Calcium-Binding Properties and ATPase Activities of Rat Liver Plasma Membranes

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ABSTRACT   Plasma membranes from rat liver purified according to the procedure of Neville bind calcium ions by a concentration-dependent, saturable process with at least two classes of binding sites. The higher affinity sites bind 45 nmol calcium/mg membrane protein with a K_D of 3 μM. Adrenalectomy increases the number of the higher affinity sites and the corresponding K_D. Plasma membranes exhibit a (Na^+-K^+)-independent-Mg^{2+}-ATPase activity which is not activated by calcium between 0.1 μM and 10 mM $CaCl_2$. Calcium can, with less efficiency, substitute for magnesium as a cofactor for the (Na^+-K^+)-independent ATPase. Both Mg^{2+}- and Ca^{2+}-ATPase activities are identical with respect to pH dependence, nucleotide specificity and sensitivity to inhibitors. But when calcium is substituted for magnesium, there is no detectable membrane phosphorylation from $[\gamma-^{32}P]$ ATP as it is found in the presence of magnesium. The existence of high affinity binding sites for calcium in liver plasma membranes is compatible with a regulatory role of this ion in membrane enzymic mechanisms or in hormone actions. Plasma membranes obtained by the procedure of Neville are devoid of any Ca^{2+}-activated-Mg^{2+}-ATPase activity indicating the absence of the classical energy-dependent calcium ion transport. These results would suggest that the overall calcium-extruding activity of the liver cell is mediated by a mechanism involving no direct ATP hydrolysis at the membrane level.

INTRODUCTION

Calcium is known to play an important role in the control of enzyme and metabolic activities of most cells and is also implicated as an intermediate in the action of several hormones (Rasmussen, 1970). Within the liver, calcium ions are involved in the regulation of the activity of soluble enzymes of the

glycogenolytic pathway (Bygrave, 1967) as well as of membrane-bound enzymes (Pohl *et al.*, 1971; Leray *et al.*, 1973). Therefore, the intracellular content and distribution of calcium would be of importance for the control of hepatic metabolism.

The plasma calcium and the intracellular calcium content are modulated, *via* carrier systems, by nutritional and hormonal factors in such a way that the intracellular calcium is maintained at a lower concentration than in the extracellular pool. In the liver, the activity of calcium in the cytosol is unknown, but data concerning the distribution pattern of calcium reveal that most of it is bound to subcellular structures (Thiers and Vallee, 1957). It has been reported from studies in perfused liver or with liver slices that a rapid exchange phenomenon between calcium of the external medium and endogenous pools occurred across the plasma membrane (Judah and Ahmed, 1963; Wallach *et al.*, 1966; Van Rossum, 1970). Calcium influx would not be closely linked to a metabolic source of energy but it could be mediated by regulated binding processes (Wallach *et al.*, 1966). On the other hand, Van Rossum (1970) found that liver slices are capable of an energy-dependent net extrusion of calcium, independent of a concentration gradient of sodium across the cell membrane, and he postulated that this activity is located at the plasma membrane level. Both influx and efflux are susceptible to change with hormonal factors (Wallach *et al.*, 1971; Chausmer *et al.*, 1972). In this respect, it has been shown that glucagon and cyclic AMP could affect calcium fluxes in perfused liver and were able to mobilize calcium from an endogenous pool (Friedmann and Park, 1968; Friedmann and Rasmussen, 1970).

The above conclusions were based on indirect evidence drawn from studies of a complex system. A classic model of calcium transport across a membrane is provided by the red cell ghost. Extensive studies by Schatzmann and Vincenzi (1969), Schatzmann and Rossi (1971) have shown that calcium can be extruded against an electrochemical gradient and that the energy is derived from the hydrolysis of ATP *via* a Ca^{2+}-activated-Mg^{2+}-ATPase located in the membrane. With this model in mind, we studied the calcium binding properties and ATPase activities of the plasma membranes isolated from rat liver. Because cyclic AMP affects calcium fluxes in the liver (Friedmann and Park, 1968; Friedmann and Rasmussen, 1970; Wallach *et al.*, 1966) and because cortisol exerts a "permissive" effect on cyclic adenosine $3', 5'$-monophosphate (cyclic AMP) action (Exton *et al.*, 1972) and modifies various membrane properties, we also studied the effect of adrenalectomy upon calcium binding to and ATPase activities of isolated plasma membranes.

Our results show that plasma membranes do bind calcium ions with high affinity, but do not possess a Ca^{2+}-activated-Mg^{2+}-ATPase activity. Adrenalectomy induces a diminution of the affinity for calcium binding.

MATERIALS AND METHODS

Materials

[45]Calcium chloride (10–25 mCi/mg calcium) was purchased from the Radiochemical Centre (Amersham, England). [γ-^{32}P] ATP (2.5 Ci/mmol) was obtained from the CEA (Saclay, France). EGTA and nucleotides (sodium salts) were from Sigma Chemical Co., St. Louis, Mo. When sodium was omitted in the assay, ATP was used as the Tris salt: it was chromatographed on a Dowex 50 W-X 8 (in the H^+ form) column (Bio-Rad Laboratories, Richmond, Calif.) and adjusted to the appropriate pH with Tris. L-histidine (free base) was from Calbiochem, San Diego, Calif. X-537 A and Salyrgan were gifts from Hoffmann-La Roche, (Nutley, N. J.) and from Hoechst (Frankfurt/Main, Germany), respectively. We also used Mersalyl Sodium from K & K Laboratories, Inc., Plainview, N. Y. Ruthenium red was from K & K Laboratories, Inc. It was used after purification according to Luft (1971). G-strophantin (ouabain) and all other reagents were from Merck Chemical Div., Merck & Co., Inc., Rahway, N. J. Bovine serum albumin (fraction V) was from Armour Pharmaceutical Company. Quartz distilled water has been used throughout.

Animals

Rats were female Wistar, about 100 g body weight. They were killed by decapitation and bled before excision of the liver. When indicated, the rats were bilaterally adrenalectomized and maintained thereafter on 0.15 M NaCl as drinking water for 5–7 days.

Preparation of Liver Microsomes and Plasma Membranes

Livers were homogenized at 4°C in 3 vol of 0.25 M sucrose containing 50 mM Tris pH 7.6, 25 mM KCl, and 5 mM $MgCl_2$. The homogenate was centrifuged at 18,000 g for 15 min. Then, the supernatant was centrifuged at 105,000 g for 2 h. The microsomal pellet was suspended in 33 mM Tris or histidine buffer, pH 7.2 and used immediately thereafter.

Plasma membranes were prepared according to the procedure devised by Neville (1968) up to step 11. This method is based upon the lysis of cells in an hypotonic medium (1 mM $NaHCO_3$) and the isolation of the membranes by floating on a nonlinear sucrose gradient. We have verified that this preparation was preferentially enriched in plasma membrane marker enzymes (adenylate cyclase, 5'-nucleotidase, ATPases) and contained very little glucose-6-phosphatase activity which is specific for the endoplasmic reticulum. Membrane preparations were suspended either in 1 mM $NaHCO_3$ or in 10 mM histidine, pH 7.4 and stored up to 6 wk in liquid nitrogen without any loss of the various activities tested. The purity of the membrane preparations was checked by electron microscopy. The membrane preparations were composed mainly of membrane sheets with bile canaliculi structures and tight junctions which are identified as originating from the plasma membranes of parenchymal liver cells (Neville, 1960). No organelles, and in particular no microsomes nor mitochondria, were found. In addition, our preparations contained vesicles. Whether membrane inversions occurred during the preparation of the membrane fragments

is not known. Proteins were determined by the Lowry method (1951) using bovine serum albumin as the standard.

Assay for Calcium Binding

Calcium binding was measured by equilibrium dialysis (Chevallier and Butow, 1971). Optimal conditions with regard to the binding assay (equilibration time, protein concentration, pH, and nature of the buffer, ratio of internal to external volume) were determined first. In our standard assay, 30–300 μg of protein in 0.25 ml of 10 mM histidine (pH 7.2) were dialyzed for 18 h at 4°C, with constant shaking, against 200 ml of the same buffer to which were added $^{45}CaCl_2$ (4 μCi) and amounts of $CaCl_2$ sufficient to vary the final concentration of calcium from 1.25 μM to 1 mM (specific activity ranged from 15,000 to 20 cpm/nmol). In order to minimize the dilution of $^{45}CaCl_2$ by calcium from endogenous sources, we found it necessary to use a high volume ratio between the dialysis medium and the bag tubing (800:1). At the end of the dialysis, samples were removed from the bags and the external medium, dissolved in 10 ml of either Instagel (Packard) or Unisolve (Koch Light Laboratories), and counted in a Nuclear Chicago Mark I Scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Controls without membrane protein were carried out in order to verify that equilibrium was reached. Under these conditions, the binding of calcium was proportional to the protein concentration up to 1.4 mg/ml.

Determination of ATPase Activities

The conditions described by Emmelot and Bos (1966) were routinely used: (*a*) "Total ATPase:" Mg^{2+}ATPase (ATP phosphohydrolase, EC 3.6.1.4) plus (Na^+-K^+-Mg^{2+}) ATPase (Na^+ plus K^+-activated phosphohydrolase): The medium contained 66 mM NaCl, 33 mM KCl, 5 mM $MgCl_2$, 25 mM Tris or histidine pH 7.4, 5 mM ATP pH 7.4 (sodium or Tris salt), with or without 10^6 cpm of [γ-^{32}P] ATP, and 40 μg protein in a total volume of 1 ml.

(*b*) Mg^{2+}-ATPase: The same conditions were used except that 1 mM ouabain was added. The activity was also tested in the total absence of sodium (and therefore in the absence of ouabain); in this case, the medium was 100 mM KCl, 5 mM $MgCl_2$, 25 mM Tris, or histidine pH 7.4, 5 mM ATP pH 7.4 (Tris salt), with or without [γ-^{32}P] ATP. The activities were similar under both conditions.

(*c*) (Na^+-K^+-Mg^{2+}) ATPase was estimated by subtracting the activity found under the conditions (*b*) from the activity found in (*a*).

(*d*) Ca^{2+}-activated-Mg^{2+}-ATPase: $CaCl_2$ was added, in concentrations varying from 0.1 μM to 10 mM as indicated in the legends to figures, to the media used in (*a*) and in (*b*). In these experiments, 0.5 mM EGTA was added in the control assay.

(*e*) Ca^{2+}-ATPase was estimated by using $CaCl_2$ instead of $MgCl_2$, under the conditions (*a*) and (*b*).

In all cases, the reaction, initiated by the addition of ATP, was run at 37°C for 10 min, and was stopped by adding ice-cold trichloroacetic acid (10% final concentration). Protein was removed by centrifugation and inorganic phosphate was estimated in the supernatant either by the method of Fiske and Subbarow (1925) or by counting the radioactivity isolated as follows: To 1 ml of the supernatant were added 0.33 ml

7.5% ammonium molybdate and 1 ml isobutanol. After extraction, an aliquot of the organic phase was counted in 10 ml Unisolve. Similar results were obtained by both methods. Appropriate blanks were performed in all cases. Results are expressed in micromoles of P_i liberated in 10 min/mg protein. Each enzymatic assay was done in duplicate.

Membrane Phosphorylation Assay

The incubation medium consisted of 50 mM Tris-HCl buffer pH 7.4 containing 3 mM NaH_2PO_4 and the specified concentrations of NaCl, KCl, $MgCl_2$, $CaCl_2$, and [γ-^{32}P] ATP. When the reaction was performed in the absence of NaCl, NaH_2PO_4 was omitted and ATP was used as the Tris-salt (pH 7.4). The total volume was 0.5 ml. The reaction, initiated by the addition of 0.3 mg membrane protein, was performed either at 4 or 37°C for the indicated time periods. At the end of the incubation, 1 ml of ice-cold 10% trichloroacetic acid containing 1 mM ATP and 1 mM KH_2PO_4 was added. The precipitated proteins were washed twice with the same mixture. It was controlled that the [^{32}P] content of the last supernatant wash solution reached negligible levels. Proteins were then dissolved in 1 N NaOH. Aliquots were counted in 10 ml Unisolve. The phosphorylated groups of membrane proteins were analyzed according to Blat and Harel (1969) on the total fraction precipitated by trichloroacetic acid: treatment by buffered hydroxylamine, which liberated acyl-phosphate groups (Hokin et al., 1965), was followed by treatment with perchloric acid at 95°C, which hydrolyzed phosphate groups from phosphohistidine and phospholysine. Samples of the two fractions liberated were counted for radioactivity. The radioactivity remaining in the proteins after these treatments was also counted ("acid-resistant phosphate"). Blanks were performed using bovine serum albumin.

Determination of Calcium Levels

Plasma and bile were diluted with the following mixture (1:21 vol/vol): 17 mM NaCl, 2.7 mM KCl, 135 mM $LaCl_3$, 104 mM HCl. Livers (0.5-g fragments) were digested in 0.3 ml concentrated H_2SO_4 and 0.5 ml concentrated $HClO_4$; the residues were adjusted to 3 ml with quartz bidistilled water. Calcium was determined with an atomic absorption flame photometer (Perkin Elmer 303, Perkin-Elmer Corp. Instrument Div., Norwalk, Conn.) using Titrisol Merck (ref 9976) as the standard.

RESULTS

Calcium Content of Liver

It is reported that the concentration of ionized calcium of animal cells is in the range of 10 nM to 10 μM in the cytosol whereas extracellular ionized calcium is around 1 mM (Rasmussen, 1970). We report here the concentrations of calcium of the whole liver, plasma, and bile from normal and adrenalectomized rats as determined by atomic absorption spectrophotometry (Table I). There are good correlations between our results and those reported in the literature (Thiers and Vallee, 1957; Peng and Gitelman, 1972). Using data from the literature for vascular, biliary, and extracellular spaces (Brauer,

TABLE I

TOTAL CALCIUM CONTENT OF LIVER, PLASMA, AND BILE
FROM NORMAL AND ADRENALECTOMIZED RATS

	Normal rats	Adrenalectomized rats
Total liver $\mu mol/g$ wet wt	0.498±0.065 (4)	0.498±0.015 (4)
Plasma *mmol/liter*	2.789±0.024 (5)	2.487±0.036 (8)
Bile *mmol/liter*	1.91; 2.28	—

Results are means ±SE; numbers in parentheses refer to the number of animals.

1963; Barber-Riley, 1965; Claret and Mazet, 1972), calculation of the total intracellular calcium concentration is theoretically possible. Our figures by themselves, however, strongly suggest a plasma-to-liver calcium concentration ratio far over one. Such a gradient is supposedly a general phenomenon of cellular systems according to Rasmussen (1970). No significant differences were found between normal and adrenalectomized rats except that the plasma calcium content was found slightly lower in adrenalectomized rats (Table I).

Calcium Binding Properties of Liver Plasma Membranes

Calcium binding was found to be a concentration-dependent, saturable process. Maximal binding was obtained at 0.5 mM $CaCl_2$ with 125 nmol of calcium bound per milligram of plasma membrane protein. Addition of 1 mM $MgCl_2$ + 0.6 M KCl diminished the bulk of calcium fixation by 90% at 10 μM $CaCl_2$. Scatchard analysis (1949) of the data revealed at least two classes of binding sites (Fig. 1 *a*), one of which was saturable, suggesting specific binding. We shall focus only on these specific and saturable sites. The influence of adrenalectomy is clearly apparent by comparing Fig. 1 *a*, *b*. Adrenalectomy diminished the slope of the curve corresponding to the high affinity sites, whereas it did not change the other portion of the curve.

Since the analysis of Scatchard plots is imprecise and inadequate when a nonspecific system is present together with a specific system, we expressed our results according to the "proportion graph" method proposed by Baulieu and Raynaud (1970). In this method, the log of the ratio of bound and total ligand is plotted as a function of the log of the total ligand concentration. The use of logarithmic scales makes it possible to study the binding capacity over a large range of ligand concentrations. The validity of our results was estimated by a computer statistical evaluation. The experimental curves were compared with theoretical curves obtained when the equations for one specific system plus one nonspecific system were used in the computer program. Fig. 2 *a*, *b* show the theoretical curves for one specific plus one nonspecific system, as compared to our experimental values. The left part of our experimental curves fits well with the existence of one specific system. The dis-
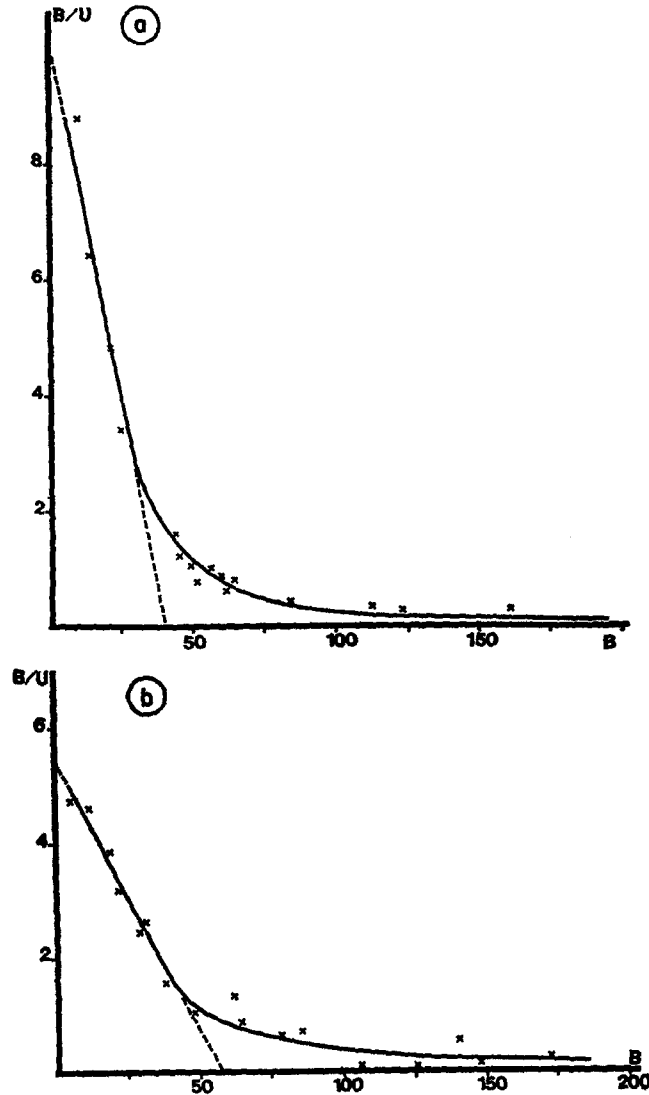
FIGURE 1. Scatchard plot for the binding of calcium by rat liver plasma membranes. Conditions as described under Materials and Methods. (a) Membranes from normal rats (183 μg membrane protein in 250 μl). (b) Membranes from adrenalectomized rats (180 μg membrane protein in 250 μl). B/U: micromolar concentrations of bound and unbound calcium, respectively.

crepancy between theoretical and experimental curves, on the right, is in favor of the existence of another specific site. We did not attempt any correlation with theoretical curves for two specific plus one nonspecific systems due to the low precision of the measurements in the presence of high calcium concentration.
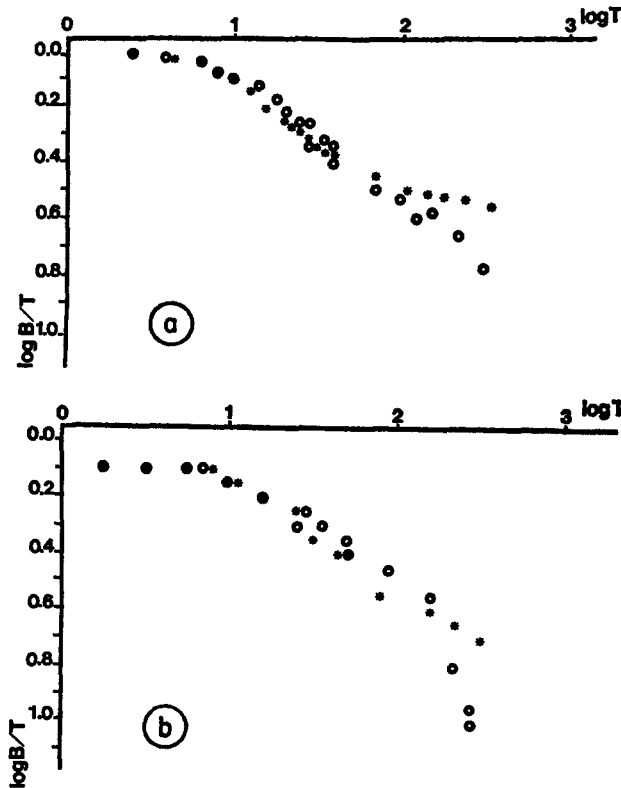
FIGURE 2.   Proportion graph of the binding of calcium by rat liver plasma membranes. Same experimental data as in Fig. 1. (*a*) Membranes from normal rats (183 µg membrane protein per assay). (*b*) Membranes from adrenalectomized rats (180 µg membrane protein per assay). B: micromolar concentration of bound calcium. T: micromolar concentration of total calcium. O: experimental value. *: theoretical value. ●: experimental value = theoretical value.

The kinetic parameters are listed in Table II, which also includes the values deduced from the Scatchard analysis. Only the values for the high affinity sites are recorded. A good correlation exists between the two methods. Several determinations have been performed over a 1-yr period using various preparations. The values for the dissociation constant (K_D) ranged between 3 and 16 μM; the amount of calcium bound per milligram protein varied from 45 to 90 nmol. In Table II, we compared experiments done on the same day. As far as the high affinity sites are concerned, adrenalectomy slightly enhanced the number of binding sites, but it diminished the affinity for calcium by a threefold factor: the limiting values for K_D were 9.8 and 29 μM and the amount of calcium bound per milligram protein was in the range of 71.6–105 nmol.

TABLE II

CALCIUM-BINDING PARAMETERS OF LIVER PLASMA MEM-
BRANES FROM NORMAL AND ADRENALECTOMIZED RATS

Membranes from	Calcium bound	Dissociation constant
	nmol/mg protein	*μM*
Proportion graph method		
Normal rats	44.5±2.03	2.9±0.2
Adrenalectomized rats	71.6±5.55	9.8±0.9
Scatchard plot		
Normal rats	56.4	4.0
Adrenalectomized rats	81.3	10.9

Only the values referring to the high affinity sites are indicated here.
Values are calculated from the curves shown in Figs. 1 and 2. Those obtained
with the proportion graph method are mean ±SE given by the computer
analysis.

Effect of ATP on Calcium Binding

When we tried to test the effect of ATP, magnesium, and oxalate on the
binding of calcium by liver plasma membranes and microsomes by the
Millipore filtration technique (Martonosi and Feretos, 1964) under the
conditions used by Hasselbach and Makinose (1961), we repeatedly observed
a diminution of the binding of calcium. This inhibitory effect of ATP and
magnesium upon calcium binding and/or accumulation could be explained
either by a chelation or by exchange between calcium and magnesium ions
at the membrane level (Sanui and Pace, 1967; Forstner and Manery, 1971),
or by an active extruding activity. However, unlike the red cell ghost prepara-
tion, the plasma membrane preparation obtained by the procedure of Neville
is heterogenous (see Materials and Methods), making it impossible to repeat
the more refined type of experiments of Schatzmann and Vincenzi (1969) on
calcium movements across the plasma membrane.

We therefore looked for a calcium-activated ATPase which could account
for calcium binding and transport in the liver plasma membranes as it does
in the erythrocytes (Dunham and Glynn, 1961; Schatzmann and Vincenzi,
1969).

Effect of Calcium on Mg^{2+}-ATPase Activity

Liver plasma membranes and microsomes possess a Mg^{2+}-ATPase activity,
part of which is (Na^+-K^+)-dependent (Emmelot and Bos, 1966). If a calcium-
activated ATPase were also present, the addition of a low concentration of
calcium to the assay system (between 0.1 $μM$ and 0.5 mM) would increase
the rate of splitting of ATP.

$CaCl_2$ was thus added from 0.1 $μM$ to 10 mM to the incubation medium in

the presence of a fixed concentration of $MgCl_2$ + ATP (5 mM each). In no case did the addition of $CaCl_2$, even at low concentrations, activate the ATPase. Fig. 3 shows that the plasma membrane (Na^+-K^+)-independent activity (or Mg^2-ATPase) was unchanged by addition of calcium up to 0.5 mM. Higher calcium concentrations were progressively inhibitory, reaching a 35% inhibition at 10 mM. In contrast, the (Na^+-K^+)-dependent activity, very sensitive to calcium, was inhibited by 80% with 10 mM $CaCl_2$. Similar results were found when the membranes were either preincubated with $CaCl_2$ before ATP-$MgCl_2$ addition, or added together with the mixture ATP-$MgCl_2$-$CaCl_2$.
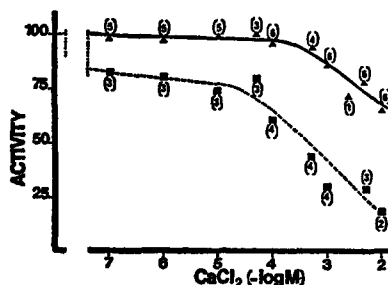

FIGURE 3.   Influence of calcium on the Mg^{2+}-ATPase and the (Na^+-K^+-Mg^{2+}) ATPase activities of rat liver plasma membranes. Conditions as described in Materials and Methods. The buffer used was 25 mM Tris pH 7.2. Activity was expressed in percent of the activity found in the presence of 0.5 mM EGTA. The mean values ±SE were 7.01 ± 0.97 (five experiments) for the Mg^{2+}-ATPase activity and 0.88 ± 0.17 (three experiments) for the (Na^+-K^+-Mg^{2+}) ATPase activity (expressed as micromoles P_i liberated/10 min/mg protein). ▲: Mg^{2+}-ATPase activity. ■: (Na^+-K^+-Mg^{2+}) ATPase activity. Numbers in parentheses refer to the number of experiments. Similar results were obtained with membranes from adrenalectomized rats.

We compared the effect of calcium upon the Mg^{2+}-ATPase and the (Na^+-K^+-Mg^{2+}) ATPase of plasma membranes and microsomes from normal and adrenalectomized rats. Table III shows that neither preparation possesses any Ca^{2+}-activated-Mg^{2+}-ATPase activity. Adrenalectomy did not modify significantly the Mg^2-ATPase nor the (Na^+-K^+-Mg^{2+}) ATPase activities, nor did it change the sensitivity to calcium ion. Similar results were found also when we used varying $CaCl_2$ concentrations as in Fig. 3. EGTA was added in the controls at a concentration of 0.5 mM, higher concentrations being inhibitory.

Since the substrate for the ATPase is known to be the Mg-ATP complex and since calcium inhibits (Na^+-K^+-Mg^{2+}) ATPase, we suspected a competition between calcium and magnesium for enzyme sites. We therefore compared the efficiency of each cation upon the various ATPase activities, in the presence of 5 mM ATP. Ca-ATP was less effective as a substrate than Mg-

TABLE III

ATPASE ACTIVITIES OF LIVER PLASMA MEMBRANES AND MICROSOMES
FROM NORMAL AND ADRENALECTOMIZED RATS

Activities	Membranes		Microsomes	
	Normal rats	Adrenalectomized rats	Normal rats	Adrenalectomized rats
Mg^{2+}-ATPase	8.78±0.63 (5)	7.81±0.91 (4)	—	—
+0.5 mM EGTA	—	7.80±0.56 (4)	0.662±0.043 (2)	0.55
+0.1 mM $CaCl_2$	8.48±0.36 (7)	7.55±0.63 (7)	0.732±0.058 (2)	0.61
$(Na^+-K^+-Mg^{2+})$ ATPase	0.91±0.15 (3)	0.57±0.18 (4)	0.02	0
+0.5 mM EGTA	—	1.12±0.19 (2)	0.04	0.02
+0.1 mM $CaCl_2$	0.91±0.10 (3)	0.40±0.02 (2)	0.02	0
Ca^{2+}-ATPase	7.67±0.41 (7)	4.22±0.14 (6)	0.337±0.023 (2)	0.380±0.05 (2)

Incubation medium: 25 mM Tris pH 7.2.
Ca^{2+}-ATPase activity was determined in the presence of ouabain.
Activities are expressed as μmol P_i liberated/10 min/mg protein.
Results are means ±SE; the number of experiments is indicated in parentheses.

ATP for maximal "total" and (Na^+-K^+)-independent activities (Fig. 4 a,
4 b), whereas it was not a substrate for the (Na^+-K^+)-dependent activity
(Fig. 4 c). Kinetic parameters of the (Na^+-K^+)-independent activity were
deduced from Lineweaver-Burk plots from Fig. 4 b: an apparent maximal
activity of 4.6 μmol P_i/mg protein/10 min was obtained with 3 mM $CaCl_2$ +
5 mM ATP, vs. 5.7 μmol P_i/mg protein/10 min with 5 mM $MgCl_2$ + 5 mM
ATP. Half-maximal activation was obtained with 0.2 mM calcium or with
0.5 mM magnesium. This higher apparent affinity of the ATPase system for
the substrate when calcium was the cofactor was observed at pH 7.4. At pH
8.2, calcium had a lower apparent affinity. This may be due to the pH in-
fluence on the formation of cation-ATP complexes.

Adrenalectomy diminished the efficiency of calcium in the ATPase reac-
tion: the activity tested in the presence of $CaCl_2$ was 87% of that tested in the
presence of $MgCl_2$ in membranes from normal rats, whereas it was only 55%
in membranes from adrenalectomized animals (Table III).

Comparison of Mg^{2+}-ATPase and Ca^{2+}-ATPase Activities

It is clear therefore that the (Na^+-K^+)-independent-ATPase activity of the
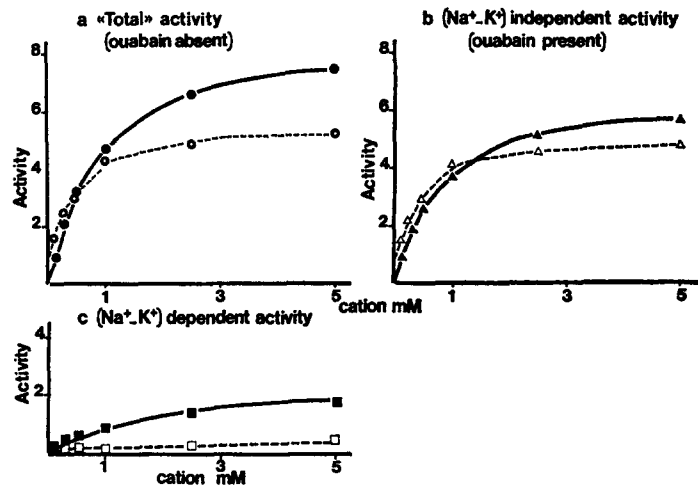plasma membranes requires either Mg^{2+} ("Mg^{2+}-ATPase") or Ca^{2+} ("Ca^{2+}-

FIGURE 4. ATPase activities of liver plasma membranes. Comparison of the effects of MgCl$_2$ and CaCl$_2$. Conditions as described in Materials and Methods. 25 mM Tris buffer pH 7.2 was used with 5 mM ATP, with either MgCl$_2$ (closed symbols) or CaCl$_2$ (open symbols), and in the presence (Fig. 4 *b*) or not (Fig. 4 *a*) of 1 mM ouabain. The (Na$^+$-K$^+$-Mg^{2+}) ATPase activity was estimated by difference (Fig. 4 *c*). Activities are expressed as μmol P$_i$ liberated/10 min/mg protein). For the (Na$^+$-K$^+$)-independent activity, the half-maximum activation was obtained with 0.2 mM calcium and with 0.5 mM magnesium. The V_{max} were 4.6 and 5.7, respectively.

ATPase'') as the cofactor. To gain further insight into the specificity of the Ca^{2+}-ATPase above described, we tried to distinguish it from the Mg^{2+}-ATPase, using a variety of parameters. The assays were performed in the presence of ouabain in order to test the (Na$^+$-K$^+$)-independent activity alone. It has been verified that the Ca^{2+}-ATPase was insensitive to ouabain (Fig. 4 *a*, *b*). (*a*) The pH dependence, tested between 6.6 and 8.5, was the same in both cases with an optimal value of 8.2.

(*b*) Substrate specificity: ATP was the best substrate, followed by GTP, ITP, UTP, and CTP for both activities (Fig. 5).

(*c*) When tested in the presence of calcium plus magnesium, each at a concentration giving half-maximal activation, the response was equal to the arithmetic mean of their effects tested separately at twice the concentration, thus showing that calcium and magnesium act on the same site (Table IV).

(*d*) The effects of inhibitors of ATPase such as Salyrgan (Hasselbach and Makinose, 1961) and ruthenium red (Watson *et al.*, 1971) were similar on both activities (Table V). It should be noted that ruthenium red, which is known to be an inhibitor of calcium binding (Vasington *et al.*, 1972), of calcium transport (Moore, 1971), and of calcium-activated-Mg^{2+}-ATPase activity (Watson *et al.*, 1971), had no effect. It did not affect the inhibitory effect of calcium upon the Mg^{2+}-ATPase activity.
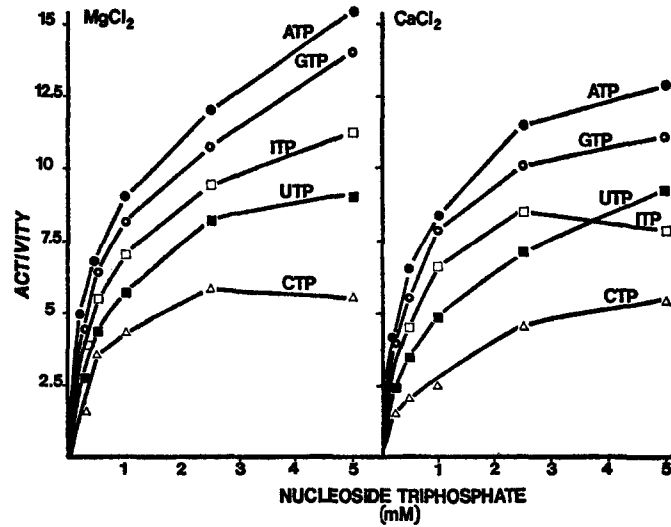
FIGURE. 5.   Comparative effects of various nucleosides triphosphate on the Mg^{2+}- and Ca^{2+}-ATPase activities of rat liver plasma membranes. Conditions as described in Materials and Methods. Incubation in 25 mM Tris pH 8.25. Left panel: activity tested with 5 mM $MgCl_2$. Right panel: activity tested with 5 mM $CaCl_2$. Activities are expressed as μmol P_i liberated/10 min/mg protein.

TABLE IV

EFFECT OF COMBINATION OF $MgCl_2$ AND $CaCl_2$ AT SUBMAXIMAL CONCENTRATIONS ON TOTAL ATPASE AND Mg^{2+}-ATPASE ACTIVITIES OF RAT LIVER PLASMA MEMBRANES

Addition to 5 mM ATP		Mg^{2+}-ATPase plus $(Na^+-K^+-Mg^{2+})$ ATPase activities	Mg^{2+}-ATPase activity
$MgCl_2$	1.4 mM	6.06±0.20	4.88±0.21
$CaCl_2$	0.6 mM	3.81±0.02	3.81
$MgCl_2$	0.7 mM + $CaCl_2$ 0.3 mM	4.25±0.28 (4.93)*	4.36±0.19 (4.35)*

Incubation medium: 25 mM Tris pH 7.25.
Activities are expressed as μmol P_i liberated/10 min/mg protein.
Results are means ±SE.
* Calculated as indicated in the text.

(e) The effect of X-537 A, an ionophore which is known to facilitate the transport of calcium through membranes (Scarpa and Inesi, 1972; Caswell and Pressman, 1972) was tested on the various ATPase activities (Table VI). It was found to have no effect at 10 μM; but at 50 μM it inhibited all the activities by the same percentage (about 39%).

These results show that the Ca^{2+}-ATPase is by no means distinguishable

TABLE V

INFLUENCE OF SALYRGAN AND OF RUTHENIUM RED ON THE
ATPASE ACTIVITIES OF RAT LIVER PLASMA MEMBRANES

Activities	Control	Salyrgan	Ruthenium red
		1 mM	60 μM
Mg^{2+}-ATPase	8.84	7.68	9.20
Mg^{2+}-ATPase + 5 mM $CaCl_2$	6.81	6.10	6.89
Ca^{2+}-ATPase	8.39	7.03	7.85

Incubation medium: 25 mM Tris pH 8.2; 1 mM ouabain.
Activities are expressed as μmol P_i liberated/10 min/mg protein.

TABLE VI

INFLUENCE OF X-537A ON THE ATPASE ACTIVITIES OF RAT LIVER PLASMA
MEMBRANES

Activities	Control	X-537 A	
		10 μM	50 μM
Mg^{2+}-ATPase*	8.56±0.22 (3)	8.48±0.04 (2)	5.19
$(Na^+-K^+-Mg^{2+})$ ATPase	1.35±0.16 (3)	1.12±0.10 (2)	0.77
Mg^{2+}-ATPase + 0.1 mM $CaCl_2$*	8.37±0.16 (2)	7.72	3.39
Ca^{2+}-ATPase*	6.94±0.03 (3)	6.70±0.4 (2)	4.68

* 1 mM ouabain.
Incubation medium: 25 mM Tris pH 7.2.
X-537 A was dissolved in ethanol. The final concentration of ethanol was 1% in all the assays.
The total ATPase activity of the control assay was arbitrarily given a value of 10 (experimental
value = 9.48) and all the activities were normalized with respect to 10. Activities are expressed
in μmol P_i liberated/10 min/mg protein.
Results are means ± SE; values in parentheses refer to the number of experiments.

from the Mg^{2+}-ATPase. In particular, calcium does not activate any discrete
nucleotidase activity.

Membrane Phosphorylation

When plasma membranes were incubated in the presence of 1 mM $[\gamma-^{32}P]$-
ATP and an equimolar concentration of $MgCl_2$, the terminal phosphoryl
group of ATP was transferred to proteins by a time and temperature-depend-
ent process (Fig. 6). The total phosphorylation was greater at 37°C than at
4°C. The labeling was multiphasic indicating that several phosphoryl groups
were formed. Analysis of the phosphate groups showed that acyl-phosphate
bonds were rapidly formed whereas acid-labile and acid-resistant phosphate
groups appeared more slowly (Fig. 7).

When calcium was substituted for magnesium, a very low level of phos-
phorylation was observed, which was close to the blank level (Fig. 6). It has
been reported that sodium inhibited phosphorylation of proteins by com-
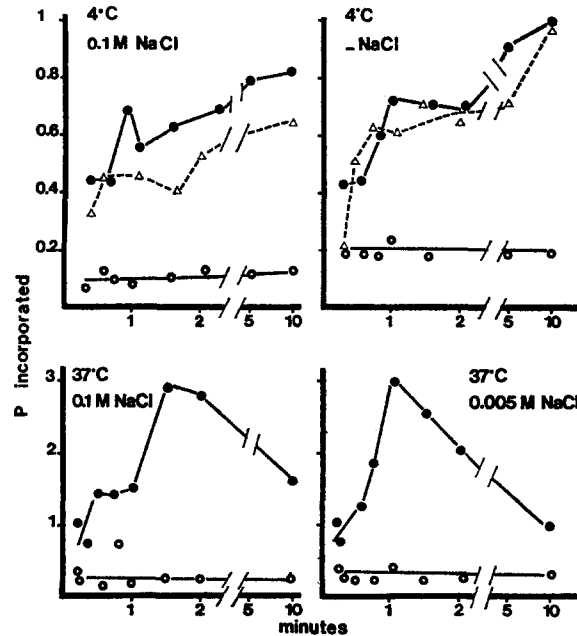peting with calcium (Judah and Ahmed, 1963). Yet, omitting the sodium did

FIGURE 6.   *Incorporation of phosphate from* [γ-^{32}P] ATP into rat liver plasma membranes. Incubations as described in Materials and Methods in the presence of 1 mM [γ-^{32}P] ATP and with the addition of 1 mM $MgCl_2$ (●), or 1 mM $MgCl_2$ + 10 μM $CaCl_2$ (△), or 1 mM $CaCl_2$ (○) at 4°C and at 37°C, with or without NaCl. P incorporated is expressed in nanomoles per milligram protein. In all conditions of incubation, when 0.125 mM EGTA was added to the assay with $MgCl_2$, phosphate incorporation was less than the incorporation observed with $MgCl_2$ or with $MgCl_2$ + $CaCl_2$, with in particular, suppression of the first peak of radioactive phosphate incorporation.

not change our results. The addition of 10 μM $CaCl_2$ to the assay performed in the presence of 1 mM $MgCl_2$ plus 1 mM ATP slightly diminished the total phosphorylation but it did not modify the general pattern (Fig. 6). Higher concentrations of $CaCl_2$ (0.1 and 1 mM) had the same effect (25% inhibition with a 30-s incubation period).

Table VII shows the repartition of the phosphate groups which were labeled in a 30-s incubation period under various conditions: presence or absence of NaCl, of KCl, and of ouabain. In all cases, when $CaCl_2$ was used instead of $MgCl_2$, phosphorylation was weak and acyl-phosphate groups seemed to be preferentially labeled. NaCl had no influence on the process.

The inhibitory effect of 10 μM $CaCl_2$ added to the assay when $MgCl_2$ and ATP were present (1 mM each) was tested at 4 and at 37°C and in the presence or absence of NaCl, over a 3-min incubation period. As an example, Fig. 8 shows that calcium diminished by 15 to 20% phosphate incorporation into acid-labile groups whereas incorporation into acid-resistant groups was not modified. The above results clearly show that (*a*) Ca-ATP is not a sub-
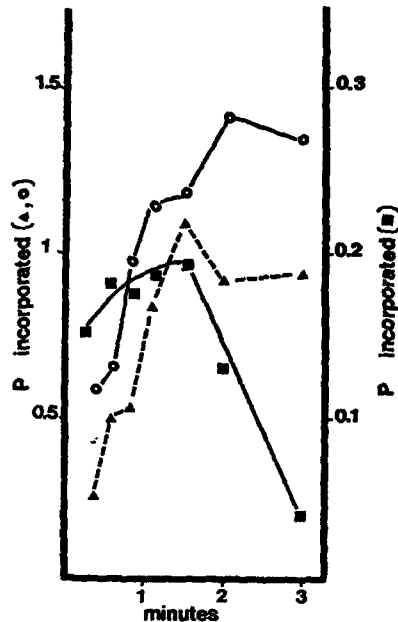
FIGURE 7   FIGURE 8

FIGURE 7. Identification of the various phosphate groups labeled with [γ-^{32}P] ATP. Incubations were run as described in Materials and Methods in the presence of 1 mM [γ-^{32}P] ATP, 1 mM MgCl$_2$, 0.1 M NaCl, at 37°C. Identification of the various phosphate groups was done according to Blat and Harel (1969). P incorporated is expressed in nanomoles per milligram protein. ■: phosphate liberated by hydroxylamine treatment = acyl-phosphate. ▲: phosphate liberated after hydrolysis in 5% perchloric acid at 95° during 15 min (hydroxylamine treatment was done before) = acid-labile phosphate. O: phosphate remaining in the proteins: acid-resistant phosphate.
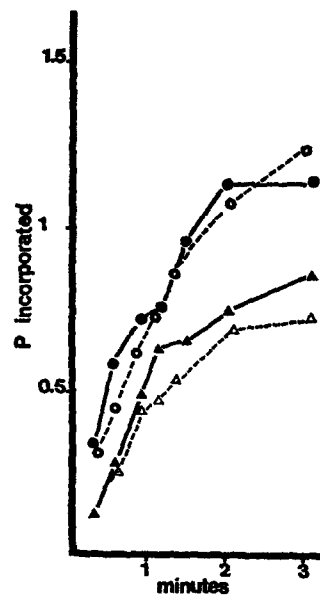
FIGURE 8. Influence of calcium on the incorporation of phosphate from [γ-^{32}P] ATP into rat liver plasma membranes. Incubations were run as described in Materials and Methods, at 37°C, in the presence of 1 mM [γ-^{32}P] ATP and 0.1 M NaCl. Closed symbols refer to incubation with 1 mM MgCl$_2$; open symbols refer to incubation with 1 mM MgCl$_2$ + 10 μM CaCl$_2$. P incorporated is expressed in nanomoles per milligram protein. ▲ △: phosphate liberated after hydrolysis in 5% perchloric acid at 95°C during 15 min (hydroxylamine treatment was done before) = acid-labile phosphate. ● O: phosphate remaining in the proteins = acid-resistant phosphate.

strate for the phosphorylation of any plasma membrane protein; (b) calcium impairs the phosphorylation by Mg-ATP.

DISCUSSION

The hypothesis of the occurrence of an active extrusion of calcium at the plasma membrane level has been suggested by the observation of a downhill gradient of calcium between the extracellular and the intracellular medium. It is known from studies with isolated perfused liver and from *in vitro* experiments on liver slices that a net calcium outflow does occur from liver and that

TABLE VII

INCORPORATION OF PHOSPHATE FROM [γ-^{32}P] ATP INTO RAT LIVER
PLASMA MEMBRANES

Experiments	Conditions	Total phosphate	Acyl-phosphate	Acid-labile phosphate	Acid-resistant phosphate
1	MgCl$_2$	1910	187	123	1600
	MgCl$_2$ + Ouabain	1359	125	107	1127
	MgCl$_2$ + KCl	1227	154	139	934
	CaCl$_2$	250	214	0	36
	CaCl$_2$ + Ouabain	115	115	0	0
	CaCl$_2$ + KCl	138	121	4.3	12.8
2	MgCl$_2$	2742	66	1495	1181
	MgCl$_2$ − NaCl	2852	115	1257	1480
	CaCl$_2$	16.6	0	5.4	11.2
	CaCl$_2$ − NaCl	69.4	14	24.4	31

The conditions were as described in Materials and Methods.
1 mg membrane protein was incubated at 37°C during 30 s with 1 mM [γ-^{32}P]ATP, 100 mM
NaCl, and 5 mM MgCl$_2$ or CaCl$_2$. When indicated the following additions were made: 1 mM
ouabain; 33 mM KCl; when KCl was added, only 66 mM NaCl was present.
Results are expressed in picomoles P$_i$ incorporated/30 s/mg protein.

this phenomenon is modulated by the cyclic AMP system and by various
hormones (Friedmann and Park, 1968; Chausmer et al., 1972). Wallach et al.
(1966) showed that calcium atoms indeed passed through the cell surface by
a complex transport phenomenon involving a carrier. Van Rossum (1970;
Van Rossum et al., 1973) postulated later that the calcium-extruding activity
of the cell is located at the plasma membrane level and is independent of the
presence of a sodium gradient; thus the energy would be directly derived from
the hydrolysis of ATP.

We report here that isolated plasma membranes from liver, prepared ac-
cording to the Neville's procedure, are able to bind calcium ions by a con-
centration-dependent, saturable process. At least two classes of binding sites
are found, one of which is highly specific with a K$_D$ between 3 and 16 μM.
K$_D$ for the calcium binding to solubilized purified proteins isolated from
structures which actively transport calcium is of the same order of magnitude
(Wasserman et al., 1968).

It is evident that the calcium-binding capacity of rat liver plasma mem-
branes has a physiological meaning. The presence of a high affinity site for
calcium could be related to the regulation of enzymic activities located in the
membranes. Local variations of calcium concentrations could modulate
either enzymatic activities or hormone actions. This is known to occur in some
systems: For example in the red cell membrane, the (Na$^+$-K$^+$)-dependent

ATPase activity and the sodium transport are regulated by intracellular calcium (Dunham and Glynn, 1961; Schatzmann and Vincenzi, 1969); in renal plasma membranes, calcium can modulate the adenylate cyclase activity by acting on both catalytic and hormonal sites (Campbell *et al.*, 1972). The adenylate cyclase activity of our liver plasma membranes is indeed very sensitive to calcium ion concentration (Leray *et al.*, 1973). Furthermore, the $(Na^+-K^+-Mg^{2+})$ ATPase is very sensitive to inhibition by low concentration of calcium as described in the present paper. The endogenous calcium content of liver plasma membranes has been reported to be 154 $\mu g/g$ dry weight (Selkirk *et al.*, 1971), which would correspond approximately to 2 nmol/mg protein. A recent study showed that a p ol of calcium was also present within adrenal membranes, which was related to the action of ACTH (Sayers *et al.*, 1972).

The presence of binding sites might be also directly related to the existence of a specific carrier involved in the translocation of calcium across the plasma membrane. In an attempt to correlate the above described binding phenomenon to the active transport postulated by Van Rossum (1970), we carried out the study of the phenomena obligatorily linked to the existence of a functional carrier.

If active calcium transport occurred, according to the model drawn from the study of sarcoplasmic reticulum, it should be mediated by a membrane-bound ATPase activity highly sensitive to change in free calcium concentration. During the process of ATP hydrolysis, a phosphoprotein should be formed, as an intermediate product in the sequence of reactions leading to calcium transport and phosphate liberation; the phosphoprotein thus formed could be an energy-rich phosphate compound (Makinose, 1972). Unlike the ATPase activities of membrane preparations which are known to possess an active transport system for calcium (Schatzmann and Vincenzi, 1969; Martonosi, 1969; Makinose, 1972; Robblee *et al.*, 1973), the Mg^{2+}-ATPase activity of isolated liver plasma membranes prepared by the procedure of Neville was not stimulated, even by low concentration of calcium ion. On the contrary, calcium inhibited the Mg^{2+}-ATPase activity (Fig. 3, Table III). Furthermore, calcium did not promote any enhanced phosphorylation of a membrane protein fraction, which would normally occur if an ATPase activity linked to calcium transport was involved (Fig. 8). It rather slightly inhibited the phosphorylation when present at low concentration together with $MgCl_2$ and ATP.

We report here (Fig. 4) that the (Na^+-K^+)-independent-ATPase activity could be studied in the presence of either Ca-ATP or Mg-ATP as the substrate, the latter leading to a maximal activity. Enzymes from other sources can also be activated either by magnesium or by calcium ions (Parkinson and Radde, 1971; Ostwald and Heller, 1972). The splitting of both sub-

strates is probably catalyzed by the same enzyme entity. Calcium can replace magnesium for the ATPase reaction, but in this case, no phosphorylation of any membrane component can be detected. The nonspecificity of the cation required for the hydrolysis of ATP raises the question of the physiological meaning of the ATPase reaction. In the presence of calcium, the ATPase activity is indeed a mere splitting of the terminal phosphate group. It thus appears that Ca-ATP is not a substrate for the formation of any phosphorylated intermediate linked to the transport of ions nor to any membrane-bound enzyme activities such as endogenous kinases. In any case, the plasma membranes possess no specific Ca^{2+}-ATPase or any specific Ca^{2+}-nucleotidase activity. The Ca^{2+}-ATPase activity displayed by the plasma membranes is not coupled to transport nor to binding of calcium, as suggested by the absence of effect of ruthenium red and X-537 A (Tables V and VI).

These results thus clearly invalidate the hypothesis of an active calcium transport capacity located at the plasma membrane level (Van Rossum et al., 1973), at least with the plasma membranes prepared according to the procedure of Neville[1]. The transport of calcium which is observed in the liver cell represents probably a part of a complex integrated system since it cannot be demonstrated in our purified membrane preparation. Whether specific components or factors required for a functional active transport process are lost or inactivated during the preparation procedure is to be considered. In the living cell, the plasma membrane could be coupled to a subjacent component with enzymatic properties such as a Ca^{2+}-activated-Mg^{2+}-dependent ATPase or to a contractile protein; such a protein seems to be present in the liver plasma membrane (Neifakh et al., 1965) and in the erythrocyte membrane (Rosenthal et al., 1970). In the Neville's plasma membranes, we showed that the loss of adenylate cyclase sensitivity to catecholamines could be readily prevented by prior adrenalectomy (Leray et al., 1972; 1973). However, as shown here (Table III), adrenalectomy does not modify the sensitivity of the Mg^{2+}-ATPase towards calcium.

A more likely possibility is that intracellular free calcium level might be kept down by a mechanism other than an active extruding activity located at the plasma membrane level itself. Mitochondria which represent a primordial locus for the accumulation of calcium (Carafoli, 1967) were proposed by Friedmann and Rasmussen (1970) as the source of calcium mobilized under physiological conditions. Plasma membranes should then act as a

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[1] As reported above, our preparation is heterogenous since lamellar fragments are present together with vesicles, which are, or are not, right-side out. The restoration of the right vesicular structure in the recovery of enzymatic activities and of functional transport properties could be of great importance. However, that the membranes used here could behave as isolated carrier proteins unable to transport but susceptible to be characterized only by their binding properties, can be dismissed since one can readily study the $(Na^{+}$-K^{+}-$Mg^{2+})$ ATPase and adenylate cyclase activities, which need the presence of ligands (substrates, ions, and hormones) on both sides.

passive carrier with ion exchange capacity. Microsomes are probably not involved in calcium movements because they do not accumulate calcium (Rossi *et al.*, 1966; personal unpublished results). Nevertheless, they possess high affinity sites for calcium binding (unpublished results). As in plasma membranes, these sites are not related to any ATPase activity specially linked to calcium transport (Table III). We would rather favor the possibility already alluded to by Judah and Ahmed (1964) that coupling of calcium and sodium fluxes in the opposite directions could be responsible for the net calcium outward transport, as described in cardiac muscle and in neurones (see Brinley, 1973, for a review). This would be in agreement with our data since no direct need for ATP splitting on the calcium transport would be necessary.

Calcium binding seems to be subject to hormonal regulation *in vivo* since adrenalectomy increases threefold the dissociation constant and slightly enhances the site number for calcium in plasma membranes. (Figs. 1, 2; Table II). It should be emphasized that the changes after adrenalectomy concern the high affinity sites, the other classes remaining unaffected. The various ATPase activities from either plasma membranes or microsomes were not significantly influenced. The major change was the diminished efficiency of calcium in the splitting of phosphate from ATP when Ca-ATP was the substrate as compared to Mg-ATP (Table III). This effect of adrenalectomy upon calcium-binding properties and ATPase activity is possibly linked to the overall action of glucocorticoids upon liver. According to Exton *et al.* (1972), glucocorticoids could exert their "permissive" effect by acting upon proteins involved in ion transport across cellular membranes. It is known that glucocorticoids can induce changes of protein and enzyme properties of membrane origin (Ballard and Tomkins, 1970; Chignell and Titus, 1966; Kimberg *et al.*, 1969). In particular, they affect the adenylate cyclase activity of isolated rat liver plasma membranes (Leray *et al.*, 1972, 1973). Variation of the calcium relationship with the liver plasma membranes after adrenalectomy, as reported here (Tables II and III), represents another parameter which should be now considered in the mechanism of action of cortisol in liver.

It is difficult to compare these data with the variations of calcium binding under hormonal influence *in vitro* which were recently described by Schlatz and Marinetti (1972 *a*). Their data are of questionable physiological significance in view of the very low K_A for calcium which they reported (Schlatz and Marinetti, 1972 *a, b*). A value of 4.0×10^3 M^{-1} would correspond to a free energy change for calcium binding of $\Delta F = -4.58$ kcal mol^{-1} at 5°C. Our ΔF values calculated from the dissociation constants which are recorded in Table II vary from -6.08 to -7.02 kcal mol^{-1} and are closer to the values reported in the case of the combination of specific ligands to high affinity sites.

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