BBA 75916

(Na^+,K^+)-ATPase ACTIVITY OF EMBRYONIC CHICK HEART AND SKELETAL MUSCLES AS A FUNCTION OF AGE

NICK SPERELAKIS

Department of Physiology, University of Virginia School of Medicine, Charlottesville, Va. (U.S.A.)

(Received December 20th, 1971)

SUMMARY

The specific activity of the membrane (Na^+,K^+)-ATPase of intact chick embryonic hearts (ventricles) was measured as a function of embryonic age. The membranes were prepared by a NaI extraction method of the 100000 $\times$ *g* fraction which selectively removes much of the ouabain-insensitive Mg^{2+}-ATPase. The specific activity of the myocardial (Na^+, K^+)-ATPase increased markedly during embryonic development from mean levels of about 3.0 μmoles P_i per h per mg protein at day 6 to 7.4 at day 16 and 11.0 at day 20 (1 day prior to hatching); the adult level was about the same as that of the 16-day-old chick. The relative activities with respect to that at day 16 (from paired experiments) averaged 43% (day 6), 56% (day 9), 73% (day 13), 140% (day 20), 115% (day 23), 126% (day 30) and 96% (adult). There was a similar increase in relative activity of the (Na^+,K^+)-ATPase from chick skeletal (leg) muscles during development. If the total protein content per unit membrane area and the turnover number remain constant, the data indicate that the surface density of the (Na^+,K^+)-ATPase molecules increases during embryonic development; thus, the cation pumping capabilities of the cells should be enhanced if the surface area/volume ratio of the myocardial cells remains unchanged. However, the pumping capabilities of the very young cells must be sufficient to maintain the known high $[K^+]_i$ and low $[Na^+]_i$ already present; their internal activities actually change only to a small extent during development. Since there is a known increase in K^+ permeability during embryonic development, thereby increasing the demand on the cation pump, the observed increase in activity of the (Na^+,K^+)-ATPase tends to compensate for this.

INTRODUCTION

Tissue electrolyte analyses gave relatively high values for the intracellular K^+ concentration, $[K^+]_i$, (*e.g.* 145 mM) and low values for $[Na^+]_i$ (*e.g.* 23–38 mM) for 8-day-old chick hearts[1]. From measurements of resting membrane potential as a function of the external K^+ concentration, the extrapolated values for the internal K^+ activity in chick embryonic ventricular muscles were 110–150 mM; the values did not vary substantially between day 2 and day 21, although there was some tendency for older hearts to yield the higher values (ref. 2 and unpublished

observations). Constant $[K^+]_i$ values have been reported also for the atrial cells of 4–18-day-old chick embryonic hearts[8]. $[Na^+]_i$ must be low (30–50 mM) in young (2–6-day-old) chick embryonic hearts because the peak overshoot potential of the action potential goes to zero and excitability fails at about 30 mM $[Na^+]_o$ (ref. 4 and unpublished observations); however, there is much bound Na^+, particularly in the mucopolysaccharide cardiac "jelly"[5] and nucleus[4,6]. Therefore, chick myocardial cells must actively transport cations before day 2.

The largest rate of increase of the transmembrane resting potential of chick ventricular myocardial cells occurs between embryonic days 2 and 8, after which the potential levels off (refs 2, 7, 8 and unpublished observations). Membrane resistance decreases due to an increase in K^+ permeability (P_K) and reaches the adult level by day 8 (ref. 2 and unpublished observations). The resulting decrease in the ratio of P_{Na}/P_K during ontogeny accounts for much of the increase in resting potential without a concomitant large increase in E_K (K^+ diffusion potential)[2]. The relationship between the arrival of cholinergic innervation at days 5–7[9] and these changes in membrane properties, including a shift from tetrodotoxin-insensitive Na^+ channels in young embryonic hearts to tetrodotoxin-sensitive Na^+ channels in older hearts, is not known[10].

The properties of the (Na^+,K^+)-ATPase of cultured and non-cultured chick embryonic heart cells at age 14–16 days were previously studied, and some were found to be correlated with the electrical properties of the intact cells[11–13]. The (Na^+,K^+)-ATPase from cultured cells had about 3- to 10-fold lower specific activity than that obtained from non-cultured hearts, but otherwise the enzymes had nearly identical characteristics[13]. The lower specific activity is consistent with the lower resting potentials and $[K^+]_i$ in cultured cells. Breaks in Arrhenius plots of enzyme activity[13] had some similarities to the effect of temperature on the membrane potentials of intact cultured heart cells[14]. The nearly complete depression of the (Na^+, K^+)-ATPase by tetracaine (2 mM)[11,13] can account for the slow partial depolarization produced in intact cells[15]. Li^+ could not substitute for Na^+ in activation of the enzyme, in agreement with the observation that Li^+ can not be actively pumped out of myocardial cells[15,16]. Hence, the hydrolytic activity of the enzyme parallels its cation transport activity. Thus, embryonic muscles can be used to study the development of specific ion pumping capabilities during ontogeny. In the present report, it was found that the activities of the myocardial and skeletal (Na^+, K^+)-ATPase increase markedly during development.

MATERIALS AND METHODS

The methods used were previously described[13]. In brief, fertilized chick eggs were incubated at 37 °C for varying periods. Some experiments were done on young posthatched chicks and on adult chickens for comparison. For each enzyme preparation, hearts were removed from a number of animals (depending on the size of the hearts) and placed in an extraction solution (at 0 °C) consisting of mannitol (0.25 M), EDTA (5 mM; as the Tris salt), deoxycholate (0.1 % w/v) and histidine (30 mM; pH 6.8). The atria and blood vessels were removed, and the ventricles were blotted, weighed and minced. In most experiments, skeletal muscles from the legs were removed from the same embryos, and their (Na^+,K^+)-ATPase activity was

determined in parallel measurements. The tissues were homogenized in fresh solution (20 ml) and centrifuged at 600 × *g* for 5 min. The pellet was discarded and the supernate centrifuged at 8000 × *g* for 15 min. This pellet was discarded and the supernate centrifuged at 100000 × *g* for 40 min. The pellet was suspended using a Dounce homogenizer in 1 mM EDTA solution (about 1 g wet weight of original tissue per ml). The suspension was diluted by 33.3 % with a solution containing NaI (6 M), EDTA (15 mM), $MgCl_2$ (7.5 mM) and Tris–HCl (120 mM) and occasionally stirred. After 30 min, this mixture was diluted with 2.5 vol. of 1 mM EDTA, and centrifuged at 35000 × *g* for 30 min. The pellet was washed twice with 1 mM EDTA (Tris salt) solution (suspended each time with the Dounce homogenizer) and suspended in 1 mM EDTA. All steps were carried out at 0 °C, and the enzyme preparation was stored at −20°C for a maximum period of 16 h (overnight). Electron micrographs of this NaI-extracted preparation showed that it consists of membranes organized primarily as spherical vesicles, 0.12–0.5 μm in diameter; many vesicles have basal lamina (basement membrane) lining their inner surface, indicating that they were derived from sarcolemma[13]. Thus, the ruptured membranes turn inside out in the process of forming vesicles. Chick myocardial cells have a sarcoplasmic reticulum, but they do not have a transverse tubular system (ref. 17 and unpublished observations).

The standard reaction mixture (total of 2 ml) for assay of ATPase activity contained 3 mM ATP (Tris salt), 3 mM $MgCl_2$, 100 mM NaCl, 8 mM KCl and 50 mM Tris–HCl (pH 7.45); these conditions give maximal activity for the chick myocardial (Na^+,K^+)-ATPase[13]. About 100 μg of membranes were added to each reaction vessel, except for the reagent blank tubes. In all cases, the assays were run in the absence and presence of ouabain (1 mM). The reaction mixture was preincubated 3 min at 37 °C, and the reaction was started by addition of the ATP and stopped by addition of 1 ml of 15 % trichloroacetic acid (0 °C). The total liberation of inorganic phosphate (P_i) was kept below 15 % of the total ATP present, because the rate of ATP hydrolysis as a function of time falls off to a lower rate when more than this fraction of the total ATP is hydrolyzed[13]. The P_i was determined by the method of Fiske and SubbaRow[18], and the protein by the method of Lowry *et al.*[19] using crystallized bovine albumin as the standard. The specific activity of the (Na^+,K^+)-ATPase, in μmoles P_i per h per mg protein, was obtained by subtracting the value in the presence of ouabain (Mg^{2+}-ATPase activity) from the value in the absence of ouabain (total ATPase activity). For this enzyme preparation, half-maximal inhibition occurred at $2.7 \cdot 10^{-6}$ M ouabain and 100 % inhibition at 1 mM; ouabain inhibited 92 % of the total ATPase activity, *i.e.* NaI extraction may selectively remove or inactivate the ouabain-insensitive Mg^{2+}-ATPase[13]. The wet weights of the original tissues and the total protein content of the final enzyme preparation were used to calculate the protein yield per 1 g of tissue; thus, the specific activity could also be expressed as μmoles P_i per h per g wet weight of tissue.

Muscles from three different ages (young, intermediate and old) were run concurrently in each experiment so that comparison could be made more easily between age groups. This is especially desirable because there is a relatively large degree of variability in absolute levels of the specific activity from one experiment to another.

RESULTS

Cardiac muscle

The specific activity of the membrane (Na+,K+)-ATPase of chick embryonic hearts (ventricles) increased markedly during embryonic development, from an average level of about 1.3 μmoles P_i per h per mg protein at day 4 to 11.0 μmoles P_i per h per mg at day 20 (Fig. 1, ○; Table I). In Fig. 1, ● presents the same data *plus* additional data from a second series of experiments on a relative scale, normalized to the mean activity present at day 16. This was done because all of our

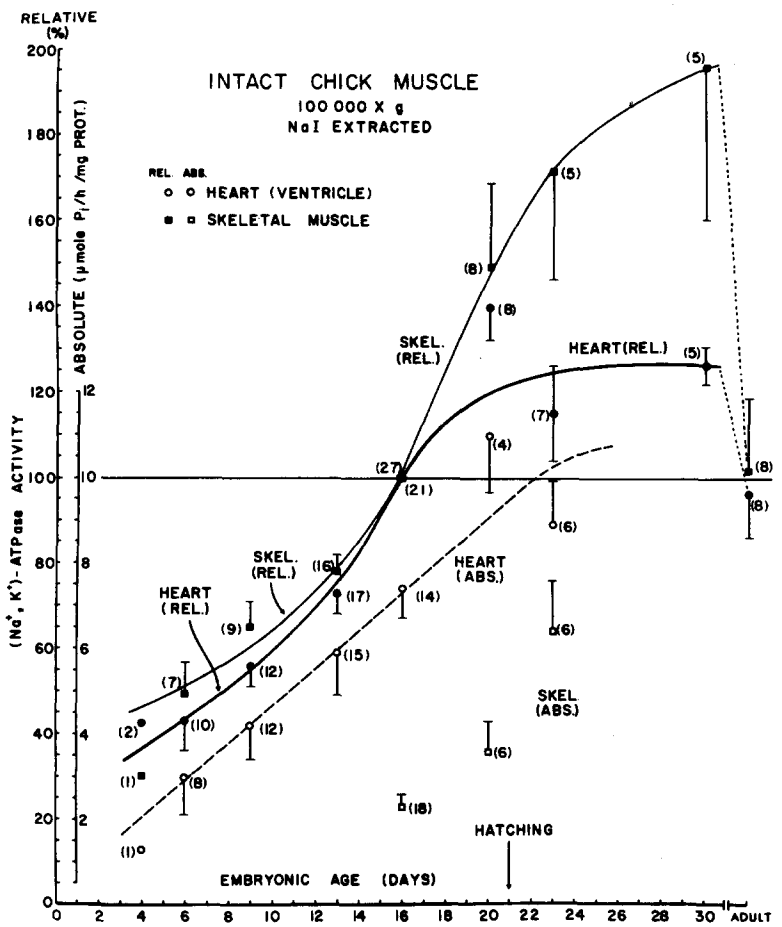

Fig. 1. Graphic representation of the activity of the myocardial and skeletal muscle (Na+,K+)-ATPase as a function of developmental age of the chick. There is a double ordinate, the first of which gives the relative activity in percent with respect to the activity at day 16, and the second gives the absolute specific activity in μmoles of inorganic phosphate (or ATP split) per h per mg protein present in the enzyme preparation. Each point plotted is the mean of the number of values indicated in parentheses; the bars give the standard errors. The (Na+,K+)-ATPase activity is the total ATPase activity (Mg^{2+} + Na^+ + K^+) *minus* the ouabain-insensitive residual activity (Mg^{2+} + Na^+ + K^+ + ouabain). Data for the heart (○ and ●) and skeletal muscle (□ and ■) are plotted; the filled symbols give the relative activity and the unfilled symbols give the absolute activity.

previously published experiments on the (Na^+, K^+)-ATPase activity of intact chick embryonic hearts or cultured heart cells were at an embryonic age of about 16 days. It is seen that the activity at days 4–6 averaged only about 43 % of that at day 16, whereas the activity at day 20 was 140 %. The percentages listed in the relative

TABLE I

SUMMARY OF DATA ON THE SPECIFIC ACTIVITY OF THE (Na^+,K^+)-ATPase OF EMBRYONIC CHICK CARDIAC AND SKELETAL MUSCLES AS A FUNCTION OF AGE

*Embryonic age** (*days*)	*Specific activity (mean ± S.E. (N))*		
	Heart muscle		*Skeletal muscle*
	Absolute (μmoles P_i per h per mg protein)	*Relative to day 16*** (%)	*Relative to day 16*** (%)
4	1.3 (1)	43 (2)	30 (1)
6	***3.0 ± 0.9 (8)	***43 ± 7 (10)	***49 ± 8 (7)
9	***4.2 ± 0.8 (12)	***56 ± 5 (12)	***65 ± 6 (9)
13	5.9 ± 1.0 (15)	***73 ± 4 (17)	78 ± 4 (16)
16	7.4 ± 0.7 (14)	100 ± 10 (28)	100 ± 12 (22) §
20	***11.0 ± 1.3 (4)	***140 ± 7 (8)	***149 ± 21 (8)
23	8.9 ± 0.9 (6)	115 ± 11 (7)	***172 ± 29 (5)
30	—	126 ± 4 (5)	***197 ± 36 (5)
Adult	—	96 ± 10 (8)	101 ± 17 (8)

* Hatching occurs on day 21.

** The relative activities listed are the averages of all of the relative activity values determined, with respect to the activity of the 16-day-old embryos, in each experiment. This column contains data from a second series of experiments (not included in the column of absolute values) which gave lower absolute values but about the same relative values.

*** Statistically different from the values at 16 days at a P level of 0.05 or less. More ages in the relative category were significantly different from the 16-day-old because of pairing before taking the mean.

§ The absolute specific activity for skeletal muscle at day 16 was 2.3 ± 0.3 (N = 18) μmoles P_i per h per mg protein.

column are somewhat different from those that would be calculated on the basis of the mean values listed in the absolute column because the former are the means of percentages calculated in each experiment. In all experiments, muscles from three different ages, 16-day-old *plus* two other ages, were run concurrently so that the activities relative to day 16 could be determined in each experiment. In nearly all cases, the activity of hearts younger than 16 days was lower and hearts older than 16 days (except in the case of adults) was higher. Because of the variability in absolute levels of the specific activity from one experiment to another, the relative activities are the most meaningful. There was an increase in the absolute or relative specific activity as a function of development time between day 4 and day 20, following which the activity levels off and actually declines somewhat. The activity of the enzyme from the adult heart is about the same as the level at day 16. Those embryonic ages whose enzyme activity was significantly different from that at day 16 are indicated by triple asterisks in Table I. The average ouabain inhibition of the total ATPase activity was 71 % (± 2 %), with the inhibition approaching 100 % in some cases.

Skeletal muscle

For comparison, the specific activity of the (Na^+,K^+)-ATPase obtained from chick embryonic skeletal (leg) muscle of the same embryos was also determined. These results are also given in Table I (relative values only) and plotted in Fig. 1 (□ and ■). It is seen that there was also an increase in specific activity during embryonic development. The absolute specific activity of the skeletal (Na^+,K^+)-ATPase was lower than that of the myocardial (Na^+,K^+)-ATPase in almost all experiments on embryos less than 16 days old; the differences were smaller in the young hatched chicks. That is, the skeletal muscle activity continued to increase during the early post-hatched period, whereas that of the heart had already leveled off. These findings are consistent with the fact that intensive electrical and mechanical activity does not begin in the leg muscles until after hatching, whereas the heart does considerable work before hatching. The average percent ouabain inhibition of the total ATPase activity was 58 % (± 2 %), the maximum inhibition being 91 %.

Protein yield

The protein yield in the final enzyme preparation varied from experiment to experiment, but there was no trend with respect to embryonic age. Since the yield was about the same for the different sized hearts, the increase in specific activity of the enzyme during development may not be explained on the basis of less contaminant proteins adsorbed to the membrane preparations as a function of embryonic age. The yield averaged 0.80 ± 0.10 mg protein per g tissue wet weight ($N = 40$) for the ventricular muscles and 0.70 ± 0.10 mg/g ($N = 30$) for the skeletal muscles. Thus, the average specific activity for 16-day hearts of 7.4 μmoles P_i per h per mg protein converts to 5.9 μmoles P_i per h per g tissue or to $1.6 \cdot 10^{-9}$ moles P_i per s per g tissue. For 23-day-old (2 days posthatched) skeletal muscle, the average specific activity of 6.4 μmoles P_i per h per mg protein (Fig. 1, □)converts to $1.2 \cdot 10^{-9}$ mole P_i per s per g. Since 1 g of myocardial tissue corresponds to about $3.3 \cdot 10^3$ cm^2 of sarcolemmal membrane (assuming cylindrical cells 10 μm in diameter and a total extramyocardial cell space of 20 %), the myocardial (Na^+,K^+)-ATPase activity is equivalent to about $5.0 \cdot 10^{-13}$ mole ATP split per s per cm^2. This is a minimal estimate since the yield is probably very low.

DISCUSSION

The results indicate that the specific activity of the (Na^+,K^+)-ATPase of chick cardiac muscle increases markedly during embryonic development, the peak levels being reached at about the time of hatching. The adult values are about the same as those for the 16-day-old embryonic chick. Assuming that the total protein content per unit membrane area remains constant, then the results suggest that the surface density of the (Na^+,K^+)-ATPase molecules, and/or turnover number per molecule, increases with age. Since there is evidence that the Na^+:K^+ pump turnover rate for cultured heart cells is low and constant (about 20 s^{-1}) under a variety of pumping conditions[20], it is more likely that the surface density of (Na^+,K^+)-ATPase molecules increases during embryonic development. Thus, the Na^+:K^+ pumping capabilities of the cells should be enhanced during embryonic development, provided that the surface area/volume ratio of the muscle cells remains unchanged. Since chick

myocardial cells contain sarcoplasmic reticulum even though they do not possess a transverse tubular system (ref. 17 and unpublished observations), the growth of a tubular system cannot complicate the present findings. Alternative possibilities, which have not yet been ruled out, to account for the present findings include large alterations in the optimal conditions for ATPase activity or changes in the lability of the isolated enzyme preparation during development.

Klein[21] also found an increase in the (Na^+,K^+)-ATPase specific activity of embryonic chick heart between day 4 and day 7; calculations from his data give values of 4.8 μmoles P_i per h per mg protein at day 7 and 1.0 at day 4. These values are in close agreement with our findings. However, he found no increase in specific activity at day 12 compared to day 7, and thereby concluded that there was an induction of enzyme occurring suddenly between days 4 and 7. However, the degree of ouabain inhibition of the total ATPase activity was very low. The specific activity of the (Na^+,K^+)-ATPase of embryonic chick brain also increases markedly from day 6 to day 12 and then levels off; since an inhibitor was not found, this suggested *de novo* synthesis of enzyme rather than its activation[22]. In developing rat brain, an abrupt increase in (Na^+,K^+)-ATPase activity occurs at birth (day 21), and the adult level is reached by the 12th postnatal day[23].

In chick skeletal muscles, the largest changes in resting membrane potential occur near the end of embryonic life, whereas in mammals, large changes occur in the early postnatal period. In embryonic chick skeletal (thigh) muscle, the resting potentials are low and not much changed between day 3 and day 15, but there is a marked increase by day 19 which approaches the adult value[24]. Consistent with this, the measured $[K^+]_i$ for 3–4-day posthatched chick breast muscle is 140 mM per l of cell water, which is close to the mean adult value of 147 mM[25]. In contrast, in the rat, the largest increase in resting potential is observed during the early postnatal period, adult values being reached by days 10–12[26,27].

The present results are consistent with the findings that the resting potentials of embryonic myocardial and skeletal muscle fibers increase with age. However, $[K^+]_i$ and $[Na^+]_i$ change only to a relatively small extent in embryonic chick hearts during development[2–4]; that is, $[K^+]_i$ is high and $[Na^+]_i$ low already in very young cells. Hence, even though the specific activity of the (Na^+;K^+)-ATPase is relatively low, the cation pumping capabilities at the early age are sufficient to maintain a high $[K^+]_i$ and low $[Na^+]_i$. The measured low K^+ permeability (P_K) of the cell membranes in the young hearts[2] would explain how a high $[K^+]_i$ can be maintained with low pumping rates. As P_K increases with embryonic age, thereby increasing the demand on the Na^+:K^+ pump, there is an increase in the activity of the (Na^+,K^+)-ATPase. Thus, there is a tendency for the pump to compensate for the higher leak. It is interesting that there is a reduction in the number of K^+ pump sites with maturation of sheep red blood cells genetically destined to become low-K^+ cells[28], and that there is a 2.3-fold increase in Na^+:K^+ pump sites in cardiac muscle of guinea pigs maintained 14 days on a K^+-deficient diet (control specific activity of about 4.5 μmoles P_i per h per mg protein)[29].

Using the previously measured[13] maximal enzyme velocity for 16-day hearts of 91 μmoles P_i per h per mg protein (or 6.1 pmoles ATP split per s per cm^2), and since 3 Na^+ are actively transported per ATP split, the Na^+ pumping rate is about $18 \cdot 10^{-12}$ mole Na^+ per s per cm^2 (or 110000 Na^+ per s per $m\mu^2$ and 37000 ATP

split per s per μm^2). This estimated Na^+ pumping rate is in reasonable agreement with the typical values measured or calculated for passive Na^+ influx down its electrochemical gradient [$J_{Na} = g_{Na}(E_m - E_{Na})/F$], since under steady-state conditions in resting cells the active Na^+ efflux must be equal to the passive Na^+ influx. With a turnover rate of 20 s^{-1}, the surface density of pump sites is 1900/μm^2 (5-fold lower if the turnover number were 100 s^{-1}, as measured for sheep red blood cells[28]). One-half of these pump sites are kept occupied by $2.7 \cdot 10^{-6}$ M ouabain (1600 molecules/μm^3). Assuming uniform distribution, the average center-to-center spacing between neighboring pump sites would be about 190 Å. The estimated density of pump sites is several orders of magnitude greater than the estimated density of K^+ channels in resting membrane (< 1 K^+ channel/μm^2)[30] and of Na^+ channels during peak activation (10–50 Na^+ channels/μm^2)[31]. Therefore, in contrast to the relatively low density of channels for passive ion movements, a large fraction of the surface area of the membrane seems to be involved in cation pumping.

ACKNOWLEDGEMENTS

This work was supported by grants from the Public Health Service (HE-11155) and from the American Heart Association. The author is pleased to acknowledge the technical assistance of Miss Debbie Wright.

REFERENCES

1 M. Harsch and J. W. Green, *J. Cell. Comp. Physiol.*, 62 (1963) 319.
2 N. Sperelakis, in W. C. De Mello, *Electrical Phenomena in Heart*, Academic Press, New York, 1972, in the press.
3 A. J. Pappano, *J. Pharmacol. Exp. Ther.*, 180 (1972) 340.
4 B. K. Yeh and B. F. Hoffman, *J. Gen. Physiol.*, 52 (1968) 666.
5 A. Thureson-Klein and R. L. Klein, *J. Mol. Cell. Cardiol.*, 2 (1971) 31.
6 R. L. Klein, C. R. Horton and A. Thureson-Klein, *Am. J. Cardiol.*, 25 (1970) 300.
7 D. Lehmkuhl and N. Sperelakis, *Am. J. Physiol.*, 205 (1963) 1213.
8 Y. Shimizu and K. Tasaki, *Tohoku J. Exp. Med.*, 88 (1966) 49.
9 A. L. Romanoff, *The Avian Embryo: Structural and Functional Development*, MacMillan Co., New York, 1960.
10 K. Shigenobu and N. Sperelakis, *J. Mol. Cell. Cardiol.*, 3 (1971) 271.
11 F. A. Henn and N. Sperelakis, *Biochim. Biophys. Acta*, 163 (1968) 415.
12 N. Sperelakis and F. A. Henn, *Am. J. Physiol.*, 218 (1970) 1224.
13 N. Sperelakis and E. C. Lee, *Biochim. Biophys. Acta*, 233 (1971) 562.
14 N. Sperelakis and D. Lehmkuhl, *Am. J. Physiol.*, 213 (1967) 719.
15 N. Sperelakis and D. Lehmkuhl, *Exp. Cell Res.*, 49 (1968) 396.
16 A. J. Pappano and N. Sperelakis, *Am. J. Physiol.*, 217 (1969) 615.
17 J. R. Sommer and E. A. Johnson, *J. Cell Biol.*, 49 (1969) 50.
18 C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, 66 (1925) 375.
19 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
20 L. Boardman, J. F. Lamb and D. McCall, *Proc. Int. Union Physiol. Sci.*, 9 (1971) 69.
21 R. L. Klein, *Biochim. Biophys. Acta*, 73 (1963) 488.
22 N. Zaheer, Z. Iqbal and G. P. Talwar, *J. Neurochem.*, 15 (1968) 1217.
23 A. A. Abdel-Latif, J. Brody and H. Ramahi, *J. Neurochem.*, 14 (1967) 1133.
24 J. Boëthius and E. Knutsson, *J. Exp. Zool.*, 174 (1970) 281.
25 J. S. Barlow and J. F. Manery, *J. Cell. Comp. Physiol.*, 43 (1954) 165.
26 S. K. Fudel-Osipova and O. A. Martynenko, *Fed. Proc. (Transl. Suppl.)*, 23 (1964) 28.
27 J. Boëthius, *Acta Physiol. Scand.*, 75 (1969) 253.
28 P. B. Dunham and J. F. Hoffman, *Biochim. Biophys. Acta*, 241 (1971) 399.
29 E. Erdmann, H. D. Bolte and B. Lüderitz, *Arch. Biochem. Biophys.*, 145 (1971) 121.
30 K. Hermsmeyer and N. Sperelakis, *Am. J. Physiol.*, 219 (1970) 1108.
31 J. Moore and T. Narahashi, *Fed. Proc.*, 26 (1967) 1655.