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PERTURBATIONS OF RAT INTESTINAL BRUSH BORDER MEMBRANES INDUCED BY Ca^{2+} AND VITAMIN D_3 ARE DETECTED USING STEADY-STATE FLUORESCENCE POLARIZATION AND ALKALINE PHOSPHATASE AS MEMBRANE PROBES

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Abstract—Rat intestinal brush border membranes (BBM) were prepared by discontinuous sucrose gradient centrifugation. This specific fraction contained alkaline phosphatase activity. A dramatic decrease in the specific activity of the BBM-bound alkaline phosphatase was observed at different concentrations of Ca^{2+} and vitamin D_3. Studies of the temperature dependence (5–40°) of alkaline phosphatase reveal a change in energy of activation (slope of the Arrhenius plot) at 23.0 ± 1.1° which was elevated to 27.8 ± 1.3° in BBM treated with Ca^{2+}, while it was depressed to 17.2 ± 1.2° in BBM treated with vitamin D_3. Membrane lipid fluidity, as assessed by the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), was significantly greater in BBM treated with vitamin D_3 and significantly lower in Ca^{2+} treated BBM. A lipid thermotropic transition temperature was observed at 22.2 ± 1.2° which rose to 28.3 ± 1.4° in Ca^{2+} treated BBM and reduced to 17.0 ± 1.2° in vitamin D_3 treated BBM. The biological significance of these results is discussed in terms of modifications in the lipid–protein interactions in BBM, induced by Ca^{2+} and vitamin D_3, and the implications in the physiological intestinal transport processes of calcium.

Cells lining the lumen of the gut may be exposed to various ions and/or lipid soluble substances originating from the diet. Interactions of these compounds with such cells are likely to modify, at least transiently, the physical state of the membrane lipids and subsequently the functions of the exposed membrane-bound enzymes [1, 2]. Calcium ions exert multiple effects on biological membranes, including the regulation of membrane-bound enzyme activities. These actions may be achieved by the interaction of Ca^{2+} with membrane lipids which affect membrane fluidity [3, 4]. The evidence that protein synthesis is not an obligatory step in initiating vitamin D-dependent calcium transport has stimulated an increasing number of studies directed towards assessing alterations in membrane properties that are mediated by vitamin D_3 [5].

In our previous studies we reported on the effect of small amphipathic compounds (e.g. cholesterol, dodecanol, or their glucosides, steroid hormones, charged drugs etc.) on the activity of some membrane-bound enzymes [6–10], suggesting that these compounds can alter the membrane fluidity, causing functional changes in the allosteric properties of integral enzymes. In the present investigation we studied the effect of Ca^{2+} and vitamin D_3 on the activity of rat intestinal brush border integral enzyme alkaline phosphatase. The physiological function of this enzyme may be related to phosphate and calcium transport, while vitamin D_3 may be involved in the regulation of Ca^{2+} and phosphate permeability of intestinal membranes as well as in the regulation of alkaline phosphatase activity.

MATERIALS AND METHODS

Albino male rats of the Wistar strain weighing180–200 g were fasted 18 hr with water *ad libitum* before removing the small intestine. Brush border as well as basolateral membranes were isolated by a method based on that described by Im *et al.* [11]. The membrane pellets were suspended in 50 mM Tris–HCl pH 7.4/0.25 M sucrose and tested immediately or stored frozen at −80°. The final protein concentration was approx. 5 mg/ml. Purity of the preparations was assessed by assay of the marker enzymes alkaline phosphatase [12] and sucrase [13] for the brush border membranes, and $(Na^+ + K^+)$ATPase [14] for the basolateral membranes. The brush border membranes showed the highest specific activities of sucrase and alkaline phosphatase, being about ten to fifteen times higher than those of the homogenate. The specific activity of the $(Na^+ + K^+)$ATPase in the basolateral membranes was about fifteen times greater than that of the homogenate. Protein concentration was estimated by the method of Lowry *et al.* [15].

Alkaline phosphatase activity was determined as described by Hugscher and West [12] in an incubation mixture consisting of, 40 mM ethanolamine–HCl buffer pH 8.0, 100 mM mannitol, 40 mM β-glycerophosphate and approximately 50 μg of brush

border membrane protein. The specific activity of the enzyme was expressed as μmoles P_i per mg protein per min.

Solubilization of brush border membranes (BBM) was performed using 0.1% (w/v) Lubrol-PX at 4° for 12 hr under magnetic stirring, in a medium consisting of 200 mM mannitol, 40 mM ethanolamine–HCl pH 8.0 and 0.36 mg of BBM protein per ml, as previously described [10, 14]. Preincubations of BBM with different concentrations of $CaCl_2$ specified in Fig. 1 were carried out for 2 hr at 37° in an incubation mixture of 100 mM mannitol, 40 mM ethanolamine–HCl pH 8.0 containing 0.120 mg BBM protein in a final volume of 0.5 ml, with continuous magnetic stirring with a magnetic bar. Alkaline phosphatase activity was estimated in portions of the membranous suspensions.

The effect of vitamin D_3 on the BBM-bound alkaline phosphatase activity was studied using "vitamin D_3 saturated bovine serum albumin solutions", due to limitations inherent in the insolubility of vitamin D_3 in water. The preparation of these solutions were made up as follows: vitamin D_3 (5.8 mg) (Serva Feinbiochemica GMBH and Co., F.R.G.) were added to 5 ml of 100 mM mannitol/40 mM ethanolamine–HCl pH 8.0 buffer solution and then sonicated under nitrogen (free of oxygen) atmosphere for 30 min. [^{3}H]vitamin D_3 (sp. radioactivity 25 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, U.K., and was brought to the desired spec. radioactivity by dilution with vitamin D_3. Bovine Serum Albumin (BSA) (975 mg) (Serva) was added to the sonicated vitamin D_3 solution and the mixture incubated for 16 hr in a water bath at 37° with continuous magnetic stirring in the presence of 100 U/ml penicillin-G and 100 μg/ml streptomycin sulfate (vitamin D_3:albumin, molar ratio 1:1). Centrifugation at 32,000 *g* for 30 min in a Type Ti-75 Beckman rotor removed vitamin D_3 unbound to BSA. The final supernatant which contained vitamin D_3 bound to BSA was named "vitamin D_3 saturated BSA solution". The 100% BSA relatively saturated vitamin D_3 solutions were usually 2.45 mM corresponding to $9.28 \cdot 10^5$ cpm. Its concentration was measured from the (known) spec. radioactivity of [^{3}H]vitamin D_3. Preincubations of BBM (0.12 mg) with different amounts of "vitamin D_3 saturated BSA solution" (0.02–0.14 ml) were carried out for 2 hr at 37° under continuous magnetic stirring in 0.5 ml final volume of 100 mM mannitol/40 mM ethanolamine·HCl, pH 8.0. Control experiments indicated that BSA, penicillin-G and streptomycin did not affect the enzyme activity and that they did not interfere with the enzymic determinations. The influence of Ca^{2+} ions and vitamin D_3 on the temperature dependence of BBM-bound alkaline phosphatase was studied by estimation of the enzyme activity at temperatures of 5–42° at 3–4° intervals after preincubation of the membranes approximately 0.13 mg, with 10 mM $CaCl_2$ and 0.12 ml of vitamin D_3 saturated BSA solutions.

Membranes were treated with the lipid-soluble fluorophor 1,6-diphenyl-1,3,5-hexatriene (DPH) (Sigma Chem. Co., St. Louis), and steady-state fluorescence polarization measurements were made in an AMINCO SPF-500 Spectrofluorometer with polarization accessory for fluorescence polarization measurements, as described by Shinitzky and Barenholz [16]. The polarization of fluorescence was expressed as the fluorescence anisotropy, r, and the anisotropy parameter $[(r_0/r) - 1]^{-1}$ was calculated using a value of $r_0 = 0.365$ for DPH [17]. The anisotropy parameter varies directly with the apparent rotational relaxation time of the probe [18] and thus inversely with the fluidity. The fluorescence anisotrophy, r, was calculated according to the equation $r = I_{VV} - I_{VH} + 2I_{VV} + 2I_{VH}$, where I_{VV} and I_{VH} are the intensities of the omitted light oriented, respectively, parallel and perpendicular to the plane of the exciting beam. Light scattering corrections were always <5% of the total signal and did not differ significantly in various preparations. Measurements were made at least in triplicate. The influence of Ca^{2+} ions and vitamin D_3 on BBM fluidity was studied

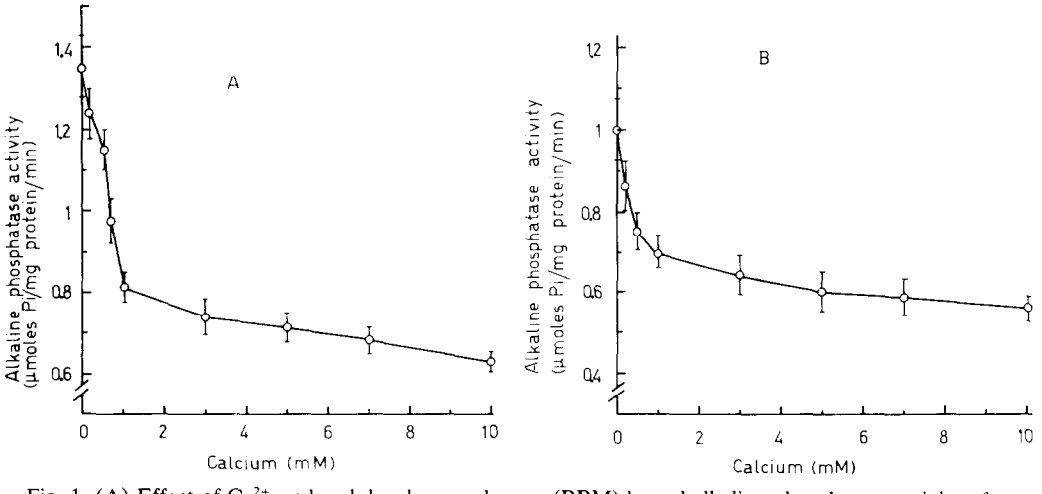

Fig. 1. (A) Effect of Ca^{2+} on brush border membranes (BBM) bound alkaline phosphatase activity of rat intestine. (B) Effect of Ca^{2+} on BBM-bound alkaline phosphatase activity in Lubrol-PX solubilized membranes. Values are the means ± S.D. on three different experiments.

by preincubation of BBM (0.3 mg/ml) with 10 mM $CaCl_2$ and 0.12 ml of vitamin D_3 saturated BSA solution for 2 hr at 37° in a buffer solution 100 mM mannitol/40 mM ethanolamine·HCl pH 8.0 under continuous magnetic stirring. The temperature dependence of $[(r_0/r) - 1]^{-1}$ was determined over the range of 4–40°. Membranes (approx. 50 μg) were warmed initially to 40° and the fluorescence polarization was estimated every 1–2° as the suspension cooled slowly to 4°. Plots of log $[(r_0/r) - 1]^{-1}$ versus $1/T$ were constructed to detect thermotropic transitions as described by Brasitus [19].

RESULTS AND DISCUSSION

The curves representing the changes of alkaline phosphatase activity at different concentrations of Ca^{2+} have biphasic character (Fig. 1A). Up to the Ca^{2+} concentration of 1 mM a sharp decrease of the enzyme activity was observed (approx. 45%) which progressively slows down (approx. 55% of its original value) at a concentration of 10 mM. The results obtained from the effect of Ca^{2+} ions on the activity of alkaline phosphatase solubilized by Lubrol-PX (Fig. 1B), i.e. decrease of the enzyme activity, reveal that Ca^{2+} ions exert their action not only by changing the physical state of the membrane, but also directly on the protein molecule.

"Vitamin D_3 saturated BSA solutions" exhibit profound effects on alkaline phosphatase activity, i.e. a linear decrease up to 50% down of its original value was observed (Fig. 2). The lack of effect of vitamin D_3 on the Lubrol-PX solubilized alkaline phosphatase activity reveals that it does not affect the enzyme directly, but the evoked functional changes in the membrane-bound enzyme are probably mediated through changes in the physical state of the membrane lipids. Indeed we already have an indication that the amphipathic dodecyl glucoside and charged drugs can affect the conformational flexibility of an integral protein by perturbing the physical state of the membrane lipids as the membrane-bound enzymes 5'-nucleotidase and acetylcholinesterase, but not the solubilized ones, are activated by dodecyl glucoside and charged drugs over a concentration range where membrane fluidity is increasing [10, 14].

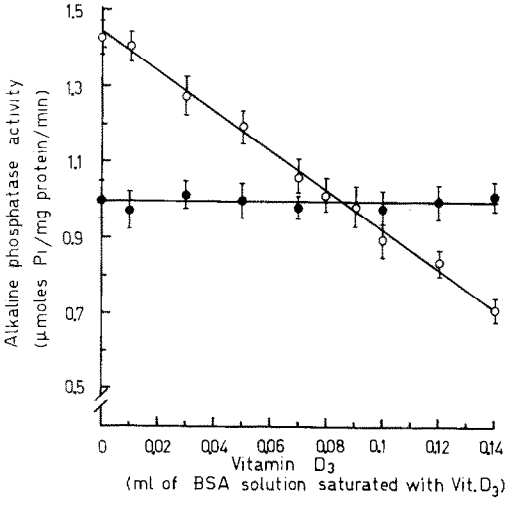

Fig. 2. Effect of "vitamin D_3 saturated bovine serum albumin solutions" on BBM-bound alkaline phosphatase activity (○—○) and on alkaline phosphatase activity in Lubrol-PX solubilized membranes (●—●). Values are the means ± S.D. of three different experiments.

The temperature dependence and the Arrhenius activation energy of brush border bound alkaline phosphatase activity in the absence and in the presence of Ca^{2+} and vitamin D_3 is shown in Fig. 3 (A, B, C) and in Table 1. Arrhenius plots of the alkaline phosphatase showed a transition temperature at 23 ± 1.1°. The elevation of the transition temperature to 27.8 ± 1.3° and the depression to 17.2 ± 1.2° in the presence of Ca^{2+} ions and vitamin D_3, respectively, may be due to modulations in the physical state of the membrane lipids. Following preincubation of BBM with Ca^{2+} and vitamin D_3, added simultaneously the transition temperature was restored at the level of untreated control membranes (23.4 ± 0.9°) (Fig. 3C). Presumably, the lipid microenvironment around the particular integral enzyme and the nature of the specific lipid–protein interactions influence the transition temperature [20]. These results are indicative of microscopic changes in the relationship between membrane-bound alkaline phosphatase and its associated lipids, induced by Ca^{2+} ions and vitamin D_3.

There is considerable evidence that Ca^{2+} ions can interact directly with phospholipids arranged in monolayers or model bilayers and thereby restrict the motional freedom or "lipid fluidity" of the arrays [21, 22]. Ca^{2+} appears to act similarly on a number of biological membranes [3, 23]. Since vitamin D_3 is required for the optimal absorption of Ca^{2+}, it is possible for vitamin D_3 to influence directly the molecular organization of brush border membranes, thus regulating the absorption of Ca^{2+} through the modulation of microvillus membrane fluidity. In favour of this possibility are the findings that the presence of detergents enhanced the rate of Ca^{2+} transport by rachitic intestine *in vitro* [24], as did filipin, a non-ionophoric polyene antibiotic [25]. The site of action of both types of compounds appears to be on the entrance of Ca^{2+} into the intestinal cell, i.e. on the brush border membrane, and in a sense these compounds mimic one of the actions of vitamin D_3. On the other hand, it has been demonstrated, *in vivo*, that 1,25-dihydroxyvitamin D increased the fluidity of intestinal brush border membranes by increasing the relative incorporation of polyunsaturated fatty acids into phospholipids and by increasing the amount of phosphatidyl-choline in the membrane. This increase in fluidity has been proposed to open cryptic Ca^{2+} channels in the membrane, stimulating Ca^{2+} flow into the cell [5, 26].

The effects of temperature on the anisotropy parameter, $[(r_0/r) - 1]^{-1}$, of DPH in BBM membranes are illustrated by representative Arrhenius plots in Fig. 4. The increase of the temperature produces concomitant diminution in the $[(r_0/r) - 1]^{-1}$ values, which means an increase of the fluidity of the membrane. However, the evolution of the fluidity was not linear; a thermotropic transition temperature was indeed observed at 22.2 ± 1.2° in untreated BBM, which separates two domains where the evol-

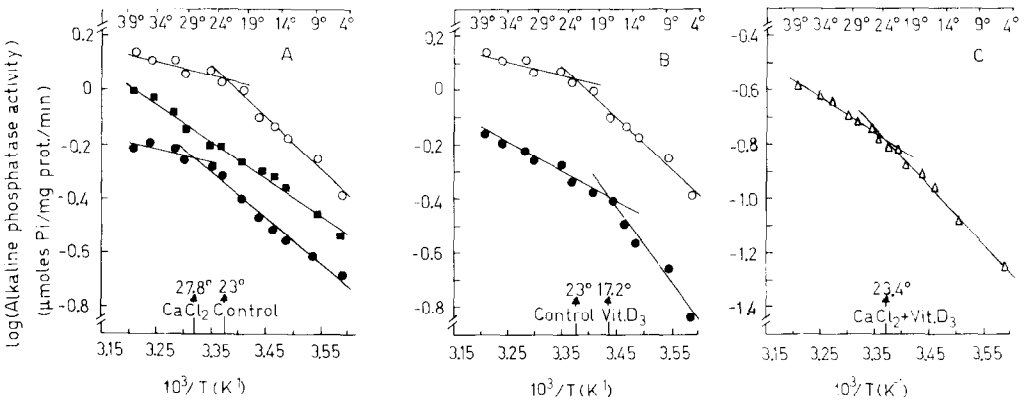

Fig. 3. Effect of temperature on the activity of BBM-bound alkaline phosphatase activity in: (A) control (○—○), Ca^{2+} treated BBM (●—●) and Lubrol-PX solubilized BBM (■—■); (B) "vitamin D_3 saturated bovine serum albumin solutions"-treated BBM (●—●); and (C) Ca^{2+} plus "vitamin D_3 saturated bovine serum albumin solution"-treated BBM (△—△). Each point represents the average value of duplicate determinations from a typical experiment which has been repeated three times. The straight lines were fitted by the method of least squares.

Table 1. Effect of calcium ions and vitamin D_3 on the transition temperature and Arrhenius activation energies of intestinal brush border membrane (BBM)-bound alkaline phosphatase activity

Preparation	Transition temperature (°)	Activation energy (Ea) (kJ/mol)	
		Above break	Below break
Control (untreated) BBM	23.0 ± 1.1	9.5 ± 0.5	37.1 ± 3.8
Lubrol-PX solubilized BBM		26.0 ± 2.7	
Calcium treated BBM	27.8 ± 1.3*	9.9 ± 0.7	35.8 ± 4.2
Vitamin D_3 treated BBM	17.2 ± 1.2*	19.5 ± 1.6*	44.3 ± 4.8*
Calcium plus vit. D_3 treated BBM	23.4 ± 0.9	24.4 ± 2.0*	39.3 ± 4.6

Values represent means ± S.D. of three independent experiments.
* Statistically significant compared with control ($P < 0.01$).

ution of the fluidity is directly proportional to the variation of the temperature. Treatment of BBM with Ca^{2+} exhibit a statistically significant ($P < 0.01$) increase in $[(r_0/r) - 1]^{-1}$ compared to untreated (control) BBM. It must be realised that the corresponding decrease in fluidity is constant over the full range of temperatures studied. Furthermore, the thermotropic transition temperature was elevated to 28.3 ± 1.4°. The corresponding data for BBM treated with vitamin D_3 are likewise best described by two straight lines at the temperature range studied and the $[(r_0/r) - 1]^{-1}$ considerably decreased compared to untreated BBM, indicating an increase in membrane fluidity. In addition, the graph was considerably shifted towards lower temperatures of 17.0 ± 1.2° compared to the corresponding plot for untreated BBM. No statistically significant differences ($P > 0.05$) in membrane fluidity appear to exist between BBM treated with Ca^{2+} plus vitamin D_3 and untreated (control) BBM, and the thermotropic transition temperature was shifted back to the control value (Table 2).

The rigidizing effect of 10 mM Ca^{2+} was readily reversed by adding the divalent cation-chelating agent EGTA (25 mM). Further, a premixed solution of $CaCl_2$ and EGTA did not increase the $[(r_0/r) - 1]^{-1}$ of the labeled BBM, and 10 mM NaCl

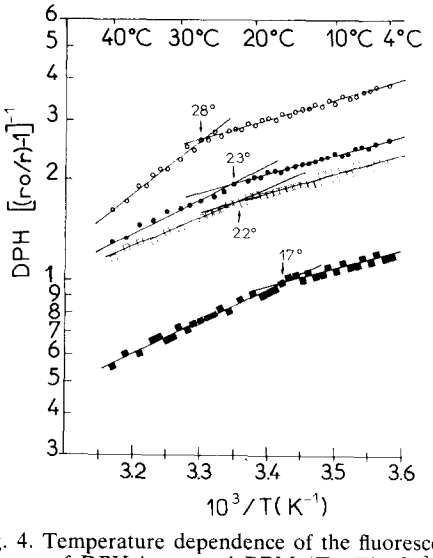

Fig. 4. Temperature dependence of the fluorescence anisotropy of DPH in control BBM (□—□), Ca^{2+} treated BBM (○—○), vitamin D_3 treated BBM (■—■) and Ca^{2+} plus vitamin D_3 treated BBM (●—●). The ordinate is the fluorescence anisotropy and the abscissa is the reciprocal of the absolute temperature. Experimental details are given in the text. This experiment is representative of three that were performed.

Table 2. Effect of calcium ions and vitamin D_3 on the fluorescence anisotropy of DPH in intestinal brush border membranes (BBM)

Preparation	Diphenylhexatriene (DPH) $[(r_0/r) - 1]^{-1}$ at 25°	Thermotropic transition temperature (°)
Control (untreated) BBM	1.66 ± 0.06	22.2 ± 1.2
Calcium treated BBM	2.71 ± 0.09*	28.3 ± 1.4*
Vitamin D_3 treated BBM	0.84 ± 0.05*	17.0 ± 1.2*
Calcium plus vit. D_3 treated BBM	1.81 ± 0.07	23.1 ± 1.1

Values represent means ± S.D. of three independent experiments.
Thermotropic transition temperatures were determined from Arrhenius plots illustrated in Fig. 4.
* Statistically significant compared with control ($P < 0.01$).

had no effect. These data suggest that the Ca^{2+}-induced rigidization of the BBM is due to the reversible binding of Ca^{2+} to discrete membrane sites and is not simply the result of an increase in ionic strength. Attempts were made to examine the temperature dependence of DPH $[(r_0/r) - 1]^{-1}$ in BBM treated with 0.1% (w/v) Lubrol-PX. These experiments were not feasible, however, because the detergent removed approx. 90% of the membrane probe and solubilized it in the ambient medium.

What is particularly significant in our present results is that the observed transition temperatures for alkaline phosphatase (Table 1) correspond closely to the thermotropic transitions of lipid-phase separations (Table 2). Moreover, solubilization of the alkaline phosphatase with the non-anionic detergent Lubrol-PX, was shown to remove the transition temperature in an Arrhenius plot (Fig. 3A). It seems reasonable, therefore, to suggest that alkaline phosphatase activity is integral membrane activity mediated by protein which exists in lipid microenvironment which is similar in composition to the bulk lipid or is influenced readily and similarly by the thermotropic changes of the bulk lipid.

The present data suggest that vitamin D_3 can modify the physical state of BBM lipids thus causing changes in the transport function of these membranes, probably by altering the conformation of enzymes or transport proteins. Since vitamin D_3 is required for the optimal absorption of Ca^{2+}, it can manifest changes in the Ca^{2+} transport either in a typical steroid fashion (i.e. by the induction of proteins directly involved in the Ca^{2+} transport process, through interaction with the nuclear genetic apparatus) or by influencing directly the molecular organization of brush border membranes.

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REFERENCES

1. I. Nemere and A. N. Norman, *Biochim. biophys. Acta* **694**, 307 (1982).
2. C. K. Hunter, L. L. Treanor, J. P. Gray, S. A. Halter and A. Hoyumpa, *Biochim. biophys. Acta* **732**, 256 (1983).
3. L. M. Gordon, A. D. Whetton, S. Rawal, J. A. Esgate and M. D. Houslay, *Biochim. biophys. Acta* **729**, 104 (1983).
4. J. Storch and D. Schachter, *Biochim. biophys. Acta* **812**, 473 (1985).
5. T. Matsumoto, O. Fontaine and H. Rasmussen, *J. biol. Chem.* **256**, 3354 (1981).
6. S. G. A. Alivisatos, G. Deliconstantinos, A. Papaphilis and G. Theodosiadis, *Biochim. biophys. Acta* **643**, 642 (1981).
7. S. G. A. Alivisatos, G. Deliconstantinos and G. Theodosiadis, *Biochim. biophys. Acta* **643**, 650 (1981).
8. G. Deliconstantinos, K. Anastasopoulou and P. Karayiannakos, *Biochem. Pharmac.* **32**, 1309 (1983).
9. G. Deliconstantinos, *Biochem. J.* **222**, 825 (1984).
10. G. Deliconstantinos and S. Tsakiris, *Biochem. J.* **229**, 81 (1985).
11. W. B. Im, D. W. Misch, D. W. Powell and R. G. Faust, *Biochem. Pharmac.* **29**, 2307 (1980).
12. G. Hübscher and G. P. West, *Nature, Lond.* **205**, 799 (1965).
13. A. Dalhquist, *Analyt. Biochem.* **7**, 18 (1964).
14. G. Deliconstantinos and G. Ramantanis, *Biochem. J.* **212**, 445 (1983).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. M. Shinitzky and Y. Barenholz, *Biochim. biophys. Acta* **515**, 367 (1978).
17. D. Schachter and M. Shinitzky, *J. clin. Invest.* **59**, 536 (1977).
18. T. A. Brasitus, K. Yeh, P. R. Holt and P. Schachter, *Biochim. biophys. Acta* **778**, 341 (1984).
19. T. A. Brasitus, *Biochim. biophys. Acta* **728**, 20 (1983).
20. E. J. McMurchie and J. K. Raison, *Biochim. biophys. Acta* **554**, 364 (1979).
21. A. D. Albert, A. Sen and P. L. Yeagle, *Biochim. biophys. Acta* **771**, 28 (1984).
22. D. P. Gregory and L. Ginsberg, *Biochim. biophys. Acta* **769**, 238 (1984).
23. C. J. Livingstone and D. Schachter, *Biochemistry* **19**, 4823 (1980).
24. D. P. A. Webling and E. S. Holdsworth, *Biochem. J.* **97**, 408 (1965).
25. A. W. Norman, J. A. Putkey and I. Nemere, *Fedn. Proc.* **41**, 78 (1982).
26. O. Fontaine, T. Matsumoto, D. B. P. Goodman and H. Rasmussen, *Proc. natn. Acad. Sci. U.S.A.* **78**, 1751 (1981).