BBA 75634

CHARACTERIZATION OF (Na^+,K^+)-ATPASE ISOLATED FROM EMBRYONIC CHICK HEARTS AND CULTURED CHICK HEART CELLS

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(Received January 12th, 1971)

SUMMARY

The (Na^+,K^+)-ATPase obtained from 14–16-day-old embryonic chick hearts (ventricles) was compared with that from trypsin-dissociated and denervated cultured chick embryonic heart cells. Two methods of enzyme preparation were studied: (1) isolation in mannitol, and (2) NaI extraction. Electron microscopy showed that the NaI preparation (100 000 $\times$ g fraction) consisted of membranes organized as vesicles. Ouabain produced 92% inhibition of the total ATPase activity in the NaI preparation compared with a 34% inhibition of the mannitol preparation. For the intact heart preparation, half-maximal inhibition occurred at $2.7 \cdot 10^{-6}$ M ouabain; K_m was about $4.6 \cdot 10^{-4}$ M ATP, and v_{max} was 91 μmoles P_i per h per mg protein. The (Na^+,K^+)-ATPase isolated from the cultured heart cells had about a 10-fold lower specific activity, but otherwise it had virtually identical characteristics. The optimal $MgCl_2$ concentration was 3 mM in the presence of 3 mM ATP, and the optimal K^+ concentration was 8 mM. Specific activity increased with Na^+ concentration and reached maximum at 50 mM. Li^+ did not substitute for Na^+. The optimal pH was 7.0–8.0, with marked depression of activity at acid pH. Ca^{2+}, Sr^{2+}, Mn^{2+}, and Ni^{2+} (2 mM) partially depressed the transport ATPase, and Ba^{2+} and Zn^{2+} almost completely depressed it. Tetracaine (2 mM) depressed the (Na^+, K^+)-ATPase. Temterature studies showed breaks in the Arrhenius plots at 25° and 15°; the Q_{10} values averaged 2.2 (25–35°) and 3.9 (15–25°) (activation energies of 14.5 and 23.1 kcal/mole, respectively). Many of these characteristics of the (Na^+,K^+)-ATPase are reflected in the electrical properties of the myocardial cell.

INTRODUCTION

Many of the electrophysiological properties of cultured heart cells grown as monolayers are similar to those of cells in intact hearts[1]. We have shown that ouabain, cocaine, and tetracaine produced depolarization and loss of electrical excitability in cultured heart cells, suggesting that the local anesthetics also inhibited active Na^+–K^+ transport; this inhibition could be rapidly reversed, concomitant with a marked hyperpolarization, by addition of either Sr^{2+} or Ba^{2+} (5–10 mM)[2]. Sr^{2+} transiently hyperpolarizes cells bathed in normal medium[3] or in high K^+ in which the resting potential should equal the K^+ diffusion potential[4], thus suggesting that Sr^{2+} stimulates the Na^+–K^+ pump. Sr^{2+} does not hyperpolarize cells depolarized

in Li^+–Ringer (Na^+-free) presumably because Li^+ cannot be actively extruded[4]. Cultured heart cells also become partially depolarized with cooling, two breaks occurring in the Arrhenius plots at about 26° and 14° (see ref. 5).

By using a lysis–sonication–centrifugation procedure[6,7], we obtained a (Na^+,K^+)-ATPase preparation from cultured chick heart cells which was inhibited by tetracaine, and this inhibition appeared to be reversed by Sr^{2+} and Ba^{2+}. These two divalent cations also increased the total ATPase activity. However, it seemed possible that the presence of some contaminating myosin-like ATPase could account for these actions of Sr^{2+} and Ba^{2+}. Similarly, the observed[6] increase in total ATPase activity when Li^+ was substituted for Na^+, combined with loss of ouabain sensitivity, could be caused by the presence of a contaminant ATPase.

The present communication is an investigation of some of the general characteristics of the (Na^+,K^+)-ATPase prepared from cultured chick heart cells and from intact embryonic chick hearts by more conventional methods. Attempts were made to correlate the effects of certain agents on the enzyme activity with some of their effects on the electrical properties of the intact cells. One advantage of cultured heart cells is that most of the enzyme isolated is probably derived from myocardial cells, and not from the vast numbers of other cells present in intact cardiac muscle.

MATERIALS AND METHODS

Fertilized chick eggs were incubated at 37°for 14–16 days. For each preparation, the hearts were removed from 30 chicks and placed in an extraction solution: histidine–mannitol–EDTA–deoxycholate containing 30 mM histidine (pH 6.8 at 0°), 0.25 M mannitol, 5 mM EDTA (as the Tris salt) and 0.1 % (w/v) deoxycholate. Any heavy-metal ion contaminants were removed from the mannitol solution by passage through an ion-exchange resin (AG 501 X 8, 20–50 mesh, Calbiochem). All steps were carried out at 0°. After the atria and blood vessels were removed, the ventricles were minced into fine pieces and transferred into 20 ml of fresh histidine–mannitol–EDTA–deoxycholate solution.

The cultured heart cells were prepared as described previously[1] from 14–16-day-old chick embryos, and cultured 4–14 days. Electrophysiological studies made on some of these cultures showed that most of the cells were myocardial cells and not fibroblasts or endothelioid cells, *i.e.* they had relatively large resting potentials, generated action potentials, and contracted. For each preparation, the culture medium from 20 cultures (40 mm diameter) was decanted and 1 ml of histidine–mannitol–EDTA-deoxycholate solution was added. Considerable loss of ATPase activity occurred if the Pucks medium was not replaced with this solution. The cells were carefully scraped off the culture dish with a Teflon-covered spatula.

Isolation of the mitochondrial and microsomal fractions was accomplished by a modification of the method of AHMED AND JUDAH[8]. The tissues were homogenized in histidine–mannitol–EDTA–deoxycholate solution using a Potter–Elvehjem homogenizer and making 8–12 passes with a motor-driven (1000 rev./min) Teflon pestle. The homogenate was centrifuged 5 min at 600 × *g* (all *g* values refer to the average force). The pellet was discarded and the supernate centrifuged 15 min at 8000 × *g*. This pellet was used for the mitochondrial fraction. The supernate was centrifuged 40 min at 100000 × *g* and this pellet was used for the microsomal fraction.

Method of isolation in mannitol

In this method, the 8000 × *g* pellet was suspended in a mannitol–Tris–EDTA solution containing 0.25 M mannitol, 30 mM Tris, and 1 mM EDTA (as the Tris salt), and stored at —20° until used. The 100 000 × *g* pellet was suspended in 20 ml mannitol solution using a Dounce homogenizer and centrifuged 40 min at 60000 × *g*. The supernate was discarded and the pellet was suspended in mannitol–Tris–EDTA solution and stored at —20°. The use of sucrose in place of mannitol did not give an enzyme preparation which had a sufficiently high degree of ouabain inhibition, as AHMED AND JUDAH[8] had found.

NaI extraction method

In this method, the 8000 × *g* and the 100000 × *g* pellets were suspended using the Dounce homogenizer in 1 mM EDTA (about 1 g of original wet tissue per ml). The suspension was diluted by 33.3% with a solution containing 6 M NaI, 15 mM EDTA, 7.5 mM $MgCl_2$, and 120 mM Tris–HCl. This mixture was held at 0° for 30 min with occasional stirring, diluted with 2.5 vol. of 1 mM EDTA to give a final NaI concentration of 0.8 M, and centrifuged 30 min at 35000 × *g*. The pellet was washed twice with 1 mM EDTA (being suspended each time with the Dounce homogenizer), suspended in 1 mM EDTA solution to give about 1 mg enzyme per ml, and stored at —20°. The enzyme preparation must be washed thorougly since the (Na^+,K^+)-ATPase is inhibited by I^- contamination[9].

Assay of ATPase activity

All salts used in the reaction system were recrystallized. Disodium ATP and tetrasodium EDTA were converted to the Tris salts by passage through an ion-exchange resin (Tris form of AG 50 W X 8, 200–400 mesh, H^+ form, Calbiochem). The standard reaction mixture (total of 2 ml) contained 3 mM ATP, 3 mM $MgCl_2$, 100 mM NaCl, 8 mM KCl, 50 mM Tris–HCl at pH 7.45, and about 100 μg enzyme. The microsomal fraction was always freshly prepared the same day. Reagent blank tubes without enzyme were always included in each experiment. In all cases, the assays were run in the absence of and presence of ouabain: 2 mM for the mannitol–Tris–EDTA method and 0.5 mM for the NaI method. The reaction mixture was preincubated 3 min at the desired temperature (usually 37°), and the reaction was started by ATP addition. The reaction was stopped by addition of 1 ml cold 15% trichloroacetic acid. The total liberation of P_i was usually below 10% of the total ATP present (6 μmoles ATP per vessel). The inorganic phosphate was determined by the method of FISKE AND SUBBAROW[10], and the protein by the method of LOWRY *et al.*[11], using crystallized bovine albumin as the standard. The results are reported as the specific activity of (Na^+,K^+)-ATPase [(Mg^{2+},Na^+,K^+)–(Mg^{2+},Na^+,K^+ + ouabain)] in μmoles P_i per h per mg protein.

RESULTS

Electron microscopy

Electron micrographs made of several of the NaI preparations isolated from intact embryonic chick hearts showed that membranes were predominant and that they were organized primarily as spherical vesicles (Fig. 1). The vesicles were of

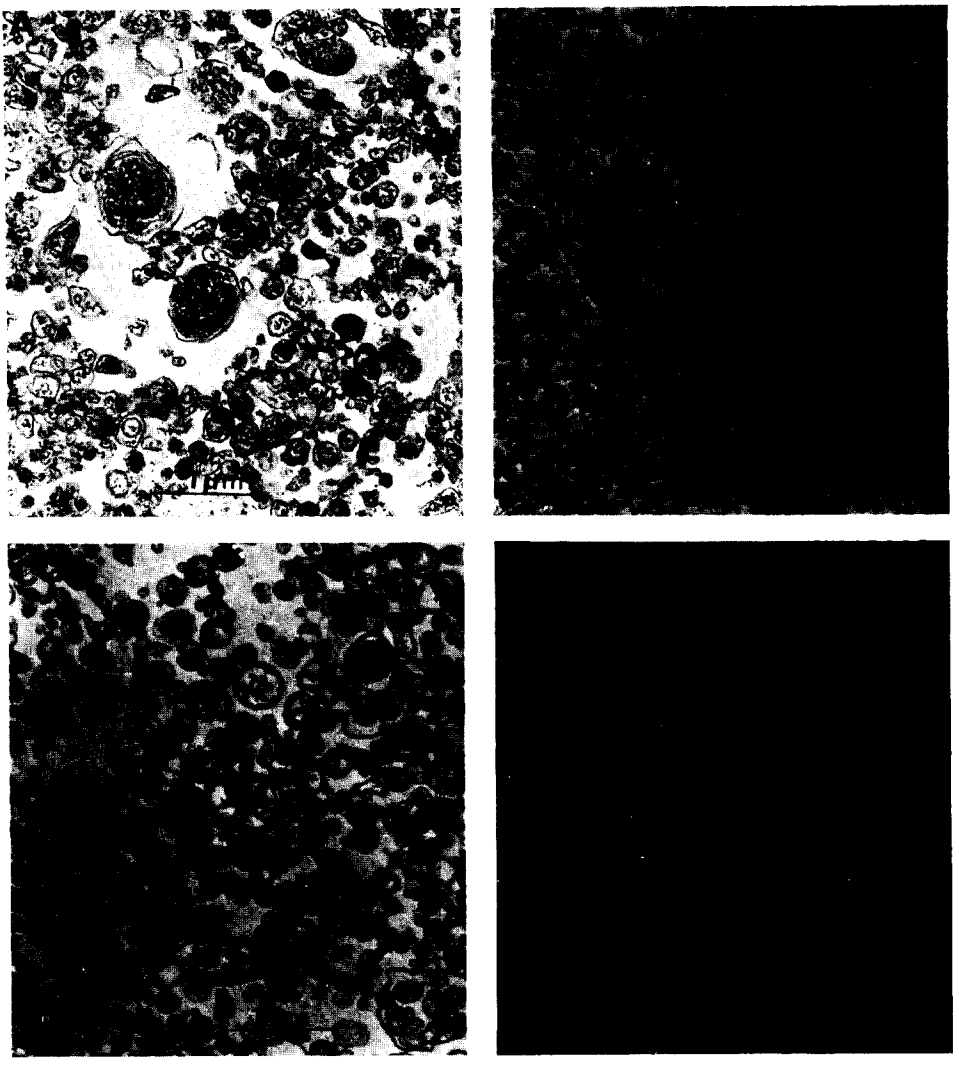

Fig. 1. Electron micrographs of enzyme preparation (100 000 × *g* fraction) from intact embryonic chick hearts isolated by the NaI extraction method and used for assay of (Na^+,K^+)-ATPase activity. The preparation was fixed with glutaraldehyde and stained with lead citrate and uranyl acetate. A. From one enzyme batch. B–D. From another enzyme batch illustrated at progressively higher magnifications. b.m., basement membrane present on inside of vesicles.

various sizes, but the majority had a diameter between 0.12 and 0.5 μm. In some vesicles, there were several layers of membrane.

Optimal ATP concentration

The optimal ATP-concentration for enzyme prepared from intact embryonic chick hearts (100000 × *g* fraction, mannitol–Tris–EDTA method) was determined. A plot of the specific activity of the (Na^+,K^+)-ATPase [(Mg^{2+},Na^+,K^+)–(Mg^{2+}, Na^+,K^+ + ouabain)] against ATP concentration is given in Fig. 2A. The

specific activity curve rose rapidly to a maximum at 3 mM ATP (equal to the $MgCl_2$ concentration). The ATP concentration used in the standard reaction mixture was 3 mM. A double reciprocal plot of these data (1/spec. act. *vs.* 1/[ATP]) gave a reasonably straight line over most of the range of substrate concentrations (Fig. 2B), indicating that the enzyme–substrate interactions obeyed Michaelis–Menten kinetics. The intercept gave a maximum velocity of 91 μmoles P_i per h per mg protein. An approximate K_m value of 0.46 mM ATP was estimated from the slope of the linear region of Fig. 2B and from Fig. 2A. This K_m value is relatively high, but is similar to the values found by others for the transport ATPase[12,13], *e.g.* $2.4 \cdot 10^{-4}$ M for that from calf cardiac muscle[13].

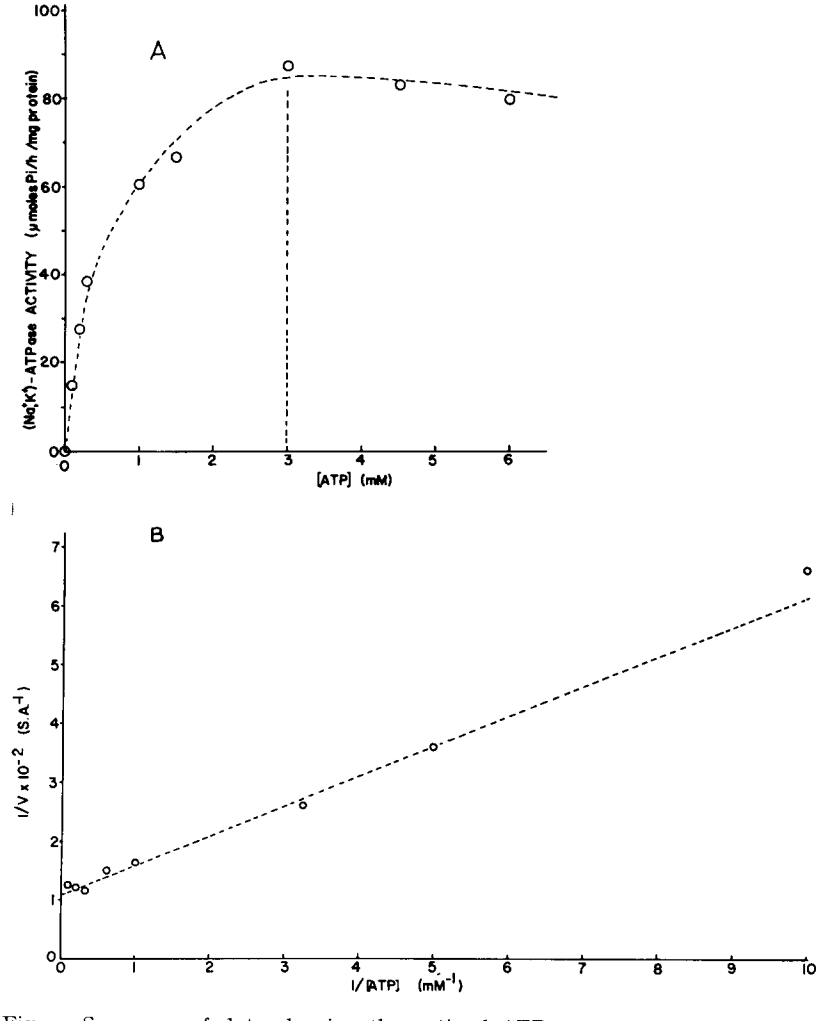

Fig. 2. Summary of data showing the optimal ATP concentration for the intact embryonic chick hearts (mannitol–Tris–EDTA preparation; 100 000 × *g* fraction; 37°). A. Plot of specific activity of the transport ATPase against ATP concentration; each point is the average of 2 experiments. B. Double reciprocal Lineweaver–Burk plot of the data given in A; curve fitted by eye. S. A., specific activity.

A plot of the amount of ATP hydrolyzed as a function of time in 2 experiments (one each on the NaI and mannitol–Tris–EDTA preparation, using 3 mM ATP) had an initial linear region with a steep slope and a bend to a second linear region of lower slope (about 59 % of the initial rate). The bend occurred at a time corresponding to a splitting of about 10 % of the total ATP present. Therefore, in most assays the reaction was stopped before 10 % of the ATP was split, and the data were discarded whenever more than 15 % was hydrolyzed.

Mg^{2+} activation

The (Na^+,K^+)-ATPase required Mg^{2+} for activation (Fig. 3). The specific activity curves, for enzyme prepared from intact embryonic chick hearts (100000 × g fraction) by both the mannitol–Tris–EDTA and NaI methods (determined in the standard reaction mixture containing optimal concentrations of K^+, Na^+, and 3 mM ATP and at 37°) rose rapidly to a maximum at 3 mM Mg^{2+}, and then decreased slightly at higher Mg^{2+} concentrations (Fig. 3). Therefore, the Mg^{2+} concentration used in the standard reaction mixture was 3 mM, and the optimal Mg^{2+}: ATP ratio was 1:1. The total ATPase activity also was optimum at 3 mM Mg^{2+}.

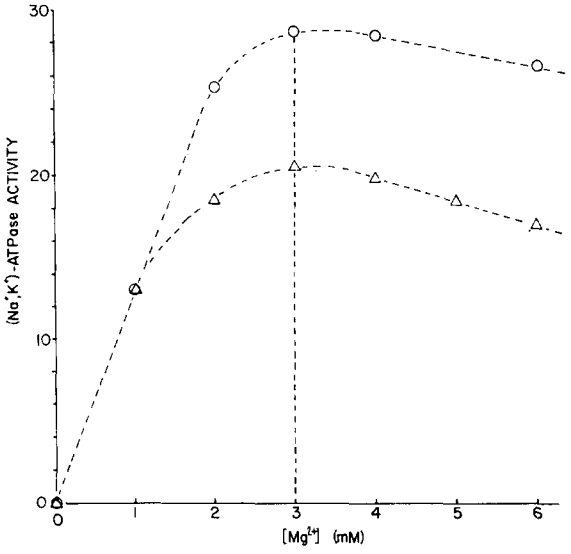

Fig. 3. Mg^{2+} activation of the transport ATPase activity obtained from intact embryonic chick hearts (100000 × *g* fraction; 37°). Reaction mixture included optimal concentrations of K^+ (8 mM), Na^+ (100 mM) and ATP (3 mM). Ordinate: specific activity (μmoles P_i per h per mg protein) of the (Na^+,K^+)-ATPase [(Mg^{2+},Na^+,K^+) – $(Mg^{2+},Na^+,K^+$ + ouabain)]. ○, NaI-extracted preparation; △, mannitol–Tris–EDTA preparation. Optimal enzyme activation occurred at 3 mM Mg^{2+}.

Optimal K^+ concentration for activation

The transport ATPase was activated by K^+, and the optimal K^+ concentration (determined in the standard reaction mixture) was 8 mM for enzyme prepared from both intact embryonic hearts (Fig. 4A) and cultured heart cells (Fig. 4B). This was also true for the transport ATPase present (at about 5- to 10-fold lower specific activity) in the 8000 × *g* fraction (Fig. 4). All data were obtained on the

NaI preparation except the curve labelled mannitol–Tris–EDTA in Fig. 4A. All the specific activity curves either remained flat or became depressed slightly above 8 mM K^+. Therefore, the optimal K^+ concentration of 8 mM was used in the standard reaction mixture.

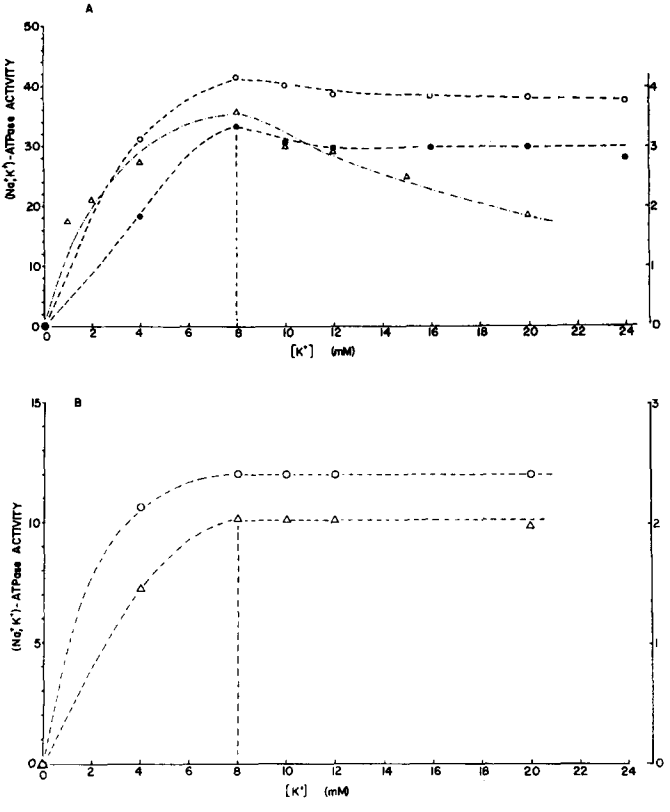

Fig. 4. Activation of the transport ATPase by K^+ (37°). Reaction mixture contained optimal concentrations of Na^+ (100 mM) and Mg^{2+} (3 mM). Ordinates: Specific activity (μmoles P_i per h per mg protein) of the (Na^+,K^+)-ATPase. A. Data for enzyme from intact embryonic chick hearts (NaI preparation) for the 100 000 × *g* fraction (○) and 8000 × *g* fraction (●; right-hand ordinate); the triangles give the mean data from 3 experiments on the mannitol–Tris–EDTA preparation, 100 000 × *g* fraction (left-hand ordinate × 0.5). B. Data for enzyme from cultured chick heart cells, 100 000 × *g* fraction (○) and 8000 × *g* fraction (△, right-hand ordinate); NaI preparation. The optimal K^+ concentration is 8 mM.

Activation by Na^+

The transport ATPase from both intact embryonic hearts (Fig. 5A) and cultured heart cells (Fig. 5B) was activated by Na^+. The specific activity curves of enzyme prepared by either method, determined in the standard reaction mixture containing 8 mM K^+, rose rapidly from levels at or near zero in 0 mM Na^+, and reached a plateau at about 50 mM. The results were similar for the transport ATPase present in the 8000 × *g* fractions (Fig. 5). Since higher Na^+ concentrations did not depress the activity, 100 mM Na^+ was used in the standard reaction mixture. There were no substantial differences in results when (a) no ion was substituted for the Na^+ omitted,

(b) Li^+ or choline$^+$ was substituted for Na^+ (sum of $[Na^+]$ + $[Li^+]$ and $[Na^+]$ + [choline$^+$] maintained constant at 100 mM), or (c) the Li^+ concentration was varied by choline$^+$ substitution ($[Li^+]$ + [choline$^+$] held at 100 mM in 2 experiments) and the results compared on the same enzyme preparation in which $[Na^+]$ + [choline$^+$]

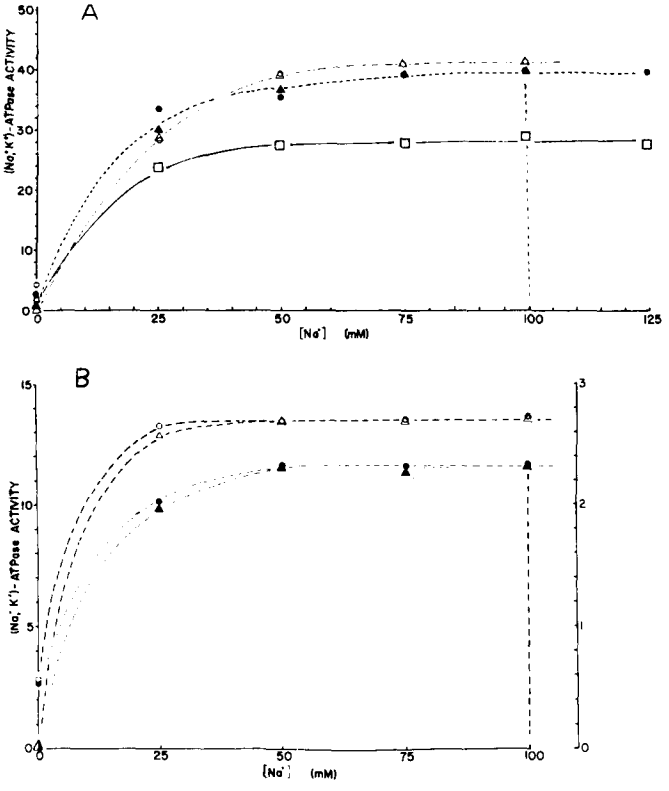

Fig. 5. Activation of the transport ATPase activity by Na^+. Reaction mixture (37°) contained optimal concentrations of K^+ (8 mM) and MgATP (3 mM). A. Intact embryonic chick hearts. NaI method: 100 000 × *g* fraction (○, △) and 8000 × *g* fraction (●, ▲, ordinate × 0.1). △, ▲, experiments in which nothing was substituted for the NaCl omitted (ionic strength varied); ○, ●, LiCl substituted for the NaCl such that $[Na^+]$ + $[Li^+]$ = 100 mM (except at 125 mM). Mannitol–Tris–EDTA method (100 000 × *g* fraction); □, give mean of data from 3 experiments in which the NaCl concentration varied by substitution with choline chloride. B. Cultured chick heart cells, NaI method, 100 000 × *g* fraction (○, △) and 8000 × *g* fraction (●, ▲, right-hand ordinate). ○, ●, Li^+ substituted for Na^+; △, ▲, no substitute for NaCl omitted.

was held constant (*i. e.* half of enzyme exposed to varying concentrations of Li^+ in complete absence of Na^+ and other half exposed to varying concentrations of Na^+, choline$^+$ being substituted in both cases). In 0 mM Na^+, 100 mM Li^+ or choline$^+$ gave a small degree (14–22 %) of activation (Fig. 5). This small activation by Li^+ and choline$^+$ in the absence of Na^+ is probably non-specific. Thus, Li^+ does not substitute for Na^+ in activation of the (Na^+,K^+)-ATPase. Because the curves were similar when no ion was substituted for Na^+ as when Li^+ or choline$^+$ were used, these data indicate that, over a wide range, ionic strength must not affect the specific activity of the (Na^+,K^+)-ATPase. This agrees with the observations on brain microsomal ATPase activity[14].

TABLE I

SUMMARY OF DATA SHOWING EFFECT OF DIVALENT CATIONS, TETRACAINE, AND DIPHENYLHYDANTOIN ON (Na^+,K^+)-ATPase OBTAINED FROM EMBRYONIC CHICK MYOCARDIAL CELLS

All data at 35° or 37°. Numbers in parentheses give the numbers of experiments. Data on divalent cations given in Fig. 9 not included in this table.

Addition	*% of control specific activity*							
	Intact embryonic hearts				*Cultured heart cells*			
	100 000 × g		*8000 × g*		*100 000 × g*		*8000 × g*	
	%	*n*	%	*n*	%	*n*	%	*n*
Ouabain (0.5 mM)*	12	(7)	60	(2)	4	(2)	—	
Ouabain (2 mM)**	62	(5)	—		70	(4)	71	(6)
Ba^{2+} (2 mM)*	19	(2)	51	(1)	8	(1)	—	
Ouabain + Ba^{2+}*	—		—		4	(1)	—	
Sr^{2+} (2 mM)	65	(7)*,**	67	(1)*	67	(2)*,**	82	(3)**
Ouabain + Sr^{2+}*	—		—		4	(1)	—	
Zn^{2+} (2 mM)*	21	(2)	—		5	(1)	—	
Ni^{2+} (2 mM)*	85	(2)	—		63	(1)	—	
Mn^{2+} (2 mM)*	53	(2)	—		61	(1)	—	
Tetracaine (2 mM)	13	(1)*	35	(1)*	48	(3)**	31	(3)**
Tetracaine + Sr^{2+}	11	(1)*	34	(1)*	48	(3)**	28	(3)**
Tetracaine + Ba^{2+}	8	(1)*	34	(1)*	—		29	(3)**
Diphenylhydantoin (2 mM)**	102	(3)	—		—		—	

* NaI method.
** Mannitol–Tris–EDTA method.

Ouabain sensitivity

In the NaI-extracted preparation, the inhibition of the total ATPase activity (100000 × *g* fraction) produced by 0.5 mM ouabain was 88 % for intact hearts and 96 % for cultured heart cells (Table I). Since a complete log dose–response curve (Fig. 6) showed that 0.5 mM ouabain was just sufficient to give maximal inhibition, this was the dose used to inhibit the transport ATPase in all experiments on the NaI preparation. The dose for half-maximal inhibition was about $2.7 \cdot 10^{-6}$ M (Fig. 6). Ouabain, at the lower concentrations, was somewhat less effective in presence of elevated K^+ concentration of 24 mM. The degree of ouabain (0.5 mM) inhibition of the 8000 × *g* fraction averaged 40 % in two experiments (Table I).

In the mannitol–Tris–EDTA preparation, of the total ATPase activity (transport ATPase *plus* other nonspecific ATPases) present in the 100000 × *g* fraction, the inhibition produced by 2 mM ouabain averaged 38 % for intact hearts and 30 % for cultured heart cells (Table I). Ouabain inhibition of the total ATPase activity of the 8000 × *g* fraction from the cultured cells averaged 29 % (Table I).

Effect of pH

The effect of pH on the (Na^+K^+)-ATPase activity is summarized in Fig. 7. The pH was varied from 4.95 to 9.00 using two buffer systems: Tris–HCl for pH 7.45–9.0 and Tris–maleate for pH 7.45–4.95. For the intact hearts (Fig. 7A), the transport ATPase (100000 × *g* fraction) had maximum activity at pH 7.45 for the NaI preparation (filled triangles) and at pH 8.0 for the mannitol–Tris–EDTA

preparation (unfilled symbols). There was a slight decrease in specific activity at higher pH, but a pronounced decrease occurred at lower pH values, such that the specific activity at pH 4.95 was only about 16–20 % of maximum. The pH sensitivity of the nonspecific ATPase was similar to that of the (Na+,K+)-ATPase.

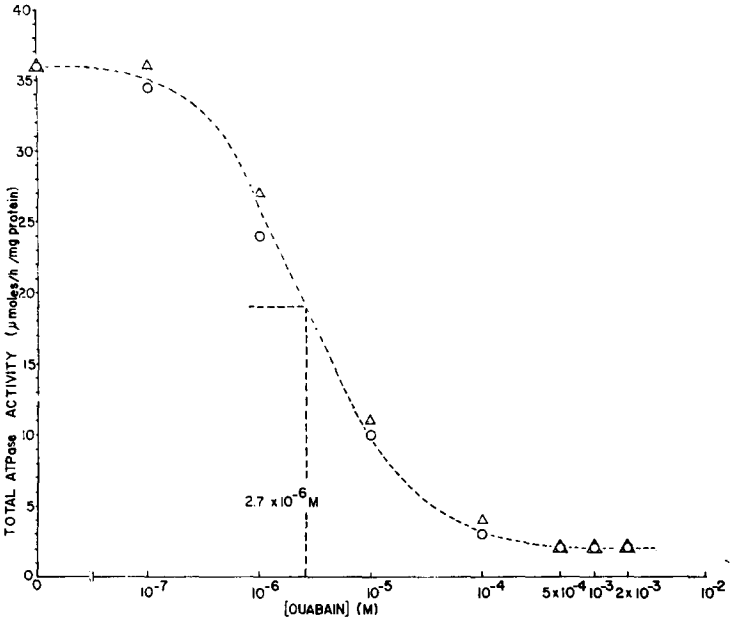

Fig. 6. Dose–response curve for ouabain inhibition of the total ATPase activity obtained from intact embryonic chick hearts (NaI extracted; 100000 × *g* fraction; 37°). Ordinate: total specific activity of ATPases present. Abscissa: ouabain concn. on a logarithmic scale. ○, data at 8 mM K^+, the optimal concentration; △, 24 mM K^+. Half-maximal inhibition occurred at $2.7 \cdot 10^{-6}$ M ouabain; 0.5 mM ouabain gave maximal inhibition of 95 %.

For cultured heart cells (Fig. 7B) the optimal pH was 7.45 for enzyme prepared by either method. Again, there was a prominent decrease in activity at low pH. However, high pH had a more pronounced inhibitory effect than the case of enzyme from intact hearts. The (Na+,K+)-ATPase activity in the 8000 × *g* fraction had a similar pH sensitivity (Fig. 7B, filled symbols).

Effect of temperature

The effect of temperature on the specific activity of the (Na+,K+)-ATPase (100000 × *g* fraction) prepared by the NaI method was determined. For the intact hearts, two breaks occurred in the Arrhenius plots, one at 25° and the other at 15°. The Arrhenius plot of the means of the data from 3 experiments is given in Fig. 8 (unfilled circles, continuous line) and clearly shows breaks at 25° and 15°. The calculated Q_{10} values were 2.3 (25–35°), 3.7 (15–25°), and 2.4 (0–15°). The corresponding activation energies are 15.0 (25–35°), 23.6 (15–25°), and 13.4 kcal/mole (0–15°). The temperature dependence of the (Na+,K+)-ATPase present in the 8000 × *g* fraction of the intact hearts was similar.

The effect of temperature on the transport ATPase of the 100000 × *g* fraction

from cultured heart cells was similar to that for intact hearts. There were also two breaks in the Arrhenius plots, at 25° and 15°, and the Q_{10} values also were similar. The Arrhenius plot of the mean data of 3 experiments showed the breaks (Fig. 8;

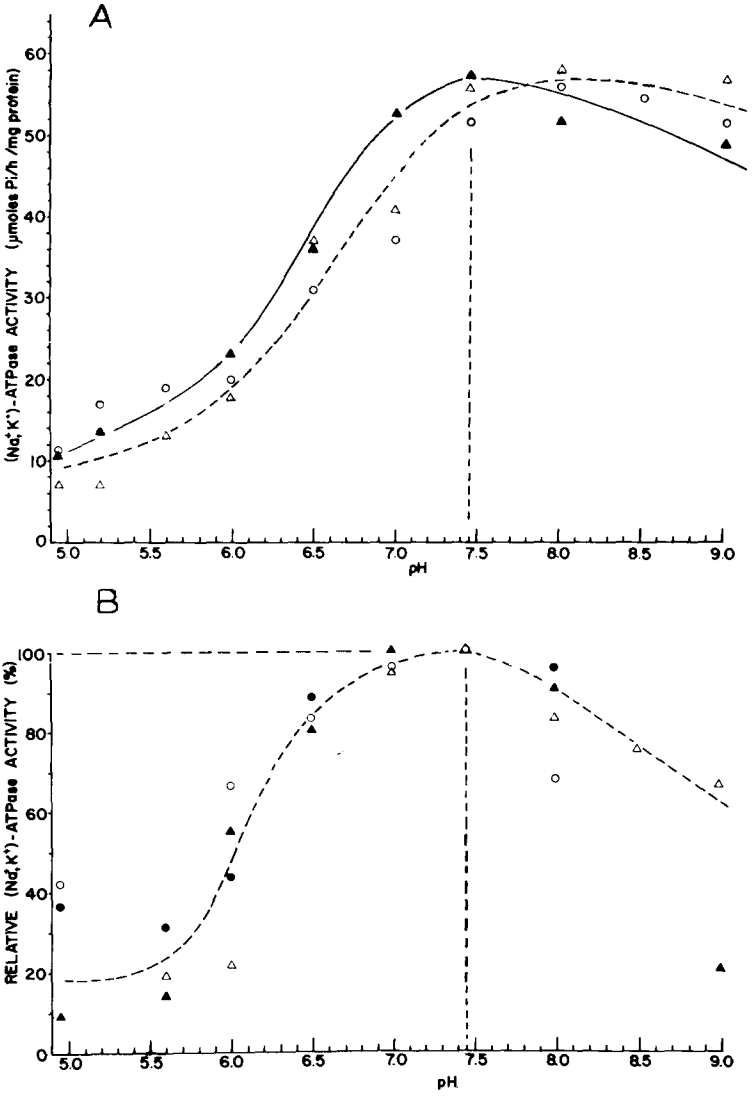

Fig. 7. Effect of pH on activity of transport ATPase (at 37°). The pH was varied from 4.95 to 9.00 using two overlapping buffer systems: 50 mM Tris–HCl (pH 7.45–9.00) and Tris–maleate (pH 7.45–4.95). The Tris–maleate buffer gave a slightly lower specific activity than the Tris–HCl buffer at pH 7.45, and all Tris–maleate values were slightly corrected by normalizing them to the Tris–HCl value. A. Intact embryonic chick hearts (100 000 × *g* fraction); NaI method (▲) and mannitol–Tris–EDTA method (△, ○, ordinate × 0.33). B. Cultured chick heart cells. Relative specific activity (in percent) of the maximum (Na^+,K^+)-ATPase activity at pH 7.45 is plotted against pH. Measurements made on 100 000 × *g* fraction (○, △) and 8000 × *g* fraction (●, ▲) of the same batch. Data from 3 experiments are plotted, one on the NaI extracted preparation (○, ●) and the mean of two on the mannitol–Tris–EDTA preparation (△, ▲).

unfilled triangles, dotted line). The Q_{10} values were about 2.1 (25–35°), 3.5 (15–25°), and 1.9 (0–15°), and the corresponding activation energies are 13.4 (25–35°), 22.5 (15–25°), and 9.46 kcal/mole (0–15°).

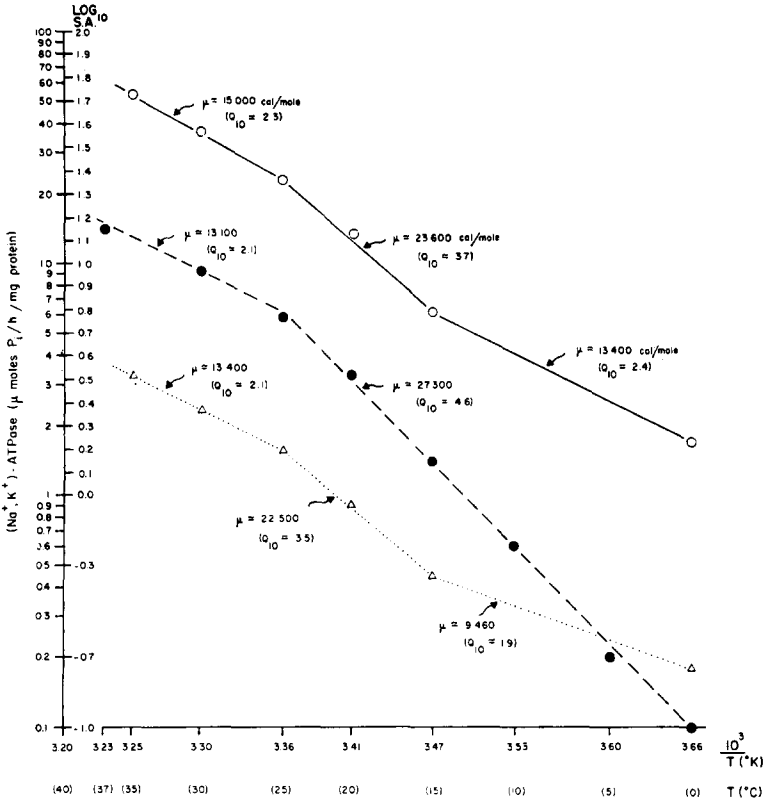

Fig. 8. Effect of temperature on transport ATPase activity in the 100 000 × *g* fraction of embryonic chick myocardial cells. Data presented as Arrhenius plots. Temperature was varied from 0 to 35° by means of thermostatically-controlled water baths; measurements made on a series of samples taken from the same enzyme batch. Enzyme prepared from intact embryonic chick hearts (○, ●) and from cultured chick heart cells (△) by NaI method (○, △) and by mannitol–Tris–EDTA method (●). Each point plotted represents the mean of 3 experiments. Ordinate: specific activity (S.A.) of transport ATPase on a logarithmic scale (corresponding logarithms also given). Abscissa: the reciprocal of the absolute temperature × 10^3 (corresponding temperatures in ° also given). In all 3 curves, a break occurs at 25° and, in 2 of the 3, a second break occurs at 15°. The Q_{10} values and the corresponding activation energies are indicated.

The temperature dependence of the (Na+,K+)-ATPase from the intact hearts (100 000 × *g* fraction) prepared by the mannitol–Tris–EDTA method was similar to that for the NaI preparation. The transport ATPase activity also had breaks in the temperature curve at 25° and 15°. The Arrhenius plot of the means of the data from 3 experiments and the activation energies are given in Fig. 8 (filled circles, dashed line); only the break at 25° is clearly seen. The Q_{10} values were about 2.1 (25–37°) and 4.6 (15–25°). The effect of temperature on the ouabain-insensitive ATPase was similar to that on the transport ATPase.

In the case of cultured heart cells, the degree of ouabain inhibition of the ATPase prepared from cultured heart cells by the mannitol–Tris–EDTA method was too small to allow the effect of temperature on the transport ATPase to be determined with accuracy. However, the ouabain-insensitive ATPase also had two breaks in the temperature curve at 25° and 15°, and the Q_{10} values were 2.2 (25–37°), 2.7 (15–25°) and 2.1 (0–15°).

To prove that the effect of temperature on the activity of the transport ATPase was not due to changes in pH of the incubation medium, the effect of temperature on the pH of the Tris buffer was measured. The buffer pH increased only a few tenths of a pH unit upon cooling from 37° to 15° (Tris–HCl has a reported change of 0.031 pK_a unit/°, ref. 15); this should have little effect on ATPase activity (Fig. 7). Thus, the slight shift in pH in the alkaline direction with cooling is much too small to account for the large effect on enzyme activity.

Inhibition by divalent cations and by tetracaine

Addition of Ca^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Ni^{2+}, or Mn^{2+} (all at 2 mM) inhibited the total ATPase activity of all enzyme preparations used (Table I). Ba^{2+} and Zn^{2+} were more potent inhibitors than Sr^{2+}, Mn^{2+}, and Ni^{2+}. Ba^{2+} or Sr^{2+} did not protect the total ATPase activity against ouabain inhibition. The data from an additional experiment is given in Fig. 9 in which the activity of the (Na^+,K^+)-ATPase from intact hearts (NaI method, 100000 × *g* fraction) was determined at various concen-

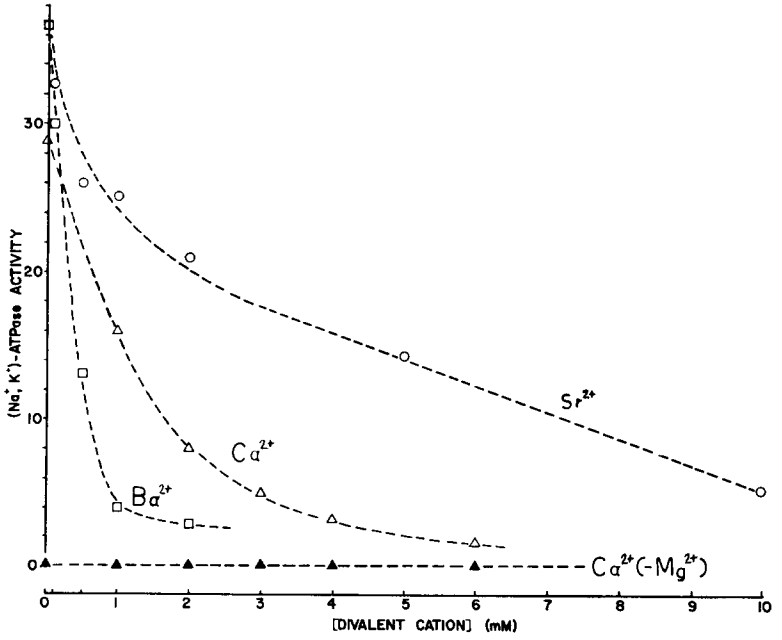

Fig. 9. Inhibition by Ca^{2+}, Sr^{2+}, and Ba^{2+} of the transport ATPase activity isolated from intact embryonic chick hearts (100000 × *g* fraction, NaI extraction method, 37°). The reaction medium contained optimal K^+ concentration (8 mM), Na^+ concentration (100 mM), and [MgATP] concentration (3 mM) (○, □, △). One Ca^{2+} experiment was done in the absence of Mg^{2+} (▲), and shows that Ca^{2+} cannot substitute for Mg^{2+} in activation of the enzyme. With full enzyme activation in presence of Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} inhibit.

trations of $CaCl_2$, $SrCl_2$, and $BaCl_2$. It is seen that Ca^{2+} does not activate the enzyme in the absence of Mg^{2+} (filled triangles). In the presence of 3 mM $MgCl_2$, $CaCl_2$ produces near-maximal inhibition at 6 mM and half-maximal inhibition at about 1.1 mM. The inhibition produced by Ca^{2+} was greater than that produced by Sr^{2+}, but less than that of Ba^{2+}. Half maximal inhibition by $SrCl_2$ occurs at about 2.8 mM, and that by $BaCl_2$ at 0.4 mM. Ba^{2+} produced near-maximal inhibition at 2 mM.

Tetracaine markedly inhibited the total ATPase activity in both fractions (Table I). The degree of inhibition produced by 2 mM tetracaine on the 100000 $\times$ g fraction (NaI preparation) was about the same as that produced by 2 mM ouabain. Tetracaine produced substantially greater inhibition of the 8000 $\times$ g fraction than did ouabain, indicating that tetracaine is probably a nonspecific inhibitor of several ATPases. Addition of $SrCl_2$ or $BaCl_2$ (2 mM) did not protect the enzymes from the inhibitory action of tetracaine (Table I).

Sodium diphenylhydantoin, an anticonvulsive and anti-arrhythmic agent postulated to affect active cation transport, had no effect on the activity of the transport ATPase isolated from the intact hearts (mannitol–Tris–EDTA method) at a high concentration of $2 \cdot 10^{-3}$ M (Table I).

DISCUSSION

The standard methods for preparation of the (Na^+,K^+)-ATPase from other tissues yields preparations from cardiac muscle which often contain large quantities of a myosin-like ATPase[16–19]. Salt extraction was used to separate transport ATPase from cardiac muscle homogenates using LiBr[20] or microsomes using NaI[13, 19–21]; this method selectively retains an insoluble (Na^+,K^+)-ATPase and removes other cardiac ATPases[21]. The present results indicate that of the two methods of preparation used, the NaI extraction yielded an enzyme preparation which had a much higher ratio of (Na^+,K^+,Mg^{2+})-ATPase to Mg^{2+}-ATPase, as evidenced by the greater ouabain inhibition. This method selectively removes or effectively suppresses other ATPase activities. Thus, prolonged cold storage to increase the ratio[13] is not necessary. However, the specific activity of the (Na^+,K^+)-ATPase often was not much better for the NaI preparation. Since electron microscopy of the NaI preparation showed that it consisted of membranes arranged as small vesicles of rather homogeneous size and without myofibrils, it would appear that this method gives a good preparation of the transport ATPase used in the active transport of Na^+ and K^+ at the myocardial cell surface[22].

The specific activity of the (Na^+,K^+)-ATPase in the 100000 $\times$ g fraction was 5–10 times greater than in the 8000 $\times$ g fraction; the lower ratios were generally obtained with the cultured cells. The activity observed in the 8000 $\times$ g fraction may represent activity present in the mitochondrial membranes and/or the presence of some contaminating microsomes. The degree of ouabain inhibition of the 8000 $\times$ g fraction was about the same as that of the 100000 $\times$ g fraction. The ouabain sensitivities of enzyme prepared from cultured embryonic chick heart cells and intact embryonic chick hearts were similar. The finding of half-maximal inhibition at $2.7 \cdot 10^{-6}$ M ouabain is similar to that for cardiac transport ATPase isolated from other sources[13,21]. The only slight diminution of the ouabain inhibition produced by elevation of K^+ concentration from 8 to 24 mM in this preparation was surprising.

Greater competition between K^+ and ouabain has been demonstrated for transport ATPase from heart[13] and other tissues[12,23,24], and ouabain inhibition of the cation pump in intact cells is prevented by $[K^+]_o$ levels of 25 mM[23,25].

The specific activities of the (Na^+,K^+)-ATPase and the ouabain-insensitive Mg^{2+}-ATPase were consistently about tenfold lower in cultured heart cells than in intact hearts. This finding is consistent with the finding of lower resting membrane potentials in cultured heart cells[1]. Since the membrane (Na^+,K^+)-ATPase is involved in active transport of Na^+ and K^+ across cell membranes[12,22,26], the ratio of Na^+:K^+ pump/leak current may be lower in cultured heart cells than in intact hearts of the corresponding embryonic age. This could mean that changes occur in the myocardial cell membranes during the culturing process. For example, the exposure of the cells to trypsin could directly affect the membrane ATPases or the denervation could produce an indirect effect. It is also possible that some of the higher activity of the membrane ATPase in the case of the intact hearts is due to the presence of neurons and other types of cells. Consistent with either of the above possibilities, preliminary results indicate that trypsin dispersion of the ventricular myocardial cells (as for preparation of cultured cells) followed by immediate isolation of the ATPase yields a preparation having low specific activity comparable to that of the cultured cells.

The transport ATPase had an absolute requirement for Mg^{2+}, K^+, and Na^+ for activation. The activity was zero or very nearly zero in the absence of any one of these cations. The optimal concentrations for activation in the presence of 3 mM ATP were 3 mM $MgCl_2$, 8 mM K^+, and >50 mM Na^+; thus, the optimal ratio of $[Na^+]/[K^+]$ was about 6. These findings are in general agreement with the findings of others[13,21,23,27]. The requirements of the enzyme for K^+ and Na^+ are consistent with the facts that in intact cardiac muscle cells low $[K^+]_o$ inhibits active Na^+ transport[25] and that the active Na^+ extrusion rate is a function of $[Na^+]_i$. The finding that Li^+ could not substitute for Na^+ agrees with the observation that Li^+ can not be actively pumped out of myocardial cells[4]. Thus, the hydrolytic activity of the enzyme appears to parallel its cation transport activity. The finding[6] that Li^+ stimulates the total ATPase activity in the sonicated preparation of cultured heart cells and that ouabain did not prevent the Li^+ stimulation, probably reflects the presence of contaminant ATPases; alternatively, sonication may alter the characteristics of the transport ATPase. SKOU[28,29] has shown that Li^+ can partially replace K^+ as an activator of the transport ATPase.

The optimal pH for the transport ATPase was 7.45 in all preparations except one in which it was 8.0. There was a pronounced decrease in activity at acid pH in all preparations. The pH sensitivity of the non-specific ATPase was similar. The pH optimum for (Na^+,K^+)-ATPase prepared from calf cardiac muscle had a sharp peak at 7.2[13], and that for mammalian erythrocytes was about 7.5[23]. Although estimates of intracellular pH in muscle give values of 5.9–6.5[30], which is below the optimum, it is possible that the extracellular pH is most influential because of stereospecificity of the membrane enzyme.

The effect of temperature on the isolated enzyme has some similarities to its effect on the membrane potentials of cultured heart cells. Partial depolarization of the intact cells is produced by cooling[5]. The two breaks in the Arrhenius plots consistently observed at 25° and 15° for all enzyme preparations agree with the breaks at about 25–27° and at 13–15° observed in plots of resting potential and

frequency of spontaneous discharge against temperature[5]. The average Q_{10} values for the myocardial (Na^+,K^+)-ATPase were 2.3 (25–35°), 3.8 (15–25°), and 2.1 (5–15°); the Q_{10} was always greatest between 15 and 25°. The fact that activation energy varies as a function of temperature is consistent with a multi-step overall enzyme reaction, such as the Na^+-dependent phosphorylation by ATP and K^+-dependent dephosphorylation steps[31]. The Na^+–K^+ pump has a Q_{10} of about 3. However, the resting potentials of cultured heart cells show little change with temperature over the range of 40–27°, but between 25 and 15° there is some depolarization ($Q_{10} = 1.5$–1.9); the cells become inexcitable at 8–13° ($Q_{10} > 3.3$)[5]. The Q_{10} for frequency of discharge is about 1.9 over 25–15°, corresponding to a Q_{10} of nearly 6 for slope of the pacemaker potential; the Q_{10} is smaller at higher temperatures and much greater at cooler temperatures[5]. The transport ATPase isolated from rat brain also had two breaks in the Arrhenius plots, one at 18° and the other at 6°, and the Q_{10} values were 12.6 (0–6°)[32], approx. 4 (6–18°), and 1.5 (18–37°); but a Q_{10} of approx. 3 between 20 and 30° was obtained for this same enzyme[14].

Tetracaine produced marked inhibition of the total ATPase activity in all enzyme preparations, as it did in the sonicated preparation of cultured heart cells[6]. The degree of inhibition of the 100000 × *g* fraction produced by 2 mM tetracaine was about the same as that produced by similar concentrations of ouabain. However, tetracaine may be a nonspecific inhibitor of several ATPases since it inhibited the 8000 × *g* fraction substantially greater than did ouabain. The results indicate that the depolarization of myocardial cells caused by local anesthetics is due to inhibition of the Na^+–K^+ pump. Local anesthetics have also been reported to inhibit active Na^+ transport in frog skin[33] and red blood cells[34].

Although requiring Mg^{2+}, the transport ATPase is inhibited by other divalent cations in approximately the following order of potency: $Ba^{2+} \simeq Zn^{2+} > Ca^{2+} > Sr^{2+} > Mn^{2+} \simeq Ni^{2+}$. Inhibition by Ca^{2+} is characteristic of the (Na^+,K^+)-ATPase[29]. The almost complete inhibition produced by 2 mM Ba^{2+} or Zn^{2+} was similar to that produced by an equal concentration of ouabain. However, half-maximal inhibition by Ba^{2+} is about $4 \cdot 10^{-4}$ M compared to the value of $2.7 \cdot 10^{-6}$ M for ouabain. The inhibitions produced by divalent cations and ouabain did not appear to be additive. The inhibition by the divalent cations may result from competition with Mg^{2+}, K^+, or Na^+ for binding sites on the enzyme[23]. Zn^{2+} and Mn^{2+} also inhibited the (Na^+,K^+)-ATPase isolated from rabbit kidney cortex[35]. Since Ba^{2+} or Sr^{2+} did not protect the total ATPase activity against ouabain or tetracaine, the presence of some contaminating myosin-like ATPase may account for the 'stimulating' and 'protective' actions of Sr^{2+} and Ba^{2+} previously reported for the sonicated preparation[6]. That is, Sr^{2+} and Ba^{2+} may activate a myosin ATPase activity in the absence of Ca^{2+} and thereby mask the inhibition of the transport ATPase produced by ouabain and tetracaine. Thus, the explanation for the action of Sr^{2+} and Ba^{2+} in rapidly stimulating the cation pump[2–4,36] remains unknown. However, Sr^{2+} and Ba^{2+} stimulate the ATPase from erythrocytes[37] and kidney cortex[35]. In addition, BAKER *et al.*[38] recently found an ouabain-insensitive Na^+–Ca^{2+} (or Na^+–Sr^{2+}) exchanging system in neurolemma in which Ca^{2+} influx is proportional to $[Na^+]_i^2$ and the system is electrogenic.

The lack of effect of diphenylhydantoin on the transport ATPase from intact embryonic chick hearts agrees with our results on the sonicated preparation of cul-

tured chick embryonic heart cells[7], and with the findings on human myocardial microsomal (Na^+,K^+)-ATPase in the presence or absence of cardiac glycoside[39]. Diphenylhydantoin also has no effect on the relatively low resting potentials of cultured heart cells[3,4]. However, it has been suggested[40] that diphenylhydantoin reverses the digitalis inhibition of myocardial (Na^+,K^+)-ATPase. Diphenylhydantoin (10^{-4} M) was reported to inhibit the (Na^+,K^+,Mg^{2+})-ATPase from brain cerebral cortex, this inhibition being additive with that produced by ouabain[41].

The use of cultured heart cells for the study of the transport ATPase offers other advantages, including the possibility for study of the development of specific ion pumping capabilities. For example, it can be determined whether some of the characteristics of the enzyme change as a function of time in culture or of prolonged maintenance of the cells in altered environments. For instance, it would be interesting to determine if the membrane can 'learn' to pump Li^+.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-aid from the American Heart Association and Grant HE-11155 from the U. S. Public Health Service.

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