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ISOLATION OF PLASMA MEMBRANES FROM EHRLICH ASCITES TUMOR CELLS

INFLUENCE OF AMINO ACIDS ON $(Na^+ + K^+)$-ATPase AND K^+-STIMULATED PHOSPHATASE

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SUMMARY

A method was developed for isolating plasma membranes from Ehrlich ascites tumor cells. The plasma membranes appeared as highly irregular shrunken sacs or ghosts. Enzymatic characterization of the plasma membranes showed them to be high in $(Na^+ + K^+)$-ATPase activity and K^+-stimulated phosphatase activity. A detailed study showed that both of these latter enzymic functions were stimulated by various amino acids. Such stimulation occurred in the 1–15 mM range of amino acids and was most effective for aromatic species, *e.g.* phenylalanine and histidine. The amino acid stimulation, which appeared to show little or no stereospecificity, was eliminated by a one carbon separation of NH_2 and COOH groups. Since the metal chelating agent EDTA was also effective in mimicking the stimulation by amino acids, and since a mild washing procedure did not render membranes insensitive to subsequent amino acid or EDTA stimulation, it is proposed that the operation of the $(Na^+ + K^+)$-ATPase (and K^+-stimulated phosphatase) is to some extent controlled by a tightly bound metal. The possible physiological function of an amino acid-regulated transport ATPase is discussed.

INTRODUCTION

The rapid growth of Ehrlich ascites tumor cells in the abdominal cavity of the mouse is dependent upon the avid uptake of sugars and amino acids from the ascitic fluid. In fact, Ehrlich cells are frequently used as a model system for transport studies because of the effectivity of transport processes and the simplicity of using a single cell suspension. Thus, a wealth of literature is available for the Ehrlich cell, examining the kinetics of solute transport and the coupling phenomena between Na^+ and non-electrolyte transport[1–4].

The plasma membrane is the principal limiting structure through which these

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transport events are operative. A study of enzyme functions, chemical structure and binding characteristics of the plasma membrane would provide fundamental information for detailed interpretation of transport processes. Earlier studies by Wallach and Ullrey[5,6] have produced a plasma membrane preparation from Ehrlich ascites cells, but their cell disruption methods typically fragmented the cell membrane into "microsomal"-sized particles. A technique by Kamat and Wallach[7] effectively separated endoplasmic membranes from plasma membranes in their microsomal fraction.

In this study we have developed a method for isolating relatively intact plasma membranes from the Ehrlich cells. The plasma membrane fraction contains an active $(Na^+ + K^+)$-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) and a K^+-stimulated phosphatase. Furthermore, both of these enzymic activities are stimulated by amino acids and we have suggested a possible role for this phenomenon in regulating amino acid and Na^+ transport.

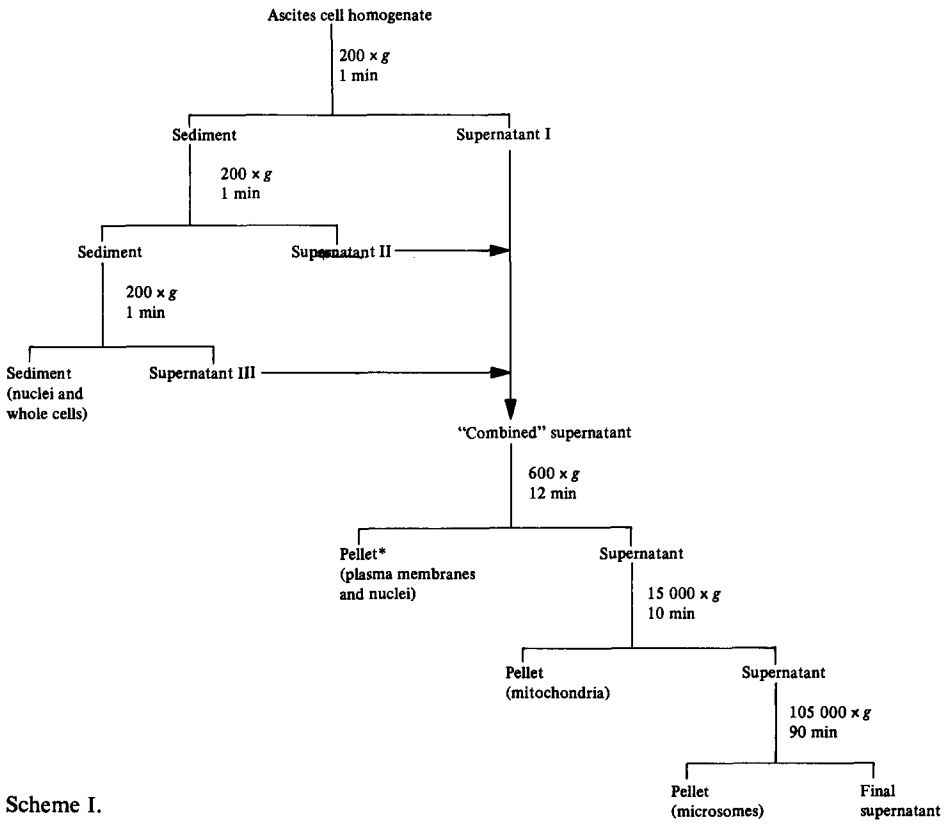
METHODS

Membrane preparation

Ehrlich ascites tumor cells were harvested from 6–9 mice which had been inoculated 6 or 7 days previously. The combined tumor cells and ascites fluid (40–65 ml) were mixed with 25 ml of 0.15 M NaCl containing 0.5 mg heparin. The cells were centrifuged then resuspended and washed three times with 0.15 M NaCl. Packed cells were resuspended in 6 vol. of cold homogenizing medium containing 18 mM Tris (pH 8.0), 25 mM NaCl and 0.5 mM $CaCl_2$. Optimal rupturing of cells was produced by 35–40 strokes with a Dounce tight pestle homogenizer. It was important to have Ca^{2+} in the homogenization and fractionation procedures in order to maintain the integrity of nuclei. Phase microscopy was used to evaluate the effectivity of cell disruption as well as all subsequent fractionation procedures. The various centrifugation steps which produced the "crude plasma membrane" pellet are shown in Scheme 1. The final "crude plasma membrane" pellet from the differential centrifugation procedure was resuspended in approximately 25 ml of a 1:1, mixture of resuspending medium and 60% sucrose. Approximately 7 ml of this resuspension was applied to each of three 30-ml centrifuge tubes previously prepared with a discontinuous density gradient consisting of successive 6-ml layers of 60, 50, 45 and 40% sucrose (all w/v). After 60 min of centrifugation at 25000 rev./min using a Spinco SW 25 rotor, a pellet and several interfacial bands were noted. The bands were harvested with a J-shaped pipette; the particulate material was collected by centrifugation and subsequently washed 2–3 times with a Ca^{2+}-free solution containing 15 mM Tris (pH 8.0) and 37 mM NaCl. In some preparations the band containing plasma membranes (Band 2; 40–45% sucrose) was resubjected to shear stress in the Dounce tight pestle homogenizer (30 strokes) in an attempt to sub-fragment large plasma membrane sacs and free residual cytoplasmic components which might be trapped within membrane folds.

Enzyme assays

For the measurement of ATPase, cell fractions were incubated at 37 °C in a volume of 1.7 ml containing 30 mM Tris (pH 8.0), 6 mM $MgCl_2$, 3–6 mM ATP and other ingredients as indicated for individual experiments. The reaction was stopped



Scheme I.

* Pellet from the 600 x g·12 min centrifugation was resuspended and washed twice in homogenizing medium for preparation of the final crude membrane pellet. It was this latter pellet which was applied to the sucrose density gradient for final purification of plasma membrane (see Methods).

with 1 ml ice-cold 15% $HClO_4$ and after centrifugation a 2-ml aliquot was taken for assay of inorganic phosphate by the method of Fiske and SubbaRow[8]. *p*-Nitrophenyl phosphatase was assayed at 37 °C in a final volume of 1.35 ml containing 30 mM Tris (pH 8.0), 3.7 mM $MgCl_2$, 3.7 mM *p*-nitrophenyl phosphate and other necessary agents as indicated. The reaction was terminated with 2 ml 0.1 M NaOH and the amount of *p*-nitrophenol liberated was monitored at 410 nm. Glucose-6-phosphatase was determined according to the method of Duttera *et al.*[9]. NADH diaphorase activity was measured at room temperature by the method of Kamat and Wallach[7]. Monoamine oxidase activity was measured using [^{14}C]tryptamine as substrate[10].

Chemical assays

Protein was measured by the method of Lowry *et al.*[11]. For lipid and nucleic acid analyses the cell fractions were precipitated and washed 3 times in cold 7.5% $HClO_4$. Fractions were then extracted with three successive 3-ml volumes of ethanol–ether (2:1, v/v) and a final extraction with 3 ml of ether. The ether and ethanol extractions were pooled for subsequent lipid analysis. The fat-free residue was extracted with 6% $HClO_4$ at 90 °C and the supernatant taken for nucleic acid analysis[12]. DNA was measured by the diphenylamine method[13] and RNA by the

orcinol method[14]. Phospholipid phosphorous in the lipid extract was assayed by the method of Bartlett[15] after hydrolysis in H_2SO_4.

Electron microscopy

Membrane fractions visualized by negative staining were first diluted with an equal volume of 2% sodium phosphotungstate, pH 7.4. A small droplet of the membrane suspension was placed on a Formvar–carbon-coated grid and excess fluid was withdrawn with filter paper.

For two separate cell fractionation procedures membranes were processed for ultramicrotomy, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed in similarly buffered 1% OsO_4. The pelleted membranes were then stained 30 min with 0.5% uranyl acetate and were subsequently dehydrated with ethanol and embedded in epoxy resin as described by Spurr[16]. Numerous sections (600–900 Å) were cut with a Sorvall MT-2 ultramicrotome and examined with a Zeiss 9 or Hitachi HU 11 electron microscope.

RESULTS

Microscopic studies

Examination by phase microscopy of the low speed pellet harvested at $600 \times g$ for 12 min revealed this to be a fraction rich in cell envelopes, but also containing an

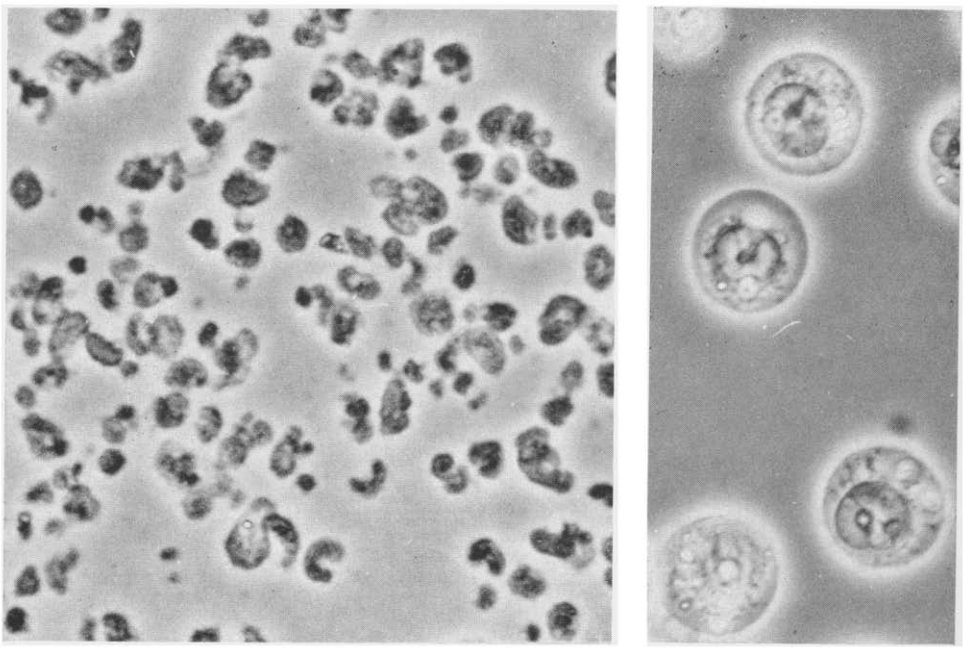


Fig. 1. Micrograph of Band 2 plasma membranes from Ehrlich ascites cells taken with phase contrast optics. Membranes appear as shrunken sacs covered by fine filamentous projections which can be seen best when adjusting the field of focus. Magnification × 880.

Fig. 2. Phase-contrast micrograph of intact Ehrlich ascites cells taken under the same conditions and magnification as for the isolated plasma membranes of Fig. 1.

abundance of nuclei and lesser amounts of other contaminants, such as small dense particles (mitochondria?) and some highly refractile particles. A high degree of purification was achieved by separation on the discontinuous density gradient where four bands and a pellet were produced.

All fractions from the density gradient were examined by phase-contrast microscopy. A micrograph of the homogeneous plasma membrane fraction (Band 2, 40–45% sucrose) is shown in Fig. 1. It is clear that the membranes, or membrane fragments, are considerably smaller than whole cells which are shown in Fig. 2 for comparison. Microscopic examination of other bands from the density gradient revealed the following: Band 1 (30–40% sucrose), mitochondria-like particles and small highly refractile particles; Band 3 (45–50% sucrose), very faint band with same plasma membranes, but containing primarily partially ruptured enucleated cells; Band 4 (50–60% sucrose), nuclei and strands of nuclear material; pellet (>60% sucrose), a few whole cells, abundant nuclei and strand-like nuclear material.

When examined at the higher resolution of electron microscopy, Band 2, or the plasma membrane fraction, revealed a characteristic morphology. Preparations negatively stained with sodium phosphotungstate appeared as irregular structures from 3 to 7 μm across the major axis. As can be seen from the reproduction in Fig. 3 the surface of the large membranous units appears to be made of many closely adjoined smaller vesicular or tubular structures. This morphological configuration visualized by negative staining may be more readily interpreted after examining thin

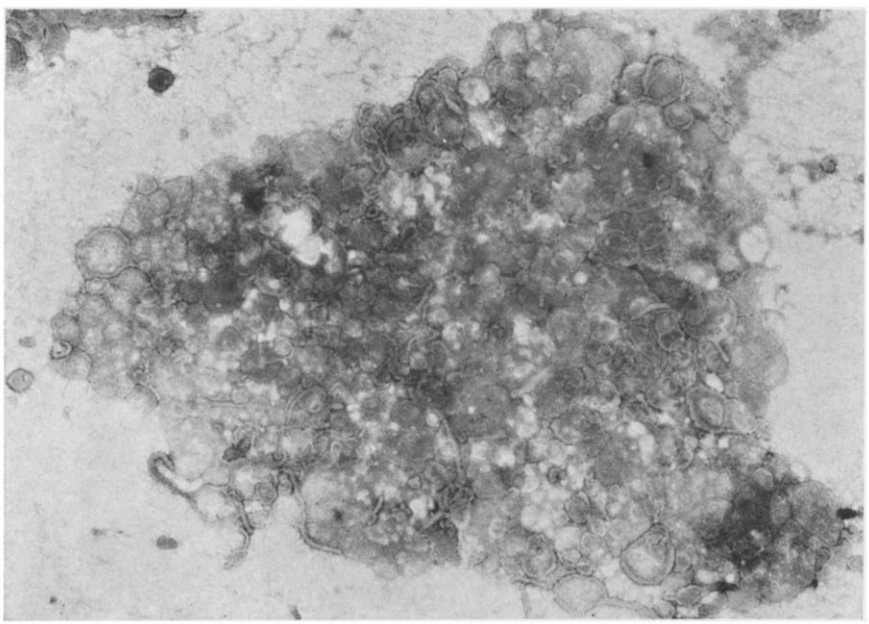


Fig. 3. Electron micrograph of plasma membrane fraction negatively stained with phosphotungstic acid. The principal unit in this field is a plasma membrane fragment which, in the pattern of negative staining, appears to be made up of smaller membranous units. This structural arrangement most likely represents the configuration of the highly irregular and tortuous surfaces of the plasma membranes. Magnification × 12200.

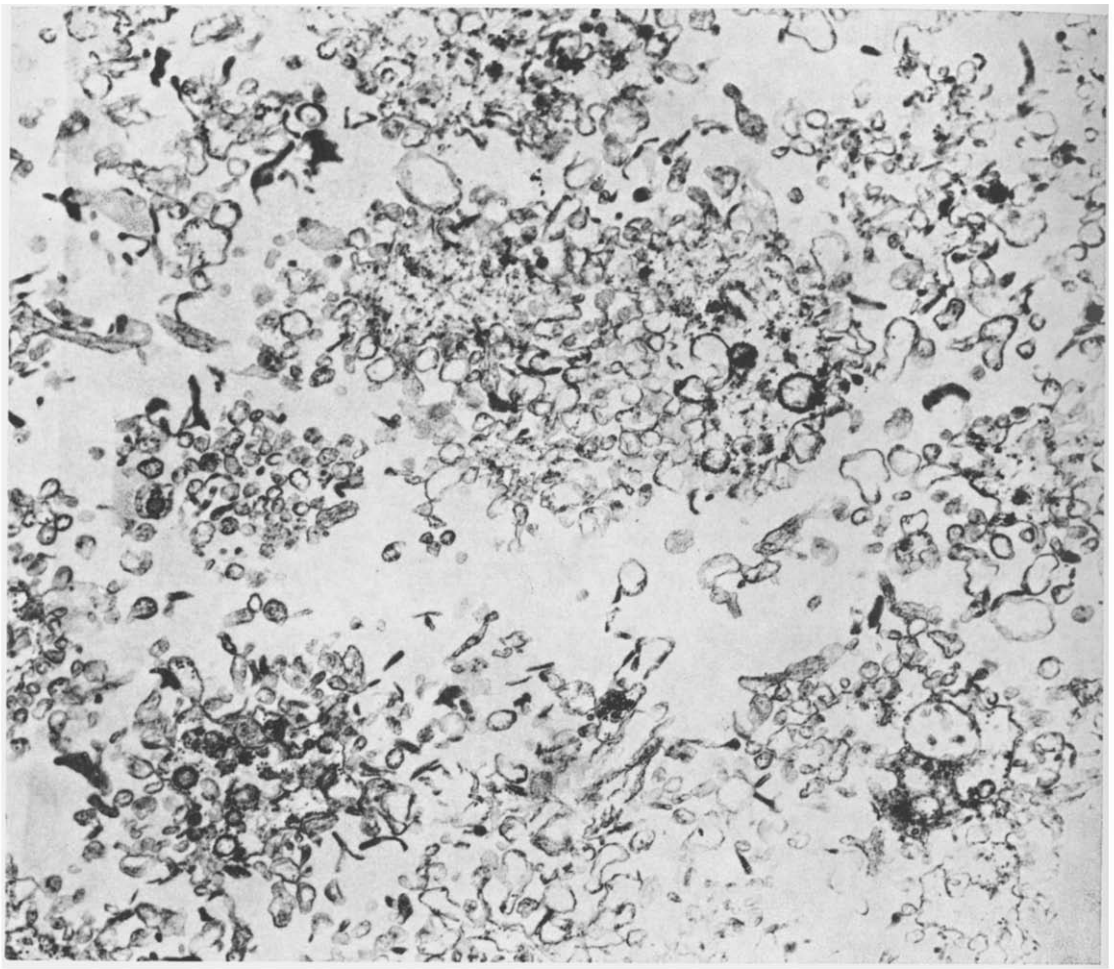


Fig. 4. Electron micrograph of thin section through the plasma membrane pellet. The individual plasma membranes appear as loosely aggregated units of many vesicular membranous profiles. This is again consistent with the highly irregular and tortuous nature of the cell surface. Within the confines of plasma membrane units one can see a few elements which represent contamination by intracellular organelles, such as endoplasmic reticulum and occasional mitochondrial fragments. Sections stained with lead and uranyl salts. Magnification $\times 16000$.

sections of pelleted membrane material as shown in Fig. 4. Again, the first impression is that of many vesicular and tubular membrane profiles; however, it is apparent that the greater limits of a much larger membrane unit may be identified. The larger membranous units can best be described as shrunken, highly crenated, membranous sacs. In thin section the finger-like projections and tortuous convolutions appear as vesicular and tubular elements with a few interconnecting membranous sheets. Within the center of the greater membrane units a few typical intracellular organelles may be identified, including ribosomes, rough surfaced endoplasmic reticulum and occasional mitochondria.

Thus several features of size and general morphology may be interpreted and summarized from the various modes of microscopic examination. Band 2 from the density gradient separation contains ghosts of the Ehrlich cell plasma membranes which appear in a contracted and highly crenated form. Furthermore, these cell envelopes contain a few residual intracellular organelles which were not set free in the homogenization procedure and thus represent possible contaminating elements.

Chemical and enzymatic characterization

Additional information concerning the characterization of membrane fractions may be provided by the various chemical and enzymatic analysis which are shown as a representative experiment in Tables I and II. From a total homogenate of Ehrlich ascites tumor cells about 0.1–0.2% of the total protein is harvested as the plasma membrane fraction (Table I).

TABLE I

COMPOSITION OF CELL FRACTIONS FROM EHRLICH ASCITES TUMOR CELLS

	Total protein (mg)	*μg/mg protein*		
		Phospholipid	*DNA*	*RNA*
Whole homogenate	625	148	64	138
Final washed 600 × *g* pellet (crude membranes and nuclei)	69	143	169	99
Plasma membranes (Band 2 from density gradient)	0.6	532	7	49
Mitochondria	63	424	25	75
Microsomes	67	132	12	478
Final supernatant	267	0	0	44

Phospholipid to protein ratio is higher in plasma membranes than in other cell fractions, indicating a high degree of membrane purification. The microsomal fraction is unusually low in phospholipid content possibly due to the great abundance of polysomes in the Ehrlich cells which sediment in this fraction and account for the high RNA content. DNA is most abundant in the low-speed washed pellet; however, a high degree of selective fractionation is accomplished on the density gradient since the plasma membrane fraction (Band 2) is relatively devoid of DNA.

Monoamine oxidase is a useful mitochondrial (outer membrane) marker[17] and the experimental results shown in Table II suggest that the plasma membrane fraction is relatively low in mitochondrial membrane activity. This is further supported by the low NADH oxidase activity of the plasma membrane fraction, since this catalytic activity is typical of inner mitochondrial membrane and, to a lesser extent, the microsomal fraction[17,18]. Glucose-6-phosphatase activity was very low for all cell fractions, and did not appear to be a useful microsomal marker enzyme in the Ehrlich cells as it is in liver. Low levels of glucose-6-phosphatase would be consistent with the metabolic and functional activities of the Ehrlich ascites tumor cells.

Activities of ($Na^+ + K^+$)-stimulated ATPase and K^+-stimulated phosphatase

TABLE II

DISTRIBUTION OF ENZYME ACTIVITIES IN CELL FRACTIONS FROM EHRLICH ASCITES TUMOR CELLS

Values given are specific enzyme activities: NADH diaphorase is in μmoles NADH oxidized per mg protein per min; monoamine oxidase is in nmoles/mg protein per h; all other values in μmoles/mg protein per h.

	NADH diaphorase	*Monoamine oxidase*	*Glucose-6-phosphatase*	*$(Na^+ + K^+)$-ATPase*	*K^+-stimulated phosphatase*
Whole homogenate	0.19	5.6	0.40	1.3	0.20
Final washed $600 \times g$ pellet (crude membranes and nuclei)	0.19	2.2	—	4.4	0.32
Plasma membranes (Band 2 from density gradient)	0.06	0.9	0.05	19.7	2.53
Mitochondria	0.90	29.7	0.81	1.7	0.07
Microsomes	0.16	1.5	0.15	0.6	0.07
Final supernatant	0.04	0.2	0.77	0	0

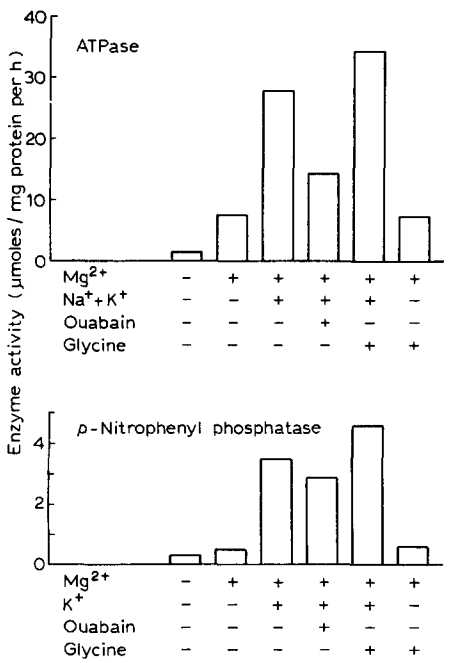


Fig. 5. General characteristics of ATPase and K^+-stimulated phosphatase (*p*-nitrophenyl phosphatase) in the plasma membrane fraction from Ehrlich ascites cells. Conditions for the enzyme assays were as described in Methods, in addition where indicated, the following concentration of reagents were used. ATPase: 55 mM Na^+, 16 mM K^+, $2 \cdot 10^{-5}$ M ouabain, and 17 mM glycine; *p*-nitrophenyl phosphatase: 25 mM K^+, $1.1 \cdot 10^{-5}$ M ouabain, and 17 mM glycine.

were clearly highest in the plasma membrane fraction. Both of these enzymes are closely associated with Na^+-transport function[19] and have been shown to be plasma membrane enzymes[7]. Except for the low speed washed pellet which included plasma membranes before purification, other cell fractions contained low to negligible amounts of Na^+ *plus* K^+-stimulated ATPase or K^+-stimulated phosphatase.

($Na^+ + K^+$)-stimulated ATPase and K^+-stimulated phosphatase

Some general characteristics of the plasma membrane ATPase and *p*-nitrophenyl phosphatase are shown in Fig. 5. A basal Mg^{2+}-requiring ATPase activity is apparent; specific activity ranged from 7.5 to 17 μmoles P_i liberated per mg protein per h. The ATPase was stimulated 3–4-fold over the basal rate by addition of Na^+ and K^+ (range 24–46 μmoles P_i liberated per mg protein per h). This stimulation by Na^+ *plus* K^+ was partially inhibited by ouabain (*e.g.* 50–60% inhibition by $2 \cdot 10^{-5}$ M ouabain).

Mg^{2+} was also required for *p*-nitrophenyl phosphatase activity. Basal enzyme activity, without K^+, was very low (range 0.1–0.8 μmole/mg protein per h) whereas the addition of 10–20 mM K^+ produced a 6–10 fold stimulation of hydrolysis rates (range 2.4–6.1 μmoles/mg protein per h). This stimulation by K^+ was only partially inhibited (approx. 20%) by $2 \cdot 10^{-4}$ M ouabain but in a few experiments it appeared that inhibition became more complete at higher levels of ouabain.

One characteristic of the ATPase and phosphatase which was of great interest concerned the effects of amino acids, such as glycine, on modifying enzyme activity. As shown in Fig. 5 glycine enhanced both the Na^+ *plus* K^+-stimulated ATPase and the K^+-stimulated phosphatase. Amino acids were without effect on the basal, Mg^{2+}-stimulated portion of enzyme activities and the amino acids had no significant effect on the ATPase of any cell fraction other than the plasma membranes.

A response for activation of ATPase by increasing concentrations of glycine is

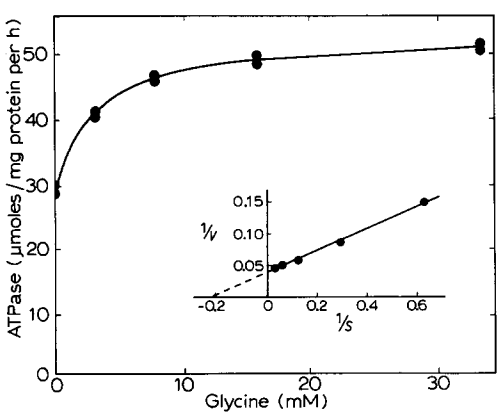


Fig. 6. Concentration-dependent effect of glycine in stimulating ($Na^+ + K^+$)-ATPase activity of Ehrlich cell plasma membranes. The points represent individual measurements of total ATPase (V_t) in the presence of 55 mM Na^+ and 16 mM K^+ in addition to the glycine levels shown. The basal rate for Mg^{2+}-dependent ATPase (V_b) with no glycine was 13.4 μmoles/mg protein per h. The insert represents a Lineweaver–Burk plot of the glycine activation curve where the reciprocal of the ($Na^+ + K^+$)-ATPase, ($1/V_t - V_b = 1/V$), is plotted against the reciprocal of glycine concentration ($1/S$).

shown in Fig. 6. In most cases the activation of both ATPase and K^+-stimulated phosphatase demonstrated saturation kinetics with an estimated K_a for glycine between 4.0 and 8.5 mM.

Various amino acids differed in their ability to stimulate enzyme activity as depicted in Table III. Most effective were the aromatic amino acids, histidine and phenylalanine. Separation of amino and carboxyl groups by more than one carbon atom (*i.e.* β or γ forms) reduced or abolished the ability to stimulate the enzyme activities. There was no significant difference between the D and L forms of either alanine or glutamic acid. In addition to the amino acids, the stimulatory effects of EDTA are also shown in Table III. Maximum activation of $(Na^+ + K^+)$-stimulated ATPase and K^+-stimulated phosphatase was produced by 0.5–1.0 mM EDTA; higher concentrations were definitely inhibitory (possibly because activity of Mg^{2+} was reduced).

To test whether the amino acids and EDTA were producing their stimulatory action by chelation of some contaminating divalent ion (*e.g.* Ca^{2+}) the following experiment was designed. Plasma membranes, prepared and washed in the usual way, were divided into three pools; one served as the control; the second was treated with 30 mM glycine (the simplest amino acid); and the third pool was treated with 0.5 mM EDTA. After a 30-min incubation at 0 °C the membranes were centrifuged, resuspended in 2 mM Tris (pH 8.0) and finally harvested by centrifugation. Relative

TABLE III

RELATIVE EFFECTS OF VARIOUS AMINO ACIDS IN STIMULATING $(Na^+ + K^+)$-ATPase AND K^+-STIMULATED PHOSPHATASE OF PLASMA MEMBRANES FROM EHRLICH ASCITES CELLS

All values are given as the percentage stimulation relative to the "control" value which is the $(Na^+ + K^+)$-ATPase or K^+-phosphatase activity for the respective columns. Numbers in parentheses are the number of membrane preparations used to compute the mean value given; for K^+-phosphatase two experiments were run in every case shown. Final concentration of amino acids was 11 mM.

	$(Na^+ + K^+)$-ATPase	*K^+-phosphatase*
Control	100	100
Glycine	132 (5)	201
L-Alanine	129 (5)	176
D-Alanine	125 (3)	151
L-Leucine	127 (5)	180
L-Glutamate	145 (4)	169
D-Glutamate	141 (2)	—
L-Lysine	158 (1)	—
L-Serine	138 (2)	—
L-Phenylalanine	161 (2)	240
L-Histidine	181 (2)	260
β-Alanine	108 (2)	93
2-Aminobutyric acid	—	113
3-Aminobutyric acid	—	99
4-Aminobutyric acid	—	104
EDTA	171 (2)	260

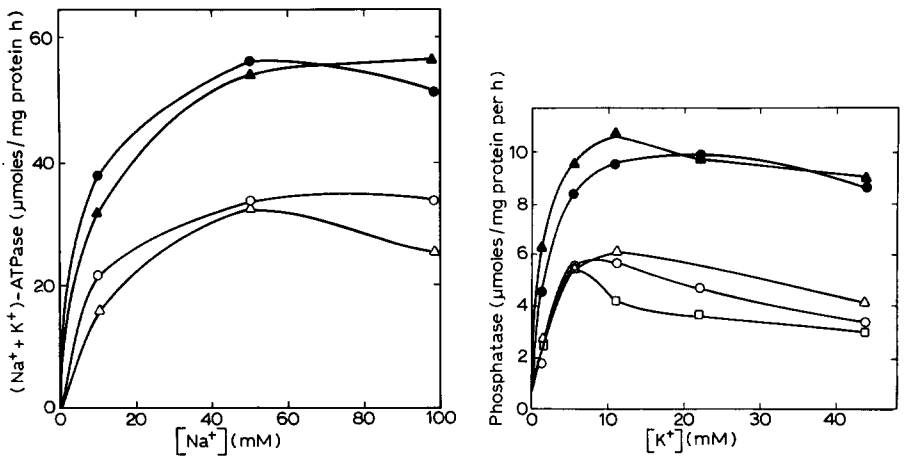


Fig. 7. Influence of Na^+ and K^+ on ATPase activity of Ehrlich ascites cell plasma membranes in the presence and absence of 8.8 mM L-histidine. Points represent ATPase activity above the value measured at 0 Na^+ concentration. 4 mM K^+ (○—○ and ●—●); 27 mM K^+ (△—△ and ▲—▲).

Fig. 8. *p*-Nitrophenyl phosphatase activity of Ehrlich cell plasma membranes under varying conditions of K^+ and Mg^{2+} with and without 10 mM L-histidine. Variations of [Mg^{2+}] were 1.4 mM Mg^{2+} (□—□); 3.7 mM Mg^{2+} (△—△ and ▲—▲), and 7.4 mM Mg^{2+} (○—○ and ●—●).

TABLE IV

EFFECT OF PREINCUBATING CONDITIONS ON THE RELATIVE RESPONSE OF ($Na^+ + K^+$)-ATPase AND K^+-PHOSPHATASE OF PLASMA MEMBRANES TO ADDED GLYCINE OR EDTA

All values are reported as the percentage stimulation relative to the K^+-stimulated activity (phosphatase) or the ($Na^+ + K^+$)-stimulated activity. Plasma membranes were either used directly or pre-incubated as shown for 30 min, then centrifuged, washed in 2 mM Tris buffer (pH 8.0) and finally resuspended for the assay shown here. For phosphatase all tubes included 22 mM K^+; for ATPase all tubes included 94 mM Na^+ and 26 mM K^+. In addition 11 mM glycine or 0.5 mM EDTA were added as shown.

	Fresh membranes	*Pre-incubated with 30 mM glycine*	*Pre-incubated with 0.5 mM EDTA*
K^+-stimulated phosphatase			
K^+	100	100	100
+11 mM glycine	224	225	268
+0.5 mM EDTA	320	329	257
($Na^+ + K^+$)-stimulated ATPase			
($Na^+ + K^+$)	100	100	100
+11 mM glycine	135	112	128
+0.5 mM EDTA	210	—	209

ATPase and *p*-nitrophenyl phosphatase activities of these three membrane pools are shown in Table IV. Activation of both the $(Na^+ + K^+)$-stimulated ATPase and K^+-stimulated phosphatase is still apparent in all three sets of preincubation and washing conditions. These results suggest that the activation phenomenon is not simply the chelation and removal of some "loosely bound" contaminating divalent metal ion.

Interactions between cation activation and amino acid stimulation of ATPase and phosphatase are shown in Figs 7 and 8, respectively. It is apparent that over a wide range of Na^+ concentration L-histidine is effective in enhancing total enzyme activity (Fig. 7). Stimulation of *p*-nitrophenyl phosphatase activity by L-histidine may be demonstrated over the full range of activating K^+ concentrations (Fig. 8). The interdependence between Mg^{2+} and other activating species is also shown in Fig. 8; it is apparent that amino acids remain equally effective over the range of Mg^{2+} concentrations shown.

DISCUSSION

One of the most frequently used procedures for the isolation of ascites cell membranes has employed intracellular cavitation with nitrogen to disrupt cells[6,7,20]. Such treatment typically fragments the cell envelopes into microsomal sized particles, thus separation from mitochondrial and microsomal elements is more tedious than for methods where the plasma membrane remains relatively intact. In the present communication we describe a method of effectively producing a plasma membrane preparation from Ehrlich ascites cells. Phase and electron microscopy suggest that the isolated plasma membranes are large fragments or ghosts. Examination of electron micrographs reveal that some contaminating "cytoplasmic" elements remain entrapped within membrane folds. However, enzymatic and chemical data suggest a rather low level of contamination and some preliminary experiments indicate that additional purification steps are feasible. For instance, we find that a rehomogenization of the plasma membrane preparation in 2.0 mM Tris buffer with a subsequent harvesting by differential centrifugation produced a further decrement (40% reduction) in NADH diaphorase and an increase (19%) in K^+-stimulated phosphatase activity.

We consistently find higher specific activity for the $(Na^+ + K^+)$-stimulated ATPase in our membrane preparation than that reported by others for Ehrlich plasma membranes[6,7,20]. This increase in activity may result from a greater membrane homogeneity in our preparation. However, the general characteristics of our ATPase system appear similar to the one studied by Wallach and Ullrey[20].

We were unable to discover a procedure which would permit the separation of the $(Na^+ + K^+)$-stimulated ATPase from the K^+-stimulated phosphatase enzyme. Moreover, since the effect of stimulatory agents, such as amino acids, were so similar for the two enzymes we would agree with others that they both may be manifestations of the same enzymic system.

The amino acid effect in stimulating the $(Na^+ + K^+)$-ATPase and the K^+-activated phosphatase of the Ehrlich cell plasma membrane is of special interest. Two questions of great concern: (a) By what mechanism do the amino acids stimulate catalytic activity and (b) Does this amino acid effect play a physiological role in transport activity of the cells[3]?

While this work was in progress two other groups published their observations

concerning the stimulation of ($Na^+ + K^+$)-ATPase by amino acids in microsomes isolated from chick brain[21] and kidney medulla[22]. Both of these groups presented evidence to suggest that the amino acid effect occurred *via* a chelation of some membrane-bound divalent ion, such as Ca^{2+}. On the basis of several points of evidence, we feel that a simple chelation-removal effect is unlikely. We have shown (Table IV) that membranes incubated and washed with amino acids or EDTA may still produce the typical response pattern to added amino acids. Thus the preincubating conditions which were sufficient to maximize the stimulatory effect were not sufficient to sequester the inhibitory metal. Furthermore, the oxyanions of ATP chelate Ca^{2+} much more effectively than amino acids[23], and one might expect a "protective substrate" effect if Ca^{2+} were the principal metal ion in question. It would appear more likely that if the activation of ($Na^+ + K^+$)-ATPase by amino acids occurred *via* a metal chelation, such interaction would involve some very tightly membrane-bound metal.

Stability, or formation, constants for most amino acid ligands and Ca^{2+} are low: on the other hand, these ligands avidly react with heavy metals like Co^{2+}, Ni^{2+} or Zn^{2+} with values ranging from 10^4–10^{10} M^{-1} for the recorded stability constants[23]. Such metals may be coordinated within the enzyme and thus restrict catalytic function at the active site or *via* an indirect allosteric action. Ternary complex formation could occur between the bidentate amino acid ligands, the metal and the enzyme ligand. The metal need not be removed providing that the stability constant between donor groups of the enzyme and two coordinate positions of the metal were sufficiently great. Although highly speculative, such an hypothesis would fit the present observations as well as those of two other studies[21,22] concerning amino acid stimulation of ($Na^+ + K^+$)-ATPase.

Whatever the precise mechanism of amino acid moderation, it is of interest to ascertain whether the observed activation of ($Na^+ + K^+$)-ATPase has a role in cellular transport function. Several problems must be considered regarding structural specificity. For instance, in the Ehrlich cell, features of amino acid transport kinetics and Na^+ dependency are functions of detailed molecular structure and the systems are highly stereospecific (*e.g.* see ref. 24). From Table III it may be seen that a Na^+-dependent amino acid such as alanine produces virtually the same responses as Na^+-independent leucine: moreover, there is little difference between D and L forms in the activation of ATPase or phosphatase. On the basis of a simple mechanism of direct coupling one might have expected the most profound stimulatory effects by the more avidly accumulated amino acids, However, the possibility of a multiple stage involvement between amino acid translocation and its effects on stimulating transport enzymes might be examined. For such a proposal the amino acid–carrier association reaction (structural specificity, *etc.*) might be sequentially separated from the activation of the energy-providing system (ATPase). For example, stimulating of the latter system by an amino acid might only occur at the inner plasma membrane interface once the amino acid has gained local access *via* the carrier system: thus structural specificity may differ for the separate sites.

Ehrlich ascites cells avidly accumulate many species of amino acids, and for some of these species there is evidence that this accumulation is energetically coupled to the passive entry of Na^+ by "co-transport". Such evidence forms the basis of the "Na^+-gradient hypothesis" which holds that through this coupling the energy required for amino acid accumulation is provided directly by the transcellular electro-

chemical potential gradient of Na^+, and possibly the inverse one of K^+ (ref. 25). There is reason to question, however, whether the coupled entry scheme can account for the total energy requirement[26,27], and whether a substantial fraction of the energy is fed into the system *via* a direct (chemi-osmotic) coupling of the amino acid transport to a metabolic reaction, *e.g.* to the splitting of ATP. In view of this uncertainty we could imagine at least two alternative mechanisms that would relate transport function to our finding that amino acids stimulate the $(Na^+ + K^+)$-ATPase. First, to the extent that the net accumulation of amino acids is directly coupled to the splitting of ATP, a specific ATPase, distinct from that connected with the $(Na^+–K^+)$-pump, might be involved. This enzyme might be activated by its proper substrate, namely the amino acid, and its function might also depend on an appropriate milieu of alkali ions, though not necessarily on ion gradients. Second, the amino acids could stimulate the ATPase connected with the $(Na^+–K^+)$-pump, which in turn maintains the ion gradients to drive the amino acid transport. We could imagine that large amounts of amino acids, while entering the cell *via* the Na^+-linked system, carry with them corresponding amounts of extra Na^+, which have to be re-extruded rapidly by the ion pump to restore the proper ion distribution and driving gradients. For this purpose the activity of the ion pump, and thus of the $(Na^+ + K^+)$-ATPase, might be regulated by the intracellular amino acid pool. Although it is not possible at the present time to unequivocally distinguish between such alternative interpretations, some scant evidence in favor of the latter one may briefly be mentioned here. The possibility of an amino acid effect on the Na^+ gradient (*e.g.* by stimulation of ATPase) would predict enhanced Na^+ transport and an apparent autocatalytic effect, or trans-stimulation, in the uptake of certain amino acids. The latter effect has been observed as an enhanced influx of glycine into ascites cells pre-loaded with glycine, although the experimental observers and others have interpreted the findings in terms of an exchange diffusion process[3,28]. Stimulation of Na^+ transport by amino acids is well documented in the intestinal epithelium[29,30]. One explanation holds that a greater Na^+ availability to the pump (*via* enhanced co-transport entry) will account for the observed stimulatory effects: however, a more specific effect of the amino acid on the ATPase can not rigorously be ruled out as the primary or support process.

The present characterization of an ATPase showing interaction between Na^+, K^+ and amino acids has thus far provoked more questions than are answered. Further experiments must be designed to test specific hypotheses concerning the nature of interaction between amino acids and active ion transport. Moreover, it will be of interest to apply such tests to membrane systems other than from the Ehrlich ascites cell.

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