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Adenine Nucleotides and Nucleosides in Chick Embryo Ventricles[1, 2] (39813)

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Introduction. In earlier studies (1), we found the incorporation of radioactive adenosine into ADP, ATP, and total adenine nucleotides to be higher in cardiac muscle cells from younger embryonic hearts (12 to 15 days) compared to older embryonic hearts (16 to 22 days). These experiments also showed a difference in the uptake mechanisms between the two groups. Adenosine kinase appears to be located on both sides of the cell membrane in the younger cells and only on the inside in the older cells (1). There are several (2–5) studies which have examined adenine nucleotides in adult mammalian heart, but the developmental changes of adenine nucleotides and nucleosides in the myocardium have received little attention. The present investigation is aimed toward understanding the importance of adenine nucleotides and thier degradative products (6) in the regulation of coronary blood flow in developing myocardium and changes in the electrophysiological events during development. This paper reports the basal levels of adenosine compounds.

Methods and material. Hubbard strain fertilized eggs were incubated at 37° in a Model 50 (The Humidaire Incubator Company, New Madison, Ohio) incubator. After appropriate incubation times (hatching occurs at 21 days), the embryonic hearts were studied at various stages of development from Day 7 to Day 22. The embryos were removed (between 2 to 10 dozen, depending on size of the heart), the thorax was opened, and the ventricles were removed and immediately dropped into cold Hanks solution (containing glucose), with Ca^{2+} (3.06 m*M*) and Mg^{2+} (0.81 m*M*). Then the ventricles were immediately transferred to 4 ml of 0.5 *N* perchloric acid and homogenized with no delay in a Polytron homogenizer at 4000 rpm at 2–4° for 90 sec. The homogenate was centrifuged at 10,000*g* for 10 min in the cold and the pellet obtained was washed once with 0.5 *N* perchloric acid. The washings were added to the previous supernatant and the pellet was dissolved in 1 *N* sodium hydroxide for protein determination according to the method of Lowry *et al.* (7). The supernatant fraction was adjusted to pH 7.0 by the addition of potassium hydroxide and the precipitate of potassium perchlorate was removed by centrifugation. The neutralized acid extract was analyzed for ATP by the luciferase method (8) and ADP and AMP were analyzed by a spectrophotometric method employing a coupled enzyme assay (9). Adenosine, inosine, and hypoxanthine were quantified by the coupled enzyme assay method of Kalckar (10) using a dual wavelength Aminco Model DW-2 spectrophotometer.

Results. Figure 1 illustrates the levels (nanomole per milligram of protein) of ATP, ADP, AMP, and their sum in 7- to 22-day-old embryonic hearts.

The values for ATP between the 7th and 10th days were unchanged, but began decreasing on Day 11 to a low on Day 15 (33% from Day 7). The concentration increased to a peak on the 18th day and dropped again to plateau between the 20th and 22nd days, just a day before hatching. These data demonstrate a biphasic change in the level of ATP during myocardial development before a steady-state is reached prior to hatching.

The levels of ADP were much lower (approximately one-third) than the ATP concentration at various ages of the embryonic heart. The levels of ADP did not change between Day 7 and Day 14, but

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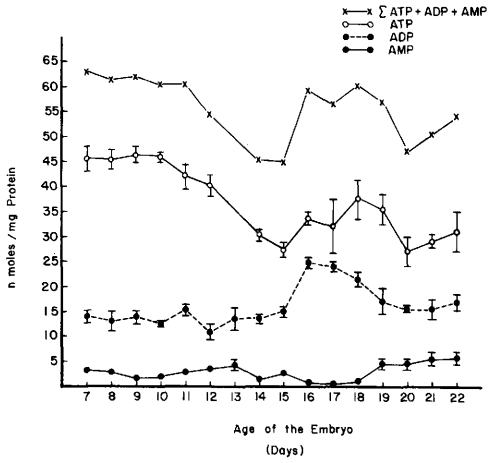

FIG. 1. Represents the levels of ATP, ADP, AMP, and their sum expressed as nanomoles per milligram of protein. These data are derived from the mean of four to six experiments in each case. Depending on the size of the heart, their numbers varied in each experiment from 16 hearts at early ages to 1 or 2 at the older ages (close to hatching). The levels of ATP were statistically significant between the ages of 7 and 15 days ($P < 0.001$) and 15 and 18 days ($P < 0.05$), ADP between the ages of 15 and 16 days ($P < 0.001$), and AMP between the ages of 13 and 17 days ($P < 0.005$) and 17 and 22 days ($P < 0.005$).

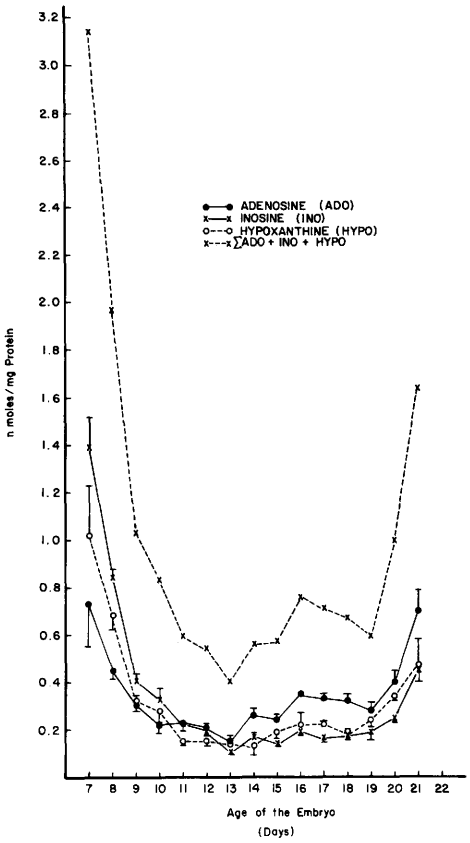

FIG. 2. Represents the mean values (from four to six experiments) for adenosine, inosine, hypoxanthine, and their sum. The values are expressed as nanomoles per milligram of protein. Other details are the same as in Fig. 1. The levels of adenosine, inosine, and hypoxanthine were statistically significant ($P < 0.02$) between 7- and 10-day-old hearts.

they increased sharply between the 15th and 16th days of development. The level of ADP decreased progressively from the 17th to the 20th days toward a steady state at the end of gestation. The increase in ADP values between Days 15 and 20 paralleled the increase in ATP and decrease in AMP during the same time period.

The levels of AMP, on the other hand, had a significant depression between 16 and 18 days, and the absolute amounts were lowest when compared with ATP and ADP concentrations. The sum of total adenine nucleotides (ATP + ADP + AMP) is most similar to the curve for ATP alone because of the higher contribution of ATP to this sum.

Figure 2 shows the levels of adenine nucleosides, namely, adenosine, inosine, and hypoxanthine, expressed as nanomoles per milligram of protein at various stages of development. The level of all three nucleosides was highest in the 7-day-old hearts. The level of adenosine decreased progressively between Days 7 and 10 and remained low until the 19th day. This period was followed by a rapid increment in the levels between Days 19 and 21.

A similar pattern was noted with inosine and hypoxanthine, except that their initial (Days 7 to 11) levels were higher and their final levels lower than those of adenosine. The curve for the sum of the adenine nucleosides has the same general shape as the curves for the individual compounds.

Table I represents the calculated values for energy potential (E) of the embryonic hearts at various stages of development. This represents the fraction of the total adenosine phosphate moieties that exist in the form of high energy phosphates. Energy potential (E) tended to decrease

TABLE I. CALCULATED VALUE FOR ENERGY POTENTIAL[a] AT VARIOUS AGES OF THE DEVELOPING MYOCARDIUM.

Age	Energy potential	Age	Energy potential
7	0.837	15	0.733
8	0.847	16	0.777
9	0.859	17	0.779
10	0.864	18	0.803
11	0.825	19	0.770
12	0.837	20	0.737
13	0.833	21	0.736
14	0.817	22	0.731

[a] Energy potential = (0.5 [ADP] + [ATP])/([ATP] + [ADP] + [AMP]).

with increasing age of the embryo.

Discussion. This study was designed to establish the levels of adenine nucleotides and nucleosides during the embryonic development of the heart. Significant concentration changes were found in the myocardium between Days 7 and 22 of incubation.

The high adenine nucleotides in the young hearts, expressed as nanomoles per milligram of protein, could partially reflect a lower protein content in young embryonic hearts, e.g., less contractile proteins. However, Guidotti *et al.* (11) reported that the protein content (milligrams of protein per milligrams wet weight) of dispersed chick heart cells is 0.131 on Day 5 and 0.165 on Day 19. This 20% increase in protein cannot explain the 1.4 (nucleotide) to 7.8-fold (nucleoside) changes we have observed at the depression of the concentration curves. The protein:DNA ratio does not change significantly during the development of chick heart (12, 13). The higher adenine nucleotide content observed in the present investigation in younger hearts is real and represents a larger pool of high-energy phosphate compounds.

Atkinson (14) proposed an independent variable that is derived from relative concentrations of adenine nucleotides. This energy potential of the adenylate pool is mathematically related to the concentrations of ATP, ADP, and AMP. If all the adenylate exists as ATP, the E value is 1.0; all as ADP, $E = 0.5$; or all as AMP, $E = 0.0$. The value of E in the present study is biphasic, as is the ATP concentration curve. There are small changes in the value of E between Days 7 and 14, with a low on the 15th day. The values of E were from 0.86 to 0.81 between Days 7 and 14 and from 0.80 to 0.73 between Days 15 and 22.

The values found early in development are consistent with the theoretical value at the metabolic steady state when ATP-generating reactions equal ATP-requiring processes, i.e., 0.85. This value has also been quoted in such diverse tissues as brain, heart, and blowfly flight muscle (15). The significant deviation downward from this value during the last 6 days of observation provide three options: (i) ATP generation is inadequate, (ii) ATP utilization is excessive, or (iii) both factors are operative.

Hughes (16) and Romanoff (17) have reported the mechanical work per gram of myocardium to be higher at Day 14 of development compared to hatch time. High levels of the adenine nucleosides were present on the 7th day falling until the 11th day. There were no significant changes noted between Days 11 and 19, the nucleosides remained low and started rising again towards the onset of hatching. The levels of inosine and hypoxanthine were higher than adenosine, which could be due to: (i) adenosine conversion to inosine and hypoxanthine, (ii) adenosine conversion to nucleotides, (iii) the activity of adenosine deaminase. Because the time between sampling and freezing was short, we believe the latter two explanations are more likely.

Adenosine has been implicated (6) as one of the agents involved in the physiological regulation of coronary blood flow. Younger hearts have a higher work load and the higher adenosine concentrations (present observation) could provide higher blood flow to the heart to meet the oxygen needs. The data of Foley *et al.* (18), showing an increase in the sum of adenosine, inosine, and hypoxanthine proportional to the increase in cardiac work in rat hearts, support the concept that adenosine is a control factor in increasing oxygen delivery to meet myocardial needs. The observed fall and rise in the levels of adenine nucleotides and nucleosides during development provide the basis for further investigations of control mechanisms involved in normal development of coronary perfusion.

Summary. The levels of ATP, ADP, AMP, adenosine, inosine, and hypoxan-

thine were measured in 7- to 22-day-old chick embryonic hearts. The nucleoside levels (adenosine, inosine, hypoxanthine) fell between Days 7 and 10, remained low until the 19th day, and increased significantly by the time of hatching. ATP concentrations showed a biphasic response with a drop at Day 15 and a peak on Day 18 approaching a steady state at the time of hatching. ADP rose between Days 16 and 18; otherwise, it remained unchanged throughout the experimental period. AMP reached its minimum level between Days 16 and 18 and increased from Day 19 to the time of hatching. The calculated value of energy potential (E) was higher in young embryonic hearts than in the older hearts. These data correlate with other reported energy demands (electrophysiological events) taking place during the embryonic development.

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1. Mustafa, S. J., Rubio, R., and Berne, R. M., Amer. J. Physiol. **228,** 62 (1975).
2. Jacob. M. I., and Berne, R. M., Amer. J. Physiol. **198** (1960).
3. Jacob, M. I., and Berne, R. M., Proc. Soc. Exp. Biol. Med. **107,** 738 (1961).
4. Liu, M. S., and Feinberg, H., Amer. J. Physiol. **220,** 1248 (1971).
5. Wiedmeier, V. T., Rubio, R., and Berne, R. M., Amer. J. Physiol. **223,** 51 (1972).
6. Berne, R. M., Amer. J. Physiol. **204,** 317 (1963).
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
8. Greengard, P., in "Methods of Enzymatic Analysis" (H. V. Bergmeyer, ed.), p. 551. Academic Press, New York (1965).
9. Adams, H., in "Methods of Enzymatic Analysis" (H. V. Bergmeyer, ed.), p. 573. Academic Press, New York (1965).
10. Kalckar, H. M., J. Biol. Chem. **167,** 429 (1947).
11. Guidotti, G. G., Luneburg, B., and Borghetti, A. F., Biochem. J. **114,** 97 (1969).
12. Doyle, C. M., Zak, R., and Fishman, D. A., J. Cell Biol. **49,** 85a (1973).
13. Doyle, C. M., Zak, R., and Fishman, D. A., Develop. Biol. **37,** 133 (1974).
14. Atkinson, D. E., Biochemistry **7,** 4030 (1968).
15. Lehninger, A. L., in "Biochemistry" p. 395. Worth, New York (1970).
16. Hughes, A. F. W., J. Roy. Microsc. Soc. **69,** 145 (1949).
17. Romanoff, A. L., in "The Avian Embryo" p. 746. Macmillan Co., New York (1960).
18. Foley, D. H., Herlihy, J. T., Thompson, C. I., Rubio, R., and Berne, R. M., Fed. Proc. **35,** 348 (1976) (Abstract).

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