3)OSO_3Na$) there is a reduction in the tendency to the dissolution process.

The curve for an ideal binary system calculated by the equation

$$\Delta G_M^I = RTN_1 \ln N_1 + RTN_2 \ln N_2$$

exhibits lesser negative values than both those mentioned before; for this curve $\Delta H_M^I = 0$ (by definition) so that the tendency for the spontaneous process is entropic with no contributions of other origin.

It can be seen that the curve corresponding to the system cholesterol–bovine albumin practically coincides with the ideal-system plot; to a first approximation, this result can be interpreted as if there were no other contribution to mutual dissolution in this system than the entropic. In other words, this result seems to favour the hypothesis of a simple process of dissolution without any specific interaction between the cholesterol and the hydrocarbon chains of amino acid residues in the protein. This could be attributed to the strong asymmetry of the hydrocarbon chains of the species involved in the dissolution process.

Through the theoretical analysis of molecular models[2], it has been postulated that strong interactions involving Van der Waals forces (16 kcal/mole) occur between hydrocarbon chains of phospholipids and cholesterol molecules in biological membranes. The plot of free energy of mixing against molar fraction for the system cetyl alcohol–cholesterol shows only a slight departure from that correspondiug to the ideal system, suggesting again in this case that the entropic contribution is the fundamental "driving force" for the dissolution process.

Considering now the plots of mean molecular area against the molar fraction of cholesterol (insets to Figs. 5 and 6) it can be seen that there is no condensing effect in any case; at surface pressures between 15 and 25 dyne/cm, the experimental points are in a straight line which implies a simple additivity rule of the areas per molecule (or per amino acid residue). Below 15 dyne/cm an expanding effect occurs. The absence of condensing effects can also be interpreted as the result of the non-existence of specific interactions between both components.

ACKNOWLEDGEMENTS

We thank Professor DELLACHA for the gift of bovine growth hormone and

Professor LAMDAN for the synthesis of sodium hexadecylsulphate. Ciba, Basle, kindly supplied [5-valine]-angiotensin, and Elanco, Argentina, provided insulin.

This work was supported in part by a grant from the Consejo Nacional de Investigaciones Cientificas y Tecnicas.

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