1966

The Metabolism of Krebs Cycle Intermediates and Malonate by Eggs and Early Embryonic Stages of Arbacia punctulata

Russell Chancellor Addison
College of William & Mary - Arts & Sciences

THE METABOLISM OF KREBS CYCLE INTERMEDIATES AND MALONATE BY EGGS AND EARLY EMBRYONIC STAGES OF *ARBACIA PUNCTULATA*

A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

of the Requirements for the Degree of

Master of Arts

By

Russell C. Addison, Jr.

October, 1966

APPROVAL SHEET

This thesis is submitted in partial fulfillment

the requirements for the degree of

Master of Arts

Russell C. Addison, Jr.

Author

Approved, October 1966:

Robert E. L. Black

Robert E. L. Black, Ph. D.

Mitchell A. Byrd

Mitchell A. Byrd, Ph. D.

Charlotte P. Mangum

Charlotte P. Mangum, Ph. D.

John B. Morrill

John B. Morrill, Ph. D.

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. Robert E. L. Black for his guidance, support, and patience throughout the course of this investigation. The author is also indebted to Dr. Mitchell A. Byrd, Dr. Charlotte P. Mangum, and Dr. John B. Morrill for their careful reading and criticism of the manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The contribution of carbon to synthetic pathways by the citric acid cycle of early embryonic stages of the sea urchin Arbacia punctulata has been investigated. Unfertilized and fertilized eggs, blastula, gastrula, and pluteus stages were fractionated into cold 10% TCA soluble, lipid, nucleic acid, and protein portions; respiratory carbon dioxide was also collected. Permeability to the intermediates acetate-2-C^{14} and succinate-2-C^{14} increased as development progressed; also, increasing percentages of label from these substrates were incorporated into nucleic acid and protein fractions at the expense of the acid soluble fraction while incorporation into lipid and carbon dioxide fractions remained low throughout development. Differing ratios of label incorporated into nucleic acid to label incorporated into protein from acetate and succinate indicate a failure to obtain equilibrium in the cycle; however, the location of rate-limiting reactions is not indicated by these data.

Malonate, an inhibitor of succinic dehydrogenase, was found to be metabolized by this system. The distribution of label from malonate-2-C^{14} is similar to the distribution of label from acetate-2-C^{14} and succinate-2-C^{14}. Results indicate that malonate may undergo cyanide-insensitive decarboxylation as an initial step in its metabolism.

THE METABOLISM OF KREBS CYCLE INTERMEDIATES AND MALONATE BY EGGS AND EARLY EMBRYONIC STAGES OF *ARBACIA PUNCTULATA*

INTRODUCTION

The terminal oxidation of nutritive materials in all aerobically respiring tissues studies passes through a common series of reactions, the tricarboxylic acid cycle (Krebs and Lowenstein, 1960). The relationships of the tricarboxylic acid cycle to other pathways are of particular interest to the present investigation. Carbon from citric acid cycle intermediates may be incorporated into nearly all classes of cellular compounds including fatty acids, sterols, porphyrins, purines, pyrimidines, amino acids, and various carbohydrates. Acetyl-CoA is the primary intermediate used in both the formation (Rittenberg and Bloch, 1944) and breakdown (Lynen and Ochua, 1953) of fatty acids. Succinyl-CoA reacts with glycine in the initial reaction of a series leading to the synthesis of protoporphyrin (Shemin and Russell, 1953). Amino acids may be formed by transamination of keto intermediates (Katz and Chaikoff, 1955) or by direct amination (Williams and McIntyre, 1955). Carbon dioxide derived from the operation of the cycle may be reincorporated into intermediates of the citric acid cycle, or form carbamyl phosphate and thereby enter the ornithine cycle (Metzenberg, Hall, Marshall, and Cohen, 1957).

Because of the relationships described above, it is possible to obtain information about the operation and relative importance of pathways related to the citric acid cycle by incubating cells in the presence of appropriate intermediates containing carbon-14 followed by fractionation and determination of the relative proportions of label incorporated into the various fractions. In the present investigation this technique has been used to detect possible changes in metabolism during early development of the sea

urchin Arbacia punctulata.

The studies of Cohen (1954) and Hultin (1953) have dealt with the synthetic aspects of the tricarboxylic acid cycle in embryonic material. Cohen incubated eggs and embryos of Rana pipiens in labeled bicarbonate and separated the material into aqueous methanol soluble, lipid, nucleic acid, and protein fractions. As development progressed increasing percentages of label were incorporated into nucleic acid and protein fractions at the expense of the methanol-soluble fraction while incorporation into the lipid fraction remained low throughout development. Chromatographic separation of the components of the methanol-soluble fraction revealed large quantities of label in aspartic and glutamic acids with lesser amounts being present in several citric acid cycle intermediates. Total incorporation of labeled bicarbonate into eggs and embryos of Psammechinus milaris increased rapidly through the blastula stage and remained constant afterward (Hultin, 1953). Incorporation into cold-TCA-soluble and ribonucleic acid fractions was greatest at the blastula stage, whereas specific activity of proteins showed a steady rise throughout the stages tested. A peak of incorporation of carboxyl-labeled acetate into proteins and fatty acids occurred at the mesenchyme blastula stage.

Several studies have dealt with specific fractions. Mohri (1964) used carboxyl-labeled acetate and measured its incorporation into lipids of the sea urchin. Incorporation followed an s-shaped curve through the prism stage, after which it declined slightly.

Monroy and Vittorelli (1962) traced the label from glucose during early development of the sea urchin Paracentrotus lividus and found activity in the amino acid alanine, glutamic acid, aspartic acid, serine, glycine, and, tentatively, proline. Although label from glucose was incorporated into

free amino acids in the unfertilized egg, indicating a functioning citric acid cycle, label was incorporated into proteins only after fertilization.

The results presented above indicate that while a considerable amount is known about the entry of intermediates into specific pathways, no one has yet determined the relative proportions of citric acid cycle intermediates which are shunted into other pathways at different stages of sea urchin development. In the present investigation methyl-labeled acetate and succinate were incubated with eggs and embryos of the sea urchin Arbacia punctulata at different stages of development followed by fractionation into acid soluble, lipid, nucleic acid, and protein portions. Such data are of interest for two reasons: 1) It is desirable to obtain an overall view of the contribution of the citric acid cycle to the synthesis of cellular constituents during early embryonic development. 2) Changes might occur in the relative proportions of carbon entering various pathways from citric acid cycle intermediates. One possible mechanism by which citric acid cycle intermediates might be shunted into different pathways during development is by decreases in rates of substrate conversion at specific points in the cycle perhaps at different times during development. Such decreases could result from decreases in the activities of specific enzymes. For example, inhibition or decrease in activity of succinic dehydrogenase might lead to a relative increase in the conversion of acetate carbon into lipids and glutamic acid whereas a decrease in activity of aconitase or isocitric dehydrogenase could increase acetate incorporation into lipids but decrease the shunting of carbon into glutamic acid. A decrease in activity of the alpha-ketoglutaric oxidase system might result in decreased shunting of acetate carbon into aspartic acid, whereas succinate carbon would continue to be incorporated into this amino acid. If there were no limiting steps

or shunting reactions in the formation of succinate from acetate, rapid equilibrium should be reached between labeled acetate and succinate when acetate is added. Once this equilibrium is reached, the same percentage incorporation of label from each substrate into proteins and nucleic acids should occur. In a similar manner, rapid labeling of citrate should occur when either acetate or succinate is added, leading to a similar percentage incorporation from each into compounds outside the cycle. In order to partially test these possibilities, the incorporation of carbon from methyl-labeled acetate and succinate was compared during development. In addition, ratios of label incorporated into proteins to label incorporated into nucleic acids have been calculated. Although the data below provide no evidence for any major changes in the shunting of carbon from the cycle into different pathways during development, differences in protein: nucleic acid ratios obtained from acetate and succinate may indicate failure to achieve equilibrium in the cycle.

In order to determine whether the relative incorporation of label from acetate and succinate into other compounds could be altered, it was intended that eggs and embryos be incubated in sodium malonate simultaneously with appropriately labeled intermediates; however, relatively high concentrations of malonate inhibited respiratory and developmental rates only slightly.

Preliminary investigation with labeled malonate indicates that failure to inhibit development and respiration may result from low permeability of malonate and metabolism of malonate to carbon dioxide. Evidence is presented that malonate is decarboxylated to acetate by a cyanide-insensitive reaction in the eggs.

MATERIALS AND METHODS

Arbacia punctulata were collected from the Eastern Shore region of the Chesapeake Bay and offshore from Beaufort, North Carolina and stored in 31 °/oo artificial sea water at 20-23° C. Eggs were released into artificial sea water prepared according to the M. B. L. formula (Cavanaugh, 1956) from reagent grade chemicals. Eggs and sperm were released by electric shock (Harvey, 1954) or potassium chloride injection (Tyler, 1949). Following washing, eggs were fertilized or jelly coats were removed by lowering pH of the artificial sea water to 4.0-4.5. Further manipulations were performed in buffered artificial sea water [0.02 M tris (hydroxymethyl) aminomethane] pH 8.0. Fertilized eggs were incubated at 23° C for 0.5, 8, 15, or 44½ hours, corresponding to fertilized egg, blastula, gastrula, or pluteus stages, in buffered sea water containing 500-1000 units/ml of penicillin and 250 units/ml of streptomycin. The eggs or embryos were washed and concentrated to a small volume, a sample was removed for counting, and 1 ml samples were added to 1 ml of buffered sea water in Warburg vessels to which was added 2 µc of acetate-2-C^{14} (10 µc/mM), succinate-2,3-C^{14} (8.5 µc/mM), or malonate-2-C^{14} (4.0 µc/mM). Oxygen consumption was recorded over a two hour period at 25° C., after which the center well alkali was removed and counted. The eggs or embryos were removed and washed five or more times with ice-cold buffered sea water. Cold trichloroacetic acid (TCA) was added to a final concentration of 10% and the embryos were homogenized by forcing through a crimped hypodermic needle. A sample, termed whole homogenate, was removed and frozen. The remaining homogenate was

fractionated. Triplicate vessels were run for most experiments.

Homogenates were fractionated by a modification of the method of Schmidt and Tannhauser (1945): 1) The acid soluble fraction was prepared by ten washes with cold 10% TCA. 2) The lipid fraction was removed with chloroform methanol (2:1 v/v), washed five times with water (Folch and Lees, 1951), and dried. 3) Nucleic acids were removed by five treatments with 5% TCA at 90° C. 4) The remaining sediment, the protein fraction, was suspended in 1.0 ml of 1N sodium hydroxide. Samples of the fractions described above were assayed for C^{14} content in a Nuclear Chicago 721 Liquid Scintillation Spectrometer. Aliquots were dissolved or suspended in 20 ml of dioxane liquifluor solution consisting of 42 ml of liquifluor (Pilot Chemicals) per liter of dioxane. Fractions were prepared as follows: 1) 0.5 ml of 10 N sodium hydroxide was added to 0.5 ml of whole homogenate then diluted to 5.0 ml with water; 0.1 ml was counted. 2) The lipid fraction was dissolved in 0.2 ml hyamine hydroxide (Nuclear Chicago) with gentle heating and counted. 3) 0.1 ml of the nucleic acid fraction was counted. 4) 0.1 ml of protein suspension was added to 0.7 ml of hyamine hydroxide and 0.1 was counted. 5) Respiratory carbon dioxide trapped in center-well alkali was rinsed into counting vessels and counted. Disintegrations per minute (DPM) were calculated from standard curves of channels ratio versus efficiency prepared for whole homogenate, 10% TCA soluble, lipid, and protein fractions.

To determine the influence of malonate on oxygen consumption, eggs or embryos were concentrated to a small volume and placed in Warburg vessels containing buffered sea water and 100, 80, 50, 20 or 0 μM/ml malonate.

To determine the effects of cyanide on carbon dioxide production from malonate labeled at the 1 or 2 carbon, 2 ml of a suspension of unfertilized,

jelly-free eggs in buffered sea water containing 1 μM/ml of KCN and 0.5 uc/ml of malonate-1-C^{14} or malonate-2-C^{14} were incubated at 25° for two hours.

The effect of malonate on carbon dioxide production from succinate carbon from the 1 and 2 positions was determined by incubation of unfertilized, jelly-free eggs in 10 μM/ml malonate and 0.5 μc/ml succinate.

The effect of malonate on respiration of homogenates of unfertilized eggs was determined by comparison of rates of respiration of homogenates containing either sodium chloride or malonate. Eggs were obtained as above and washed and jelly coats removed in calcium-free sea water [Moore's formula (Cavanaugh, 1956)]. Eggs were packed by hand centrifugation in an equal volume of solution containing 0.94 M sucrose, 0.05 M Tris, and 0.011 M ethylenediaminetetraacetic acid, pH 7.5. The eggs were then homogenized by a hand-operated Tanbroeck glass homogenizer at 0-5° C. The final concentrations of other constituents in μM/ml in Warburg vessels were: DPN 0.22; ATP 0.83; Cytochrome c 0.02; KCl 8.3; phosphate 1.7; MgCl 16.6; and sodium malonate 8.3. Blanks contained 8.3 μM/ml sodium chloride. The final concentrations of egg material was approximately 25% by volume. In a second experiment vessels contained malonate at 4.2, 8.3, 16.7, or 33.3 μM/ml. Blanks contained 33.3 μM/ml sodium chloride.

The breakdown of malonate to carbon dioxide was investigated in homogenates using an incubation mixture similar to the above but with the following changes: 1) Thiamine pyrophosphate was present at 2.2 μM/ml. 2) 1.0 μM/ml of KCN or KCl (blank) was present. 3) The vessels contained either sodium malonate-2-C^{14} (1.6 μc/uM) or sodium malonate-2-C^{14} (4.0 μc/uM) + 0.5 μc/ml. Respiratory carbon dioxide was collected over a one hour period and counted as above.

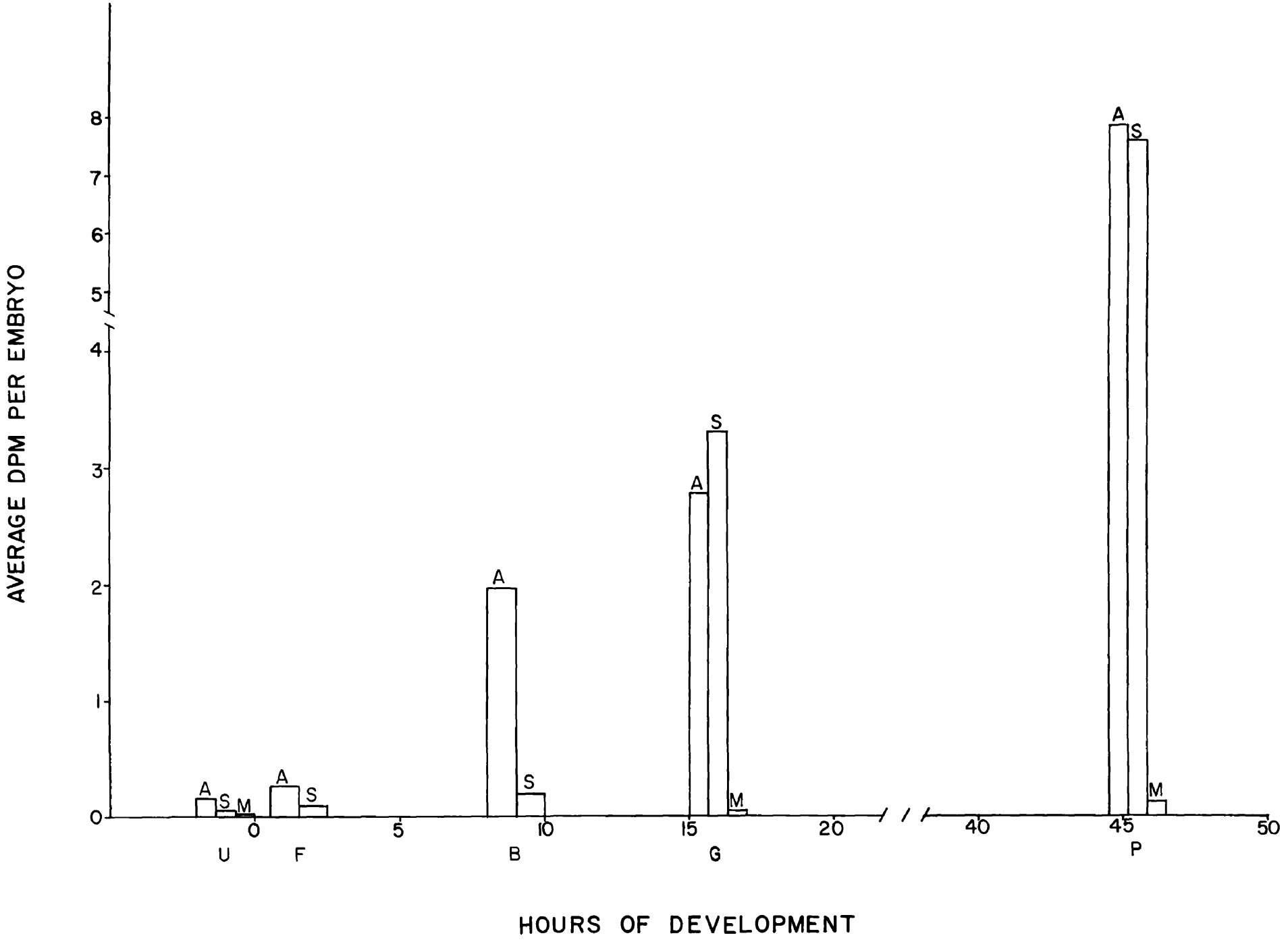
RESULTS

Fractionation Experiments

The uptake of acetate, succinate, and malonate into sea urchin eggs and embryos is shown in Figure 1. Total uptake has been calculated by summing the radioactivity recovered in the various fractions. This procedure was necessitated by the difficulties encountered in counting the whole homogenate, the counts from which were consistently lower than those obtained from the fractionated material. Probably this is due to incomplete solubility of the whole homogenate. The amount of label taken up per embryo rises throughout development in all three cases, but the uptake of malonate is always considerably less than that of acetate or succinate. This may be due in part to the difference in specific activities of the three compounds even though the specific activity of each intermediate was consistent throughout the experiments.

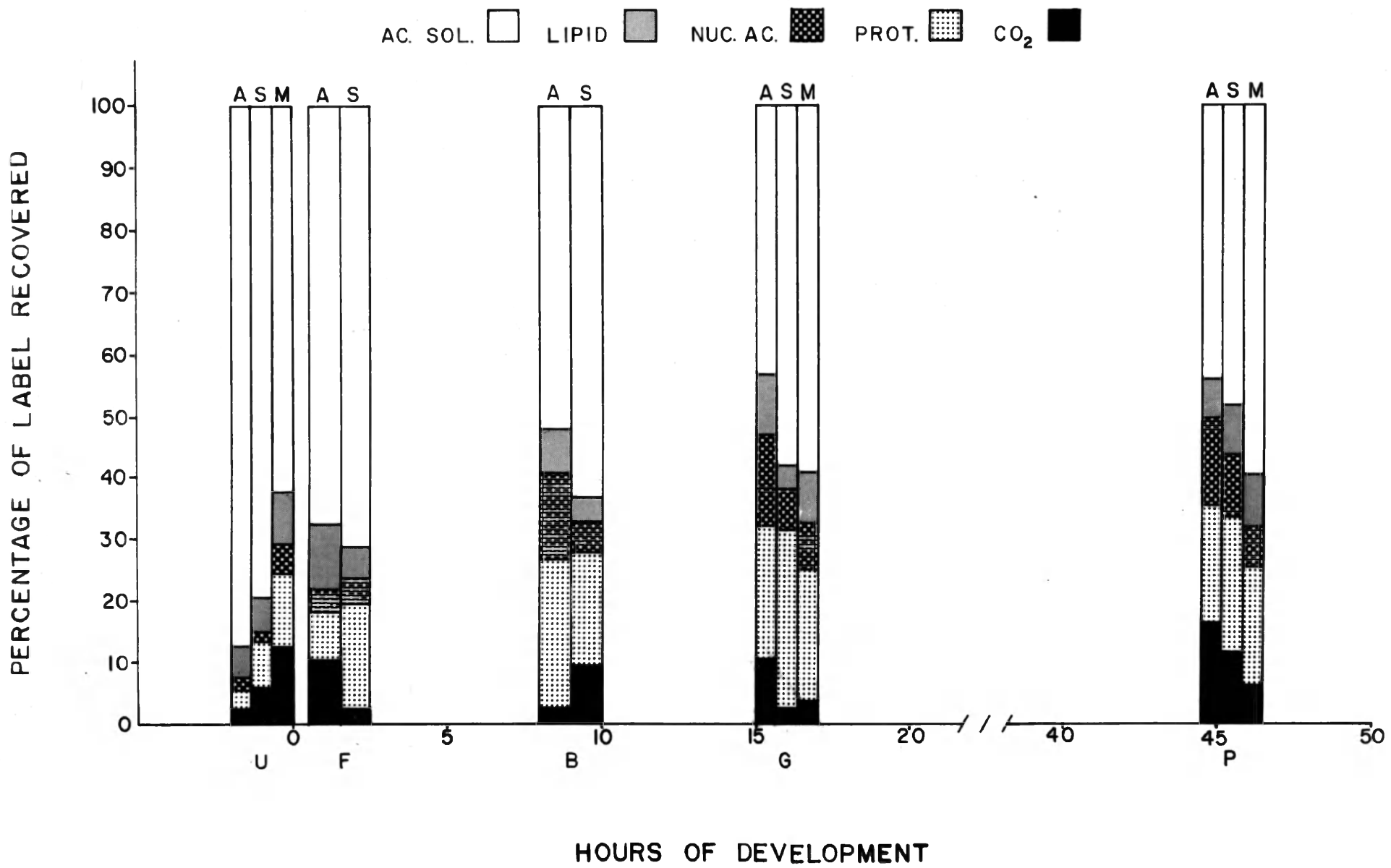
Oxygen consumption per embryo in the presence of acetate and succinate is given in Table I. In the experiments using acetate and succinate, oxygen consumption is lowest in the unfertilized egg, rises through the gastrula stage, and decreases slightly in the pluteus stage. Oxygen consumption of the two stages labeled with malonate is considerably less than comparable stages labeled with succinate or acetate. The reason for decreased oxygen consumption is unknown; the concentration of malonate was probably too low to affect oxygen consumption to a significant degree. Although the large number of embryos labeled with malonate might possibly decrease oxygen consumption per embryo in the pluteus stage, this could not apply to the gastrula stage as a similar number of embryos labeled with succinate showed no decrease of oxygen consumption, compared with a much smaller number of embryos labeled with acetate at the same stage.

Figure 1. Total uptake of label from 1 μc/ml of acetate, succinate, and malonate into sea urchin eggs and embryos during a two-hour incubation period. Most values are averages from three batches of eggs. Abbreviations used are as follows: A, acetate-2-C^{14}; S, succinate-2-C^{14}; M, malonate-2-C^{14}, U, unfertilized eggs; B, blastula; G, gastrula; P, pluteus.


AVERAGE DPM PER EMBRYO
8
7
6
5
4
3
2
1
0
A
S
M
A
S
A
S
A
S
M
A
S
M
0
5
10
15
20
40
45
50
U
F
B
G
P
HOURS OF DEVELOPMENT

The percentage distribution of recovered label from acetate, succinate, and malonate is shown in Table II and Figure 2. This data is taken from the same experiments as the data from Table I. Carbon dioxide was collected during the two hour incubation period only; consequently, the percentage of carbon dioxide recovered should be assumed to be the lower limit of incorporation into carbon dioxide. This may also account for the erratic percentages found in the carbon dioxide data. At the stages tested, increasing percentages of the label are incorporated from acetate and succinate into nucleic acids and proteins at the expense of the acid soluble fraction as development progresses. The percentage incorporation of label into lipids from acetate and succinate is rather variable at different stages due, perhaps, to losses during fractionation and washing of the material. The percentage incorporation into lipids does not increase as does incorporation into nucleic acids and proteins but remains at a low level throughout the stages tested. Generally a higher percentage of label is incorporated into lipids from acetate than from succinate. This is particularly true in the blastula and gastrula stages when very low percentages of label are incorporated into lipids from succinate. Approximately eight percent of the label taken up is incorporated from malonate into lipids in the three stages tested. Since any label which remained in acetate, succinate, or malonate would be recovered in the acid soluble fractions, the data show a progressively greater incorporation of the label into the structural components of the cell during development. The situation is somewhat different with respect to malonate, the percentage of which incorporated into lipids, nucleic acids, and proteins of the unfertilized egg is higher than that incorporated from acetate or succinate at the same stage. Further, the increase in percentage incorporated into these constituents rises to a lesser degree than does the percentage incorporated from acetate or succinate.

Figure 2. Per cent uptake of label into various fractions of sea urchin eggs and embryos from acetate, succinate, and malonate. Most values are averages from three batches of eggs. Abbreviations used are as follows: A, acetate-2-C^{14}; S, succinate-2-C^{14}; M, malonate-2-C^{14}; U, unfertilized eggs; F, fertilized eggs; B, blastula; G, gastrula; P, pluteus.


AC. SOL.
LIPID
NUC. AC.
PROT.
CO_2
PERCENTAGE OF LABEL RECOVERED
100
90
80
70
60
50
40
30
20
10
0
A S M
A S
A S
A S M
A S M
0
5
10
15
20
40
45
50
U
F
B
G
P
HOURS OF DEVELOPMENT

Similar percentages of label are incorporated into nucleic acids from acetate and succinate in unfertilized eggs, but the percentage incorporation from acetate is noticeably higher than from succinate at the blastula, gastrula, and pluteus stages.

The incorporation of label from acetate and succinate into proteins rises quickly after fertilization and in later stages accounts for the greatest percentages of acid-insoluble label. Generally a higher percentage of label is incorporated into protein from succinate than from acetate, particularly in the early stages.

The ratio of percent label incorporated into proteins to percent label incorporated into nucleic acids has been calculated (Table II). It is readily seen that with all added substrates a greater percentage of label is incorporated into proteins than into nucleic acids. The protein:nucleic acid ratios obtained from acetate and succinate differ somewhat during the stages tested. The ratio from acetate was low before fertilization but reached a maximum in the fertilized egg; it then declined through the gastrula stage and remained constant at the pluteus stage. The protein : nucleic acid incorporation ratio from succinate was highest in the unfertilized egg and decreased through the blastula stage; the ratio increased at the gastrula stage but decreased again at the pluteus stage. The ratio of label incorporated into protein to label incorporated into nucleic acids obtained from acetate is always considerably lower than the same ratio obtained from succinate. If the assumptions given above are valid, it seems probable that rate-limiting steps occur in the formation of citrate from succinate and in the formation of succinate from acetate.

The sequence by which the methyl carbon of acetate enters nucleic acids without traversing the Krebs cycle is not clear. Hultin (1953) stated that

Label from acetate-1-C^{14} enters purine via carbon dioxide; however, in order for labeled carbon dioxide to be derived from the methyl carbon, this carbon must first traverse the entire cycle. If this were the case then equilibrium with succinate carbon should result, as noted above. It is clear that more data on the labeling of specific compounds from acetate are needed.

A comparison of the data in Table II and Figure 2 with percentages of label from carbonate incorporated into various fractions of the developing frog embryo (Cohen, 1954) may prove instructive. In both the present study and that of Cohen the lipid fractions received a relatively small portion of label throughout development, but in the present study incorporation into lipids on a percentage basis was several times as large as incorporation into lipids in the frog. In both studies the nucleic acids incorporated increasingly greater percentages of label as development progressed, but the percentages were always much higher in the amphibian, possibly because carbonate may be incorporated more directly into purines. In this connection, it is interesting to note that the ratio of percentages incorporated into proteins to percentages incorporated into nucleic acids in the amphibian embryo is always less than one and increases constantly throughout the stages tested. It is of interest to make a further comparison of the present data with those of Hultin (1953), who studied the incorporation of label from carbonate and acetate-1-C^{14} into several fractions of developing embryos of the sea urchin Psammechinus milaris. The specific activity of the lipid fraction was found to be low throughout the gastrula stage when labeled with carbonate. Label incorporated into the ribonucleic acid fraction and also hypoxanthine increases through the blastula stage and drops in the gastrula stage whereas the label incorporated into proteins to counts incorporated into the ribonucleic acid fraction is always less than one and rises progressively

during development. When acetate-1-C^{14} is used as the labeled substrate the patterns of labeling are somewhat different. The label incorporated into fatty acids and proteins rises sharply after fertilization while the labeling of ribose nucleotides remains rather low throughout development. The ratio of label incorporated into proteins to label incorporated into RNA nucleotides is greater than one at all times and rises as development progresses. Neither Cohen (1954) nor Hultin (1953) indicate the relative degree of shunting from the citric acid cycle during development.

Malonate-Effects On Intact Eggs and Embryos

In assessing the contribution of Krebs cycle intermediates to other compounds it was of interest to attempt to partially block the cycle at some point, after which the distribution of label from acetate or succinate could be redetermined. Malonate was chosen for the blocking agent since it is a competitive inhibitor of succinic dehydrogenase in most systems (Krebs and Lowenstein, 1960). In initial experiments the extent or respiratory inhibition was tested with malonate with the results shown in Table III. Concentrations of malonate below 0.08 M had either no effect or a slight depressing effect on respiration while a concentration of 0.10 M always caused some increase in oxygen uptake. Malonate at 0.10 M caused only a slight inhibition of development.

The results shown above leave the question of malonate inhibition of the citric acid cycle largely unanswered. The effect of malonate on the citric acid cycle was therefore investigated by determining the extent to which malonate inhibited the production of labeled carbon dioxide from succinate-1-C^{14} or succinate-2-C^{14}. Both labeled substrates were used in order to test the possibility that succinate was being decarboxylated to propionate via a two reaction sequence which releases the carboxyl carbon

of succinate as carbon dioxide (Lane and Halenz, 1962; Beck, 1962). If malonate inhibition of these reactions differs from its inhibition of succinic dehydrogenase, then one might detect differences in the rate of labeled CO_2 formation from succinate-1- and succinate-2-C^{14} in the presence of unlabeled malonate. If, on the other hand, succinate is being oxidized by the citric acid cycle and if succinic dehydrogenase is inhibited by malonate, labeling of carbon dioxide from both succinate-1-C^{14} and succinate-2-C^{14} should decrease and the percentage decrease should be approximately the same for both substrates. The results shown in Table IV indicate that the production of labeled carbon dioxide from both succinate-1-C^{14} and succinate-2-C^{14} was inhibited approximately fifty percent by 0.1 M malonate. This result appears to indicate that succinate is being metabolized by the citric acid cycle which contains a malonate-sensitive succinic dehydrogenase. Failure of malonate to inhibit to a greater extent may be due to a number of factors; among them, the relative impermeability of the egg to malonate and the rapidity of metabolism once malonate enters the cell, both of which would delay or prevent the establishment of inhibitory concentrations of malonate.

Data on the distribution of label from malonate (Table II and Figure 2) indicate that malonate is metabolized by the eggs. The similarity of the distributions of label from malonate, succinate, and acetate appear to indicate that carbon from malonate undergoes very few preparatory reactions before it enters the citric acid cycle. Nakada, Wolfe, and Wick (1957) proposed that malonate (as malonyl CoA) was decarboxylated to acetate (as acetyl CoA) as one of the initial steps of its metabolism. To test the possibility that decarboxylation was occurring, an experiment was performed in which the label incorporated into carbon dioxide from malonate-1-C^{14} and

malonate-2-C^{14} in the presence or absence of cyanide was measured. Cyanide inhibits Krebs cycle dehydrogenases by preventing the reoxidation of reduced coenzymes (Krebs and Lowenstein, 1960). The results in Table V show that while the amount of label incorporated into carbon dioxide from malonate-1-C^{14} is unchanged by the presence of cyanide, the amount of label incorporated into carbon dioxide from malonate-2-C^{14} is approximately halved by the presence of cyanide. This indicates that the first step in malonate metabolism is cyanide insensitive and may involve the release of carbon dioxide from the carboxyl carbon. A possible explanation is that malonate was decarboxylated to acetate; in this case, half the label from malonate-1-C^{14} would be lost in this initial decarboxylation, whereas all the label from malonate-2-C^{14} would remain in the acetate produced in at least one turn of the cycle. Partial inhibition of the cycle would thus reduce the label released as carbon dioxide from malonate-2-C^{14} by a greater percentage than the label produced from malonate-1-C^{14}.

Malonate-Effects On Homogenates

Nakada, Wolfe, and Wick (1957) found that most rat tissues would decarboxylate malonate in slices but that the malonate decarboxylase system was destroyed by the cell rupture process. It was, therefore, of interest to determine the effects of malonate on homogenates of unfertilized eggs. The effects of malonate on oxygen consumption are shown in Table VI. In all experiments at least a slight increase in oxygen consumption is noted when compared with a blank containing sodium chloride; however, there is a large amount of variation in the amount of increase between experiments. The results from experiment 3 show that the concentration of malonate over the range tested has a slight positive effect. There is a possibility that

the chloride (33.3 uM/ml) contained in the blanks could inhibit oxygen consumption but this appears unlikely. It is also possible that the increase in oxygen consumption may not be due to the oxidation of malonate but to the oxidation of other materials which is somehow facilitated by the presence of malonate.

In view of the increase in oxygen consumption obtained with homogenates in the presence of malonate, an experiment to determine the effects of cyanide on the labeling of carbon dioxide from malonate-1-C^{14} and malonate-2-C^{14} was performed (Table VII). The amount of label converted to carbon dioxide is very small in comparison to the amount of label converted in intact eggs. As in the intact eggs the presence of cyanide did not appear to affect production of label from malonate-1-C^{14}, but no counts were found in either cyanide inhibited or noninhibited vessels from malonate-2-C^{14}. This would be most easily explained by an initial cyanide-insensitive decarboxylation to acetate, possibly followed by oxidative metabolism. If oxidative metabolism occurred the label released was too limited to detect under the conditions employed.

DISCUSSION

This study has been concerned with the shunting activity of the citric acid cycle in the egg and developing embryo of the sea urchin Arbacia punctulata. The data indicate that throughout early development a major percentage of the total activity of the cycle is concerned with this shunting activity which provides carbon for the synthesis of cellular constituents. Although no specific sites of blockage have been identified in the cycle at the stages tested, the differing ratios of label incorporated into proteins and nucleic acids from acetate and succinate indicate that carbon from succinate and acetate fail to reach equilibrium in the cycle. Such blocks would be expected if the cycle is to provide a major source of carbon for other compounds. No evidence is provided in the present study for any changes in the nature of rate-limiting reactions during development.

Malonate was found to be metabolized by sea urchin eggs and embryos. The details of the metabolism of malonate are not clear but the data presented seem to indicate an initial cyanide-insensitive decarboxylation followed by metabolism by the citric acid cycle. It is interesting to speculate on the possibility of malonate formation in the egg by a reversal of such a decarboxylation. If such were the case, malonate might serve as an internally produced respiratory inhibitor. Although the occurrence of free malonate in Arbacia embryos has not been shown, Cohen (1963) has demonstrated an accumulation of labeled malonate in lethal amphibian hybrids (R. pipiens ♀ x R. sylvatica ♂) when incubated in labeled carbonate. Several pathways for the production of malonate exist. In animals the enzyme succinyl-β-ketoacyl

CoA transferase may form malonate from malonyl CoA by transfer of the CoA to acetoacetate (Menon and Stern, 1960). Malonate may also be produced by decarboxylation of oxaloacetate in the presence of metmyoglobin from horse liver (Vennesland, Evans, and Francis, 1946) and pig heart (Vennesland and Evans, 1944). Spencer and Lowenstein (1962) have proposed that oxaloacetate is decarboxylated to malonate in high speed supernatent fluids of rat mammary gland. The bacterium Pseudomonas aeruginosa may form malonate from malonic semialdehyde (Nakamura and Bernheim, 1961) while members of the Mycobacterium form malonate by degradation of barbituric acid (Hayaishi and Kornberg, 1952).

A number of questions are raised as a result of this investigation. The specific compounds which receive label from acetate, succinate and especially malonate in the sea urchin embryo have not been identified. Questions such as the differing ratios of label incorporated into proteins and into nucleic acids from acetate and succinate may be answered only by this type of study. Such a study would also be likely to furnish information concerning the failure of acetate and succinate to equilibrate in the cycle.

The question has not been answered as to whether more than one system in the sea urchin embryo is capable of preparing malonate for further metabolism. The localization of the system or systems by fractionation of homogenates, provided a procedure could be found for obtaining sufficient activity from the system(s), might serve to elucidate this question. Several different methods of malonate utilization occur in other systems. Pseudomonas fluorescens converts malonate to acetate by means of a cyclic mechanism whereby malonate first reacts with coenzyme A to form malonyl CoA, which is then decarboxylated to acetyl CoA. Another malonate molecule then combines with

the coenzyme A releasing acetate (Hayaishi, 1955). Malonate has also been found to react with coenzyme A in the presence of ATP and magnesium ions in human placenta (Hosoya and Kavada, 1958). The formation of malonyl CoA from malonate may lead to incorporation of malonate into fatty acids. In many tissues acetate first reacts with coenzyme A to form acetyl CoA which is then carboxylated to malonyl CoA (Brady, 1958). The malonyl CoA is decarboxylated subsequent to the release of coenzyme A accompanied by the formation of a malonyl-enzyme complex (Lynen, 1959). Malonyl-CoA may also be formed by transfer of coenzyme A to malonate (Menon and Stern, 1960).

SUMMARY

The uptake and distribution of label from acetate-2-C^{14} and succinate-2-C^{14}, two citric acid cycle intermediates, and malonate-2-C^{14} into acid soluble, lipid, nucleic acid, and protein fractions and respiratory carbon dioxide has been determined. Total uptake of acetate, succinate, and malonate was lowest in the unfertilized egg and rose continuously as development progressed. The uptake of malonate was always lower than that of acetate and succinate.

Following fractionation of the unfertilized egg a large majority of label from acetate and succinate was found in the cold 10% TCA soluble fraction; however, as development progressed through the gastrula stage, increasing percentages of label were incorporated into the lipid, nucleic acid, and protein fractions. Incorporation into acid-insoluble components at the pluteus stage was similar to that in the gastrula stage. Increases in percentages incorporated into acid insoluble components as development progressed are due mainly to increases in the amount of label incorporated into nucleic acids and proteins; incorporation into lipids and respiratory carbon dioxide is somewhat erratic and generally low. Much of the carbon entering the citric acid cycle is shown to become incorporated into cellular constituents rather than oxidized to carbon dioxide; therefore, the citric acid cycle appears to be acting largely in the capacity of a shunting device. The differing ratios of label incorporated into proteins to label incorporated into nucleic acids obtained from acetate and succinate suggest a failure to obtain equilibrium in the cycle. No sudden changes occur in these ratios

throughout the stages tested.

Malonate, an inhibitor of succinic dehydrogenase, has been shown to be metabolized. The distribution of label from malonate into various fractions is quite similar to that of acetate or succinate except that a greater percentage of label is incorporated into the acid insoluble fractions in the unfertilized egg from malonate and the increase in percentage incorporation into these components at later stages is considerably less than for acetate and succinate. These data compared with those obtained from acetate and succinate lend themselves to the interpretation that much of the carbon from malonate traverses the citric acid cycle. Several experiments have been conducted to determine the relationships of malonate to the citric acid cycle. The oxygen consumption of eggs and embryos was increased by the presence of 0.1 M malonate but not by lower concentrations. Malonate was shown to decrease the amount of label incorporated into respiratory carbon dioxide from succinate-1-C^{14} and succinate-2-C^{14} by approximately equal percentages in the unfertilized egg. Cyanide was found to decrease the amount of label incorporated into carbon dioxide from malonate-2-C^{14} but not from malonate-1-C^{14} in unfertilized eggs. In homogenates of unfertilized eggs malonate increased oxygen uptake but the amount of increase over the blank was only slightly affected by the concentration of malonate. Cyanide had no effect on the amount of labeling of carbon dioxide from malonate-1-C^{14} in homogenates of unfertilized eggs, but the amount of label given off was extremely small. These data are compatible with the metabolism of malonate by an initial cyanide insensitive decarboxylation followed by metabolism via the citric acid cycle.

TABLE I

OXYGEN CONSUMPTION OF EGGS AND EMBRYOS DURING INCUBATION WITH LABELED SUBSTRATES

Oxygen consumption is expressed as average (usually of three samples) oxygen consumed in µl per hour per 10^4 eggs or embryos. The number of embryos is given in thousands of embryos per vessel.

Labeled Substrate	Stage	Oxygen Uptake	Number of Embryos
Acetate	Unfertilized egg	0.6	733
	Fertilized egg	2.1	114
	Blastula	3.1	154
	Gastrula	3.8	84
	Pluteus	3.4	70
Succinate	Unfertilized egg	0.6	987
	Fertilized egg	2.0	552
	Blastula	3.4	337
	Gastrula	4.0	418
	Pluteus	3.8	122
Malonate	Unfertilized egg	**	1,184
	Gastrula	2.7	417
	Pluteus	2.8	284

** Oxygen consumption was not obtained from this experiment.

TABLE II

DISTRIBUTION OF LABEL IN EGGS AND EMBRYOS AT VARIOUS STAGES

Total uptake was calculated by summation of the disintegrations per minute incorporated into the various fractions and is given as disintegrations per minute per thousand eggs or embryos. Per cent incorporation was calculated using the figure for total uptake as one hundred per cent. Figures marked * were not used in making Figures 1 and 2 or calculating averages.

Stage	Total Uptake	% Incorporation					% Protein
		Acid Sol.	Lipid	Nuc. Acid	Protein	CO_2	% Nuc. Ac.
				Acetate-2-C^{14}			
Unfertilized egg	124	85.3	5.5	2.2	3.0	4.0	1.4
	162	88.4	4.5	2.0	3.2	2.0	1.6
	160	88.6	5.1	2.1	3.2	1.0	1.5
	149	87.4	5.0	2.1	3.1	2.3	1.5
Fertilized egg	276	64.1	11.5	4.7	9.3	11.8	2.0
	316	76.4	7.0	3.2	5.8	7.6	1.8
	276	62.9	11.8	4.3	9.2	11.8	2.1
	289	67.8	10.2	4.1	7.6	10.4	1.9
Blastula	309*	54.8*	9.0*	5.5*	19.3*	11.4*	3.5*
	1,752	50.6	7.6	14.5	23.8	3.5	1.6
	2,171	53.7	6.4	13.6	24.2	2.0	1.8
	1,961	52.2	7.0	14.1	24.0	2.8	1.7

TABLE II (cont.)

Stage	Total Uptake	% Incorporation Acid Sol.	Lipid	Nuc. Acid	Protein	CO_2	% Protein % Nuc. Ac.
			Acetate-2-C^{14} (cont.)				
Gastrula	2,749	40.4	11.6	14.6	23.6	18.7	1.6
	2,397	48.6	6.9	12.5	17.9	14.0	1.4
	2,067	41.0	11.4	17.0	21.9	8.8	1.3
	2,737	43.3	10.0	14.7	21.6	10.4	1.5
Pluteus	7,665	40.6	5.3	11.7	23.6	18.7	2.0
	7,556	47.0	8.6	12.5	17.9	14.0	1.4
	8,646	44.7	5.3	15.4	18.0	16.5	1.2
	6,824*	50.8*	14.0*	11.1*	24.1*	0.0*	2.2*
	7,956	44.1	6.4	13.2	19.8	16.4	1.5
			Succinate-2-C^{14}				
Unfertilized egg	49	83.4	4.4	1.2	9.0	1.9	7.5
	46	82.7	3.6	2.3	9.3	2.0	4.0
	37*	71.5*	5.2*	7.5*	11.3*	1.3*	1.5*
	47	83.0	4.0	1.8	9.1	1.9	5.1
Fertilized egg	82	67.8	7.1	2.1	18.5	4.4	8.8
	86	71.1	3.0	2.8	21.1	2.1	7.5
	85	74.7	5.2	7.5	11.3	1.3	1.5
	84	71.2	5.1	4.1	17.0	2.6	4.1
Blastula	219	68.7	4.0	5.3	17.0	5.0	3.2
	202	61.6	4.4	6.0	16.7	11.3	2.8
	90*	59.3*	3.5*	3.4*	21.0*	12.6*	6.2*
	210	65.2	4.2	5.6	16.8	8.2	3.0

TABLE II (cont.)

Stage	Total Uptake	% Incorporation					% Protein / % Nuc. Ac.
		Acid Sol.	Lipid	Nuc. Acid	Protein	CO_2	
		Succinate-2-C^{14} (cont.)					
Gastrula	3,316	59.0	3.7	5.8	27.1	4.3	4.7
	3,236	58.6	3.9	7.9	29.2	0.5	3.7
	3,496	56.7	3.8	6.2	31.3	2.0	5.1
	3,314	58.1	3.8	6.6	29.2	2.3	4.4
Pluteus	7,084	51.9	6.2	10.7	23.5	7.8	2.2
	8,164	45.0	6.9	10.3	20.7	17.1	2.0
	7,794	48.5	10.3	9.9	20.6	10.7	2.1
	7,680	48.5	7.8	10.3	21.6	11.8	2.1
		Malonate-2-C^{14}					
Unfertilized egg	3	58.8	5.4	2.2	16.8	16.8	7.6
	3	65.8	11.6	7.3	6.6	8.7	0.9
	3	62.2	8.5	4.7	11.7	12.8	2.5
Gastrula	45	58.8	8.4	7.2	22.9	2.7	3.2
	40	58.4	6.5	8.8	21.6	4.7	2.5
	38	60.9	9.8	6.2	20.2	2.9	3.3
	41	59.4	8.2	7.3	21.6	3.4	3.0
Pluteus	131	57.2	9.1	7.1	20.6	5.9	2.9
	123	58.3	8.3	6.2	20.0	7.2	3.2
	114	63.6	7.6	6.6	16.9	5.3	2.6
	123	59.7	8.3	6.6	19.2	6.1	2.9

TABLE III

OXYGEN CONSUMPTION OF EGGS AND EMBRYOS IN THE PRESENCE OF VARYING CONCENTRATIONS OF MALONATE

The number of eggs is given in thousands of eggs per ml. The concentration of malonate is given in µM/ml. Oxygen consumption given as µl of oxygen consumed per 10^4 embryos per hour.

Stage (No. Embryos)	Malonate Concentration	Oxygen Uptake
Unfertilized egg (96)	0	0.1
	0	0.7
	100	0.9
	80	0.6
	50	0.6
	20	0.7
Gastrula (141)	0	1.9
	0	1.8
	100	3.0
	80	1.5
	50	1.1
	20	1.6
Pleuteus (183)	0	2.7
	0	3.2
	100	3.5
	80	3.1
	20	3.5

TABLE IV

INFLUENCE OF MALONATE ON THE PRODUCTION OF LABELED CARBON DIOXIDE FROM SUCCINATE-1-C^{14} AND SUCCINATE-2-C^{14}

Each vessel contained 450,000 unfertilized eggs. Malonate concentration is given in μM/ml; disintegrations per minute (DPM) is given per thousand eggs for an incubation period of one hour.

Labeled Substrate	Malonate Concentration	DPM in CO_2	Per Cent of Control
Succinate-1-C^{14}	0	5.75	100
	100	3.16	54
	100	2.44	42
Succinate-2-C^{14}	0	2.04	100
	100	1.09	53
	100	1.19	58

TABLE V

EFFECT OF CYANIDE ON THE PRODUCTION OF LABELED CARBON DIOXIDE FROM MALONATE IN INTACT UNFERTILIZED EGGS

DPM refers to the total number of disintegrations per minute incorporated into carbon dioxide from each vessel over a two hour period.

Expt. No.	Substrate	Inhibitor	DPM in CO_2	Per Cent of Control
1	Malonate-1-C^{14}	None	482	100
		Cyanide	559	116
		Cyanide	438	91
	Malonate-2-C^{14}	None	114	100
		Cyanide	68	59
		Cyanide	39	34
2	Malonate-1-C^{14}	None	727	100
		Cyanide	655	90
		Cyanide	743	102
	Malonate-2-C^{14}	None	112	100
		None	105	
		Cyanide	47	43
		Cyanide	59	54

TABLE VI

EFFECT OF MALONATE ON OXYGEN CONSUMPTION OF HOMOGENATES OF UNFERTILIZED EGGS

Concentrations of malonate are expressed as µM/ml. Oxygen uptakke is expressed as µl of oxygen consumed per 10^5 eggs per hour. Numbers in parentheses indicate an average of readings from that number of vessels.

Expt. No.	Inhibitor	Concentration	Oxygen Uptake	Per Cent of Control
1	None		2.7	100
	Malonate	8.3	3.4	129
	Malonate	8.3	3.3	123
	None		2.0	100
	Malonate	8.3	2.2	110
	None	(2)	2.7	100
	Malonate	4.1 (2)	4.1	148
	Malonate	8.3 (2)	4.2	155
	Malonate	16.6 (2)	4.6	168
	Malonate	33.3 (1)	5.2	190

TABLE VII

EFFECT OF CYANIDE ON FORMATION OF LABELED CARBON DIOXIDE FROM MALONATE IN HOMOGENATES OF UNFERTILIZED EGGS

Each vessel contained homogenate of 940,000 eggs. DPM is expressed as the total number of counts recovered as carbon dioxide per hour per vessel.

Substrate	Inhibitor	DPM in CO_2
Malonate-1-C^{14}	None	38
	Cyanide	46
Malonate-2-C^{14}	None	0
	Cyanide	0

BIBLIOGRAPHY

Beck, William S. 1962. Methylmalonyl CoA isomerase, p. 581-587. In Sidney P. Colowick and Nathan O. Kaplan, (ed.), Methods in Enzymology, Vol. V. Academic Press, Inc., New York.

Brady, Roscoe O. 1958. The enzymatic synthesis of fatty acids by aldol condensation. Proc. Nat. Acad. Sci. 44: 993-998.

Cavanaugh, Gail M. (ed.). 1956. Formulae and Methods IV of the Marine Biological Laboratory Chemical Room. Marine Biological Laboratory, Woods Hole, Massachusetts. 61 p.

Cohen, Stanley. 1954. The metabolism of $C^{14}O_2$ during Amphibian development. J. Biol. Chem. 211: 337-354.

Cohen, S. 1963. Carbon dioxide fixation and the accumulation of malonic acid in amphibian hybrids (R. pipens ♀ x R. sylvatica ♂). Exp. Cell Res. 29: 207-211.

Folch, Jordi, and M. Lees. 1951. Proteolipids, a new type of tissue lipoproteins their isolation from brain. J. Biol. Chem. 191: 807-817.

Harvey, Ethel Browne. 1954. Electrical method of determining the sex of sea urchins. Nature (Lond.) 173: 86.

Hayaishi, Osamu. 1955. Enzymatic decarboxylation of malonic acid. J. Biol. Chem. 215: 125-136.

Hayaishi, Osamu, and Arthur Kornberg. 1952. Metabolism of cytosine, thymine, uracil, and barbituric acid by bacterial enzymes. J. Biol. Chem. 197: 717-732.

Inoya, Morimasa, and Nobuka Kavada. 1958. Malonate metabolism in human placenta. J. Biochem. (Tokyo) 45: 363-365.

Hultin, Tore. 1953. Incorporation of C^{14} labeled carbonate and acetate into sea urchin embryos. Ark. Kemi 6: 195-200.

Katz, Joseph, and I. L. Chaikoff. 1955. Synthesis via the Krebs cycle in the utilization of acetate by rat liver slices. Biochem. Biophys. Acta 18: 87-101.

Krebs, H. A., and J. M. Lowenstein. 1960. The tricarboxylic acid cycle. p. 129-203. In David M. Greenberg (ed.), Metabolic Pathways, Academic Press, New York.

Lane, M. Daniel, and Donald R. Halenz. 1962. Mitochondrial Propionyl Carboxylase, p. 576-581. In Sidney P. Colowick and Nathan O. Kaplan (eds.), Methods in Enzymology, Vol. V. Academic Press, Inc., New York.

Lynen, F. 1959. Participation of acyl-CoA in carbon chain biosynthesis. J. Cell. Comp. Physiol. 54: Supp. 1: 33.

Lynen, Feodor, and Severo Ochoa. 1953. Enzymes of fatty acids metabolism. Biochim. Biophys. Acta 12: 299-313.

..., Govind K., and Joseph R. Stern. 1960. Enzymatic synthesis and metabolism of malonyl coenzyme A. J. Biol. Chem. 235: 3393-3397.

Metzenberg, R. L., Leo M. Hall, Margaret Marshall, and Philip Cohen. 1957. Studies on the biosynthesis of carbamyl phosphate. J. Biol. Chem. 229: 1019-1025.

Mohri, H. 1964. Utilization of C^{14}-labeled acetate and glycerol for lipid synthesis during the early development of sea urchin embryos. Biol. Bull. 126: 440-455.

Monroy, Alberto, and Maria Vittorelli. 1962. Utilization of C^{14} glucose for amino acids and protein synthesis by the sea urchin embryo. Jour. Cell. Comp. Physiol. 60: 285-288.

Shinda, Henry I. Jack B. Wolfe, and Arne N. Wick. 1957. Degradation of malonic acid by rat tissues. J. Biol. Chem. 226: 145-152.

Nakamura, Keji, and Frederick Bernheim. 1961. Studies on malonic semialdehyde dehydrogenase from Pseudomonas aeruginosa. Biochim. Biophys. Acta 50: 147-152.

Rittenberg, D., and Konrad Bloch. 1944. The utilization of acetic acid for fatty acid synthesis. J. Biol. Chem. 154: 311-312.

Schmidt, Gerald, and S. J. Thannhauser. 1945. A method for the determination of desoxyribose nucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. 161: 83-89.

Shemin, David, and Charlotte S. Russell. 1953. δ-Aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. J. Am. Chem. Soc. 75: 4873-4874.

Spencer, Alice F., and John M. Lowenstein. 1962. The supply of precursors for the synthesis of fatty acids. J. Biol. Chem. 237: 3640-3648.

Tyler, Albert. 1949. A simple noninjurious method for inducing repeated spawning of sea urchins and sand dollars. The Collecting Net 19: 19-20.

Vennesland, Birgit, and E. A. Evans, Jr. 1944. The formation of malonic acid from oxaloacetic acid by pig heart preparation. J. Biol. Chem. 156: 783-784.

Vennesland, Birgit, E. A. Evans, Jr., and Arista M. Francis. 1946. The action of met myoglobin, oxygen and manganese on oxaloacetic acid. J. Biol. Chem. 163: 573-574.

Williams, Virginia R., and Russell T. McIntyre. 1955. Preparation and partial purification of the aspartase of Bacterium cadaveris, J. Biol. Chem. 217: 467-477.

VITA

Russell Chancellor Addison, Jr.

Born in Lynchburg, Virginia, February 18, 1942. Graduated from Amherst County High School Amherst, Virginia, June, 1959. Received Bachelor of Science degree from The College of William and Mary, Williamsburg, Virginia, June, 1963. Teaching assistant in the Department of Biology from September, 1963 to February, 1965.