# DEVELOPMENT OF ENZYME SYSTEMS IN CHICKEN EMBRYO HEART TISSUE

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

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March 24, 1981

This thesis for honors recognition has been approved for the Department of Biology.

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ACKNOWLEDGEMENTS

I would like to thank Dr. James Manion for his assistance in the selection of this topic and organism of study. I would also like to thank Mr. Alfred Murray for his assistance in determining the proper method for the statistical analysis of the data. And finally, Dr. John Christenson, without whose many hours of help in design and implementation this project could not have been completed.

## ABSTRACT

The development of some enzymes and enzyme systems necessary for energy metabolism in the hearts of developing chicken embryos was studied from day 7 through day 12. There was a 36% decrease in the protein present per g of wet tissue weight during this time period. A 242% increase in ETS activity per g of tissue and a 436% increase per mg of protein present was found. LDH activity was found to decrease 64% relative to mass and 44% relative to protein content. The overall glucose utilization decreased 83% when related to the mass of the hearts and 89% when related to the protein content. Thus, there was an increase in the dependence on the enzyme systems which require oxygen and are more efficient. There was also a decrease in the activity of the enzyme systems which do not require oxygen.

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# INTRODUCTION

This study deals primarily with how developing chicken embryo hearts obtain the required energy for development. Specifically, it deals with differential rates at which several enzyme systems are formed.

The embryonic lung consists of a vascular plexus located just beneath the shell. This plexus, the chorio-allantoic vascular plexus, has its origin in two extra-embryonic membranes. These are the chorion, or serosa, and the allantois. The chorion begins to form at approximately 30 hrs of incubation. It is actually a fold of tissue which begins at the cephalic extent of the embryo and forms caudally. At approximately 72 hrs of incubation, a similar fold begins to form at the caudal extent of the embryo and forms cephalically. At approximately 96 hrs of incubation, these two folds fuse. This fusion results in the formation of two separate membranes. The innermost, called the amnion, contains fluid which bathes the embryo during development. The outermost membrane is the chorion. Upon formation, the chorion grows rapidly, enveloping the yolk sac and embryo. The chorion eventually envelops all of the extraembryonic membranes and comes to lie just beneath the egg shell membrane.

Simultaneously, at 72 hrs of development the allantois begins. Initially it is nothing more than an outgrowth of the cloaka or hind gut. At approximately 96 hrs of development, the allantois pushes out of the body of the embryo and begins to expand into the extraembryonic cavity within the shell. By 240 hrs, or 10 days, the allantois has expanded until it encompasses the entire embryo and its yolk sac. In this process the allantois fuses with the chorion, forming the chorioallantoic membrane. It is within this membrane that the chorioallantoic vascular plexus forms. Thus, by day 10 the embryonic lung is complete.

The hypothesis being tested is as follows. The embryo becomes better able to obtain oxygen from the external environment as the embryonic lung develops. As this occurs, the embryo should become more dependent upon the metabolic pathways which require oxygen, since these aerobic pathways are much more efficient in obtaining energy for the embryo. This would represent a transition from dependence upon anaerobic glycolysis ending in the formation of lactic acid, to dependence upon aerobic glycolysis, the TCA cycle and the electron transport system with $CO_2$ and $H_2O$ as the final products.

## LITERATURE REVIEW

When dealing with how chicken embryos obtain oxygen from the atmosphere, several factors come into play. Two questions to be considered are: What is the exact mechanism of gas exchange, and how does this mechanism and its efficiency change with time? A brief explanation of the complexity of this problem is as follows. Oxygen must travel through the egg shell and egg shell membranes before reaching the capillary plexus in the chorioallantoic membrane where oxygen exchange occurs.

This gas exchange requires the loss of water from the egg to the surrounding air. The rate of loss is approximately 0.4 ml per day, or about 8.4 ml during the entire incubation period (Wangensteen and Rahn, 1970-71).

Two gases are exchanged in great amounts. One is oxygen, with a total amount of about 5 l per 21 days. The other is carbon dioxide, of which slightly more than 5 l is evolved during the same time period.

It has been shown that no active transport is involved. Simple diffusion is responsible for the gas exchange (Wangensteen and Rahn, 1970-71). The rate of this diffusion is dependent upon the differences in oxygen tension between

the environmental air and the air spaces within the shell. It is also dependent upon the difference in oxygen tension between the air space and the mean chorioallantoic oxygen tension (Tazawa and Mochizuki, 1977).

The limiting factor in the diffusion process varies with age. Prior to 11 days of incubation, the limiting factor seems to be the resistivity to oxygenation of the chorioallantoic capillary plexus (Tazawa and Mochizuki, 1977). When comparing observed oxygen consumption with predicted values, deviations were observed early in development. These deviations were attributed to the fact that the allantois, and thus the chorioallantoic capillary plexus, was not completely developed. However, after day 11 the observed and expected values were in concordance (Wangensteen and Rahn, 1970). Later in development, the permeability of the shell becomes the limiting factor. The permeability does not change with time and is not a limiting factor during the earlier stages of embryological development (Wangensteen and Rahn, 1970-71).

The rate of gas diffusion varies with time and is different for different gases. Due to its smaller molecular size, oxygen is able to diffuse into the egg at a faster rate than carbon dioxide can diffuse out. In fact, given equal tension gradients, only 78% as much carbon dioxide can diffuse out as oxygen can diffuse in (Wangensteen and Rahn, 1970-71). These findings have been substantiated in other studies where blood was analyzed just before it

entered the chorioallantoic vascular plexus and as it left. The oxygen tension was found to increase 250%, while the carbon dioxide tension decreased 28% (Tazawa and Mochizuki, 1976).

As previously stated, the rate of diffusion is dependent upon the oxygen tension differences or gradient established between the atmosphere and the internal environment of the egg. A more accurate statement would be that the rate of diffusion is dependent upon the tension gradient established between the atmospheric oxygen and carbon dioxide and that of the arterial blood of the chorioallantoic plexus. This distinction is an important one since it is the arterial blood that enters the chorioallantoic plexus and establishes the gradient with the atmosphere. This is also true because the arterial and venous oxygen tensions can vary by as much as 800% in relative saturation (Tazawa and Mochizuki, 1977).

As development progresses, greater amounts of carbon dioxide accumulate due to the different diffusing rates for $O_2$ and $CO_2$. Thus, the embryo gradually becomes hypoxic (Tazawa and Mochizuki, 1977; Tazawa et al., 1976). As the hypoxia becomes progressively more severe, a lower arterial oxygen tension is found. This in turn favors the maximum possible diffusing rate given the limited permeability of the shell (Wangensteen and Rahn, 1970-71).

In addition to the diffusion rate, there are several other factors which affect the oxygen supply to the embryo. Three of these factors are the affinity of the blood for

oxygen, the oxygen-carrying capacity of the blood, and the rate of blood flow. As development proceeds, the embryonic blood demonstrates an increased affinity for oxygen (Tazawa et al., 1976). An increase in the oxygen-carrying capacity within the blood was also found (Tazawa et al., 1976).

Corresponding with the increasing oxygen capacity is an increase in carbon dioxide in the blood. These increase with age and show a positive linear correlation with the mass of the embryo (Tazawa and Mochizuki, 1977). These findings supported those of an earlier study by Girard, who found the oxygen concentration of fully oxygenated blood to increase from 5 ml of oxygen per 100 ml of whole blood at day 7 to 15 ml of oxygen per 100 ml of whole blood at day 19 (Girard, 1971).

In trying to explain this increase in oxygen affinity and carrying capacity of whole blood, several hypotheses have been proposed. One hypothesis suggests there is a change in the form or type of hemoglobin found in the embryonic red blood cells. However, starch block electrophoresis showed no detectable change (Bartels et al., 1966).

A second hypothesis put forth to explain these increases proposed that increases in hemoglobin content and hematocrit were responsible. A significant increase was found to occur between days 7 and 15 (Girard, 1971; Dawes and Simki, 1969 /cited by Girard, 1971/). Another study found the hemoglobin concentration to increase 49% within the embryonic blood between days 9 and 18 (Tazawa et al., 1971).

These studies also showed that low oxygen affinity observed in the early embryonic period favors the unloading of oxygen into the tissue. This supplies the younger embryos, which have a higher metabolic rate per weight, with relatively large amounts of oxygen. However, as the embryo grows and the diffusion rate across the shell becomes a factor, the increasing oxygen affinity and capacity of the embryonic blood allows the embryo to survive the hypoxia which occurs later in development (Tazawa et al., 1976).

An additional factor favoring the survival of the later embryos is their highly efficient utilization of the oxygen in their blood. Chicken embryos are believed to utilize as much as 80% of the oxygen transported by their blood (Tazawa and Mochizuki, 1976).

The increase in the $CO_2$ tensions observed by Tazawa and Mochizuki causes the serious problem of respiratory acidosis. This is caused by the carbon dioxide reacting with water to form deprotonated carbonic acid and free hydronium ions. A decrease in the pH of the embryonic blood of 0.215 units between days 5 and 19 was found in one study (Girard, 1971). A second study found a decrease of 0.213 pH units between the 11th and 17th days (Tazawa et al., 1971).

The actual increase in the amount of acid produced is greater than the above data would indicate. The effects are minimized due to the development of a buffering system within the blood. There is a significant rise in buffering capacity between the 7th and 15th days of incubation. This

increase helps to counteract the decrease in pH (Girard, 1971). This increase in buffering is the metabolic compensation in response to the developing respiratory acidosis. This response parallels the increase in acidity but never fully compensates for it (Girard, 1971).

The pH of the blood and its ability to supply the available oxygen to the embryo are of vital importance in determining how the embryo oxidizes the food available to it. In response to this latter issue, this study was undertaken to relate the activity of the various enzyme systems to the available $CO_2$.

## METHODS AND MATERIALS

The eggs were incubated in a still air incubator at 38°C for 7, 9, 11 and 12 day periods. The hearts were then excised from the embryos and maintained at -5°C until used.

The tissue samples were homogenized in a 0.25 M phosphate buffer (pH=7.4) at a ratio of 10 ml buffer per g of tissue. The homogenate was then centrifuged at 8200 g for 1 min. The supernatant was decanted and centrifuged at 10,000 g for 20 min to isolate the mitochondria. The final supernatant was decanted and saved for determining the enzyme activity of the cytoplasmic fraction. The remaining mitochondrial pellet was then suspended in 1 ml of 0.25 M phosphate buffer (pH=7.4) and used to determine the electron transport activity.

The protein content of the cytoplasmic fraction was determined by using the Bio-Rad[a] standard assay procedure. The stock assay reagent was diluted 1 to 5. 0.1 ml of the cytoplasmic fraction was added to 5.0 ml of the diluted solution. The optical density was read at 595 nm against

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[a]Bio-Rad protein assay reagent obtained from Bio-Rad Laboratories, Richmond, California.

a blank of 0.1 ml of buffer and 5 ml of diluted solution. The optical density was then plotted against a standard curve to determine the protein concentration in mg/ml.

Glucose utilization was determined by adding 100 ul of 0.05 M glucose and 0.025 M phosphate buffer to 50 ul aliquots of 0.08 M $MgCl_2$, 0.025 M NAD, 0.01 M ATP, 0.025 M $KHCO_3$, and 150 ul of the cytoplasmic fraction. The above fractions were then divided into the zero time controls and the experimental group. Also, a group containing all of the above-listed reagents except the cytoplasmic fraction served as a control. To the zero time controls, 0.5 ml of 2% zinc sulfate and 0.5 ml of 1.8% barium hydroxide were added to precipitate all proteins out of solution, then water was added to make a 5.0 ml final volume. The experimental and control groups were incubated at 38°C for 30 min and then treated like the zero time controls. The fractions were then centrifuged and the supernatants analyzed for glucose by adding 0.25 ml of supernatant to 2.5 ml of glucostat reagent.[a] The tubes were then incubated at 37°C for 30 min and the optical density at 450 nm determined.

Lactate dehydrogenase activity was determined by adding 100 ul of the cytoplasmic fraction to 2.5 ml of substrate composed of 2% sodium pyrophosphate, 0.61% lactic acid and, 0.39% NAD at a pH of 8.8. The change in optical density was then recorded at 30 sec intervals for 5 min at 340 nm.

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[a]Obtained from Sigma Chemical Co., St. Louis, MO.

To measure the activity of the electron transport system for days 9, 11 and 12 a 50 ul sample of the mitochondrial suspension (100 ul for day 7) were added to a 2.0 ml aliquot of color reagent composed of 2,6 dichlorophenol indophenol, $K_2HPO_4$ and water. To this 200 ul of 0.5 M sodium succinate were added. The decrease in optical density was then recorded at 30 sec intervals for 3 min at 600 nm.

For each day of the study, the trial which showed the lowest overall activity was taken as the expected value and all of the other trials for that day were compared to this value. The method of comparison was a student t distribution using a two-tailed test with a confidence level of 0.90.

## RESULTS

Data was collected from chicken embryos after 7, 9, 11 and 12 days of incubation for activity of the electron transport system, lactate dehydrogenase, glucose utilization and the protein content per g of tissue.

The amount of protein present in mg of protein per g of tissue was recorded for the above days.

| Days of Incubation | Protein Content mg/g |
|---|---|
| 7 | 28.09 |
| 9 | 26.41 |
| 11 | 20.83 |
| 12 | 17.91 |

Table 1. Protein content in chicken embryonic hearts at different stages of development

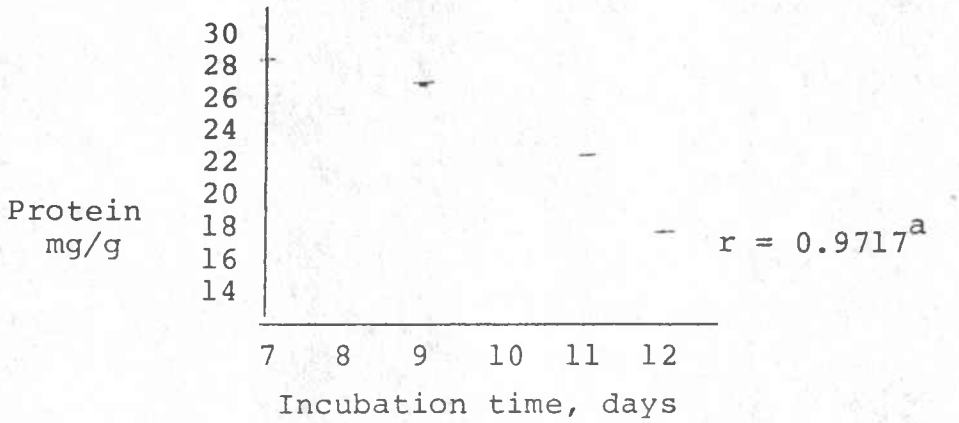


Fig. 1. Protein content in chicken embryonic hearts at different stages of development

[a]Coefficient of correlation

Percent differences for comparing the above data are shown in Table 2.

| Incubation Time | Day 7 | Day 9 | Day 11 | Day 12 |
|---|---|---|---|---|
| Day 7 | -- | -5.9 | -25.9 | -36.2 |
| Day 9 | | -- | -21.1 | -32.2 |
| Day 11 | | | -- | -14.0 |
| Day 12 | | | | -- |

Negative sign implies a decrease between the first and second values

Table 2. Percent differences of protein concentration (mg protein/g wet tissue) at different stages of embryological development in chicken embryo hearts

The relative change in the electron transport system over the 5-day period is expressed as a change in optical density per min per g of tissue and a change in optical density per min per mg of protein.

| Incubation Time | Day 7 | Day 9 | Day 11 | Day 12 |
|---|---|---|---|---|
| Change per min per g of tissue | $38 \pm 24$ | $98 \pm 29$ | $113 \pm 39$ | $130 \pm 32$ |
| Change per min per mg protein | $1.36 \pm 0.86$ | $3.7 \pm 1.1$ | $5.44 \pm 1.58$ | $7.3 \pm 1.8$ |

Table 3. Electron transport system activity at different stages of development in chicken embryo hearts. These values are expressed as mean ± standard deviation.

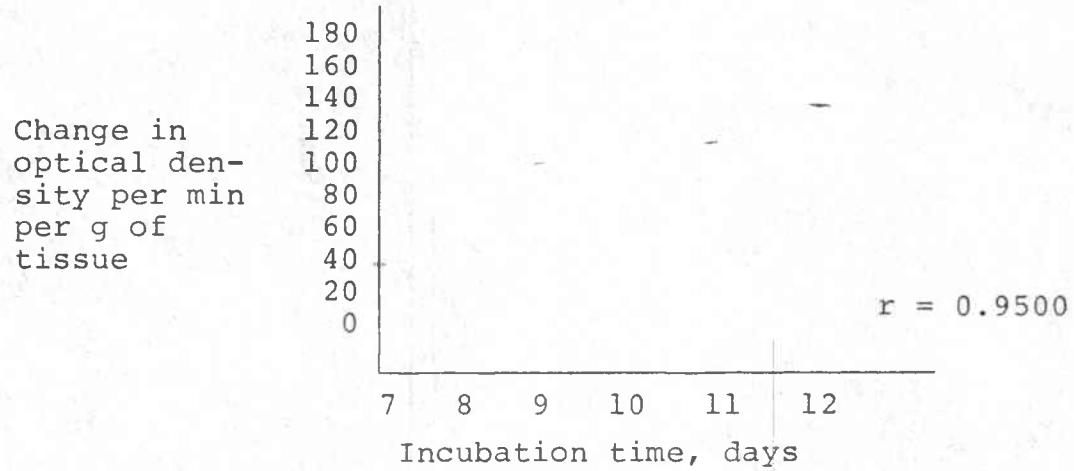


Fig. 2. Electron transport system activity per g of tissue at various stages of development in chicken embryo hearts

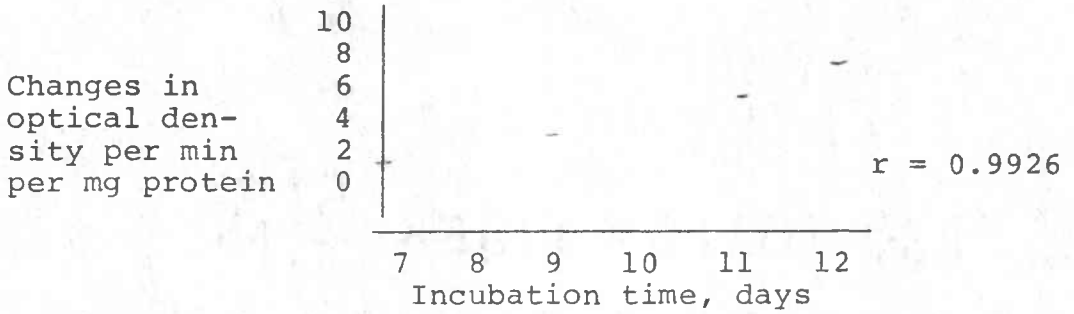


Fig. 3. Electron transport system activity per mg of protein at different stages of development in chicken embryo hearts

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The Z scores calculated using a two-tailed test showed that the differences between any of the timed intervals were significant.

The percentage differences between the above data are shown in Table 4.

| Incubation Time | Day 7 | Day 9 | Day 11 | Day 12 |
|---|---|---|---|---|
| Day 7 | -- | +158.7 (+172.1) | +198.1 (+300.0) | +242.7 (+436.1) |
| Day 9 | | -- | +15.2 (+45.8) | +32.0 (+95.4) |
| Day 11 | | | -- | +15.0 (+34.0) |
| Day 12 | | | | -- |

Positive sign refers to an increased activity

Table 4. Percent differences for electron transport system data at different stages of development in chicken embryo hearts

Percentage difference relative to mass of tissue
(Percentage difference relative to protein content)

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For lactate dehydrogenase the relative activities were found for days 7, 9, 11 and 12 and are expressed as a change per min per g of tissue and/or a change per min per mg protein.

| Incubation Time | Day 7 | Day 9 | Day 11 | Day 12 |
|---|---|---|---|---|
| Change per min per g of tissue | $70 \pm 41$ | $37 \pm 13$ | $42 \pm 21$ | $25 \pm 16$ |
| Change per min per mg protein | $2.5 \pm 1.5$ | $1.4 \pm 0.5$ | $2.0 \pm 1.0$ | $1.4 \pm 0.9$ |

Table 5. Lactate dehydrogenase activity at different stages of development in chicken embryo hearts

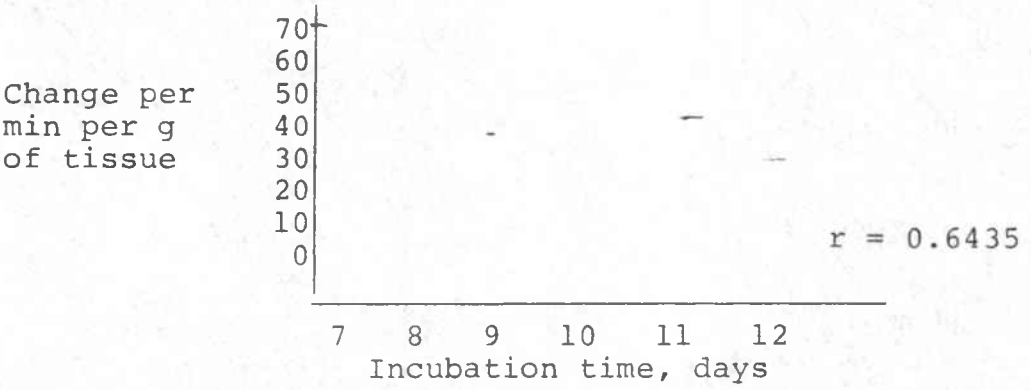


Fig. 4. Lactate dehydrogenase activity per g of tissue at different stages of development in chicken embryo hearts

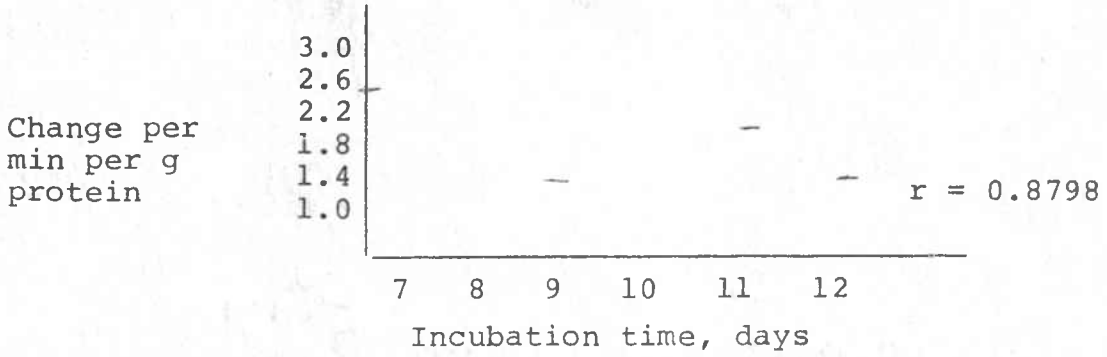


Fig. 5. Lactate dehydrogenase activity per mg of protein at different stages of development in chicken embryo hearts

Percentage differences for these findings were as follows.

| Incubation Time | Day 7 | Day 9 | Day 11 | Day 12 |
|---|---|---|---|---|
| Day 7 | -- | -47.1 (-44.0) | -40.0 (-20.0) | -64.3 (-44.0) |
| Day 9 | | | -13.5 (-42.9) | -32.4 (-00.0) |
| Day 11 | | | | -40.5 (-30.0) |

Table 6. Percentage differences for LDH activity at different stages of development in chicken embryo hearts

Percentage difference relative to mass of tissue
(Percentage difference relative to protein content)

Glucose utilization was determined in the cytoplasmic fraction relative to mass and protein content.

| Incubation Time | Day 7 | Day 9 | Day 11 | Day 12 |
|---|---|---|---|---|
| m moles of glucose used per min per g of tissue | 3.03E-3 ±4.3E-4 | 2.90E-3 ±7.9E-5 | 8.50E-4 ±4.5E-5 | 5.30E-4 ±7.1E-5 |
| m moles of glucose used per min per mg protein | 1.69E-4 ±2.4E-6 | 1.39E-4 ±3.7E-6 | 3.23E-5 ±1.7E-6 | 1.89E-5 ±2.5E-6 |

Table 7. Glucose utilization data at different stages of development in chicken embryo hearts. These values are expressed as mean ± standard deviation.

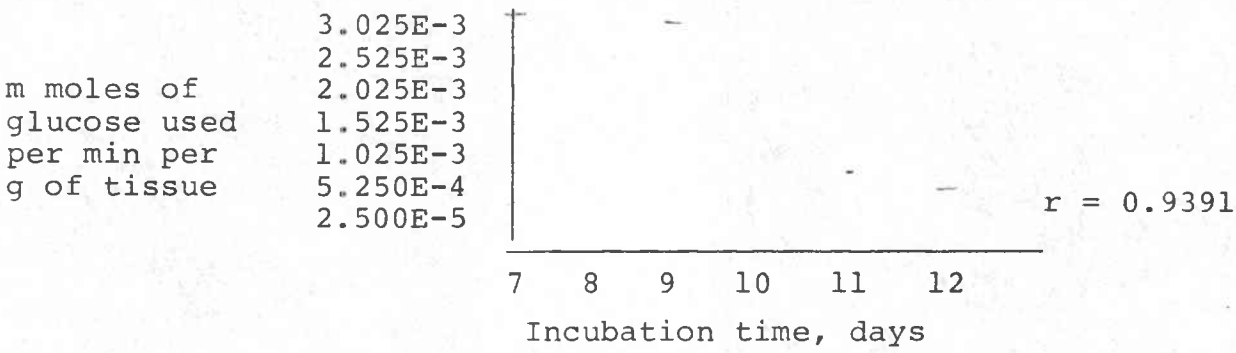


Fig. 6. Glucose utilization relative to tissue mass at different stages of development in chicken embryo hearts.

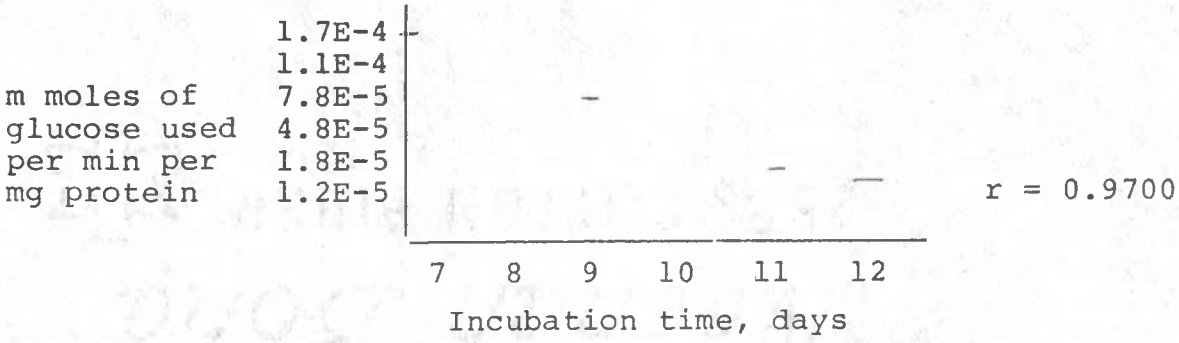


Fig. 7. Glucose utilization relative to protein content at different stages of development in chicken embryo hearts

The percentage differences for these are as follows.

| Incubation Time | Day 7 | Day 9 | Day 11 | Day 12 |
|---|---|---|---|---|
| Day 7 | | -4.29 / (-17.8) | -72.0 / (-80.9) | -82.5 / (-88.8) |
| Day 9 | | | -70.7 / (-74.6) | -81.7 / (-41.5) |
| Day 11 | | | | -37.7 / (-41.5) |

Table 8. Percent differences for glucose utilization at different stages of development in chicken embryo hearts

Percentage difference relative to mass of tissue
(Percentage difference relative to protein content)

The Z scores were calculated using a two-tailed test with a confidence level of 0.90; they showed that the differences between each of the days were significant except for the difference relative to change per g of tissue between days 11 and 12.

## DISCUSSION AND CONCLUSIONS

Since no previous work was found in this study area, there were no findings with which to compare the results of this study. Therefore, for each day of study the trial which showed the lowest overall activity was taken as the expected value and all of the other trials for that day were compared to this value. The method of comparison was a student t distribution using a two-tailed test with a confidence level of 0.90. Any trial with values that were significantly different from this value were discarded from further analysis. However, in all cases the majority of the trials fit within the acceptance region of the distribution. I realize that by using this method of analysis, a biasing of the results has occurred. However, since the same method was used for each day of the results, the effects of utilizing this method should be minimal. The reason for this is that the main concern of this study was to compare the difference between various stages of development. Therefore, any biasing should have minimal effects since it would affect all trials equally and not significantly change the differences between each of the stages of development.

## Protein Content

The protein content per g of wet tissue was found to decrease with time from 28.09 mg per g of tissue at day 7 to 17.91 mg per g of tissue at day 12. This decrease represents a 36% decrease over this 5-day period. This decrease is inversely related to the incubation period and has a coefficient of correlation of $r = 0.9717$, and is referred to later.

## Electron Transport System

The activity of the electron transport system increased 242% between days 7 and 12 on the basis of activity per g of tissue. The greatest increase was between days 7 and 9. This increase corresponds with the period of most rapid development for the allantois and thus the chorioallantoic plexus. The corresponding change when related to total cytoplasmic protein was 436%. However, in this case the greatest amount of change occurred between days 11 and 12. Thus we see an increase in the potential activity of an enzyme system which requires oxygen increasing as oxygen becomes more readily available.

## Lactate Dehydrogenase

Total lactate dehydrogenase activity decreased 64% per g of tissue during the 5-day period of incubation under study. There was a decrease of 44% between days 7 and 12 when total activity was related to the protein content. In both of these relationships, we see that the greatest amount of change

in activity occurred between days 7 and 9. This corresponds approximately with the period of greatest development of the chorioallantoic vascular plexus. This would relate to the time period when an increase in oxygen availability would be greatest. However, neither of these changes shows the linearity present in the other enzyme systems studied. The coefficients of correlation were -0.6485 and -0.8798 relative to the total mass of tissue and total protein content, respectively. Even without this linearity, or steady relationship between activity and time, this data does show an overall decrease of lactate dehydrogenase activity. This is significant since this enzyme catalyzes the conversion of pyruvate to lactate, the last step of anaerobic glycolysis.

Glucose Utilization

Between days 7 and 12, the ability of the cytoplasmic fraction to utilize glucose decreased 82%. This change is from 3.03 umoles glucose per min per g of tissue to 0.53 umoles glucose per min per g of tissue. In this case, the time period of greatest change was between days 9 and 11, where a 68% decrease in glucose utilization activity was observed. When this activity change is related to total cytoplasmic protein present, a decrease in glucose utilization of 88.8% is seen. This represents a change from 0.169 umoles of glucose used per min per mg protein at day 7 to 0.0189 umoles of glucose per min per mg of protein at day 12. Once again, the greatest change in activity is seen between

days 9 and 11. In both of these cases, the data shows a great degree of linearity with coefficients of correlation of -0.9391 and -0.9700 for the activity changes relative to tissue mass and protein content, respectively. Thus, a relationship between change in activity and time is implied. This is important since we see a significant correlation between time and increased availability of oxygen for the embryo.

A general trend established in all of the above cases is an increase in the activity of the aerobic pathways and a decrease in the rate of anaerobic glycolysis as incubation time increases. Since the aerobic pathway is much more efficient, this would seem to be a definite advantage for the embryo. If we assume that the embryo will utilize as much of the oxygen as possible, this increase would be expected in light of the 300% increase in oxygen concentration in the embryonic blood between days 7 and 19 (Girard, 1971).

A second reason for the importance of this conversion to aerobic pathways is the final end product of the pathway. The end product of anaerobic glycolysis is lactate. If excessive amounts of lactate accumulated within the embryo, the resulting decrease in pH would be hazardous to it. This would be critical because it would worsen the respiratory acidosis that the embryo experiences later in development (Girard, 1971; Tazawa et al., 1971).

The end products of aerobic glycolysis are $CO_2$ and $H_2O$. Both of these may, to a great extent, diffuse out of the egg. The fact that over 5 l of $CO_2$ diffuse out of the egg during the 21-day incubation period attests to this (Wangensteen and Rahn, 1970-71). However, the onset of respiratory acidosis does imply that all of the $CO_2$ which is evolved does not diffuse out of the egg. Thus, we see that the end products of aerobic glycolysis are not totally harmless to the embryo.

As was stated in the introduction, the chorioallantoic vascular plexus is completed by approximately day 10 of development. Thus, the period of greatest development within the time frame of this study would occur between days 7 and 9 or days 7 and 11. Therefore, it is not surprising to see that the greatest increase in ETS activity occurred between days 7 and 9. The greatest decrease in the rate of glucose utilization occurred between days 9 and 11.

A second finding pertaining to ETS activity is that the greatest change in activity per mg of protein occurred between days 11 and 12. During this 1-day period there was a 34% increase in activity per mg of protein. During this same time period, there was a continued decrease in the rate of anaerobic glycolysis.

Thus we see that once the $O_2$ is available, a proportionately greater amount of the cellular protein is committed to these more efficient pathways. In this way, both building block materials and the energy used in synthesizing these proteins

are conserved. However, one should not assume that this accounts for all the change in protein content. Between days 3 and 16 of incubation, there is a 63% decrease in hyaluronidase activity within the hearts of chicken embryos (Orkin and Toole, 1978).

One can assume that some of the decrease in protein content is the result of lesser amounts of the enzymes involved in the differentiation of the heart being present at these later dates. Thus, other factors beyond the scope of this project come into play. One general conclusion can be made. As the embryo becomes better able to obtain oxygen from the external environment, there is a statistically significant increase in the activity of the electron transport system and aerobic pathways. Also during this same time period, there is a statistically significant decrease in the activity of anaerobic pathways.

All of these changes show a significant correlation when plotting activity against incubation time. Therefore, since there is a significant increase in oxygen concentration during this time interval, the above relationship seems justified.

Subsequent studies should extend to embryos younger than 7 days and those older than 12 days. However, due to the lack of time and funds, this could not be done at this time.

Another study area could include the effects of the onset of respiratory acidosis reported by Girard, 1971 and Tazawa et al, 1971. This would be important for two reasons. First, to see what effect the lower pH has on enzyme activity. Second, to see how this lower pH affects the transport and release of oxygen in the blood to the various tissues of the embryo.

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