AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented August 7, 1964

Title STUDIES OF SOLUBLE AND PARTICULATE ISOCITRATE-NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE-OXIDOREDUCTASE ACTIVITIES DURING DEVELOPMENT OF THE CHICK EMBRYO.

Abstract approved
(Major professor)

Isocitrate-NADP oxidoreductase activity in mitochondrial and soluble fractions was determined for hearts, livers and brains of chick embryos at various stages of development. One-half to two-thirds of the activity in brain and liver homogenates is located in the soluble fraction. Supernatant activity increases in these organs to a maximum during the latter one-third of development. This increase is correlated with the accumulation of lipids in these tissues and is presumed to furnish reduced NADP for fatty acid synthesis.

Most of the activity in heart homogenates is located in the mitochondrial fraction. Particulate activity increases in all three organs during development. This increase is correlated with a general rise in capacity for oxidative metabolism. Possibly the particulate enzyme also functions in reductive synthesis. Heat

inactivation studies indicate the particulate and soluble isocitrate-NADP oxidoreductase activities have different isozymic make-ups.

A low level of isocitrate-NAD oxidoreductase activity was also demonstrated in some preparations. In no preparation was this activity demonstrated to be greater than ten percent of the NADP dependent activity.

The RNA, DNA and nitrogen contents of hearts, livers and brains of chick embryos at various stages of development were also determined.

STUDIES OF SOLUBLE AND PARTICULATE ISOCITRATE-NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE-OXIDOREDUCTASE ACTIVITIES DURING DEVELOPMENT OF THE CHICK EMBRYO

by

WILBER WINSTON BAKER

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

August 1964

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Date thesis is presented August 7, 1964

Typed by Nancy Kerley

ACKNOWLEDGEMENT

The author wishes to express his gratitude to Dr. R. W. Newburgh for his constructive criticism, his generous assistance and his instructive guidance through the experimental work and writing of this manuscript.

My thanks to Dean Vernon H. Cheldelin and the other staff members for many stimulating discussions and to the Science Research Institute for providing the necessary physical and technical assistance.

My thanks to my wife for her encouragement and to my fellow students for many useful suggestions and discussions.

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LIST OF ABBREVIATIONS USED IN THIS THESIS

NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
NADP	Nicotinamide-adenine dinucleotide phosphate
NAD	Nicotinamide-adenine dinucleotide
ADP	Adenosine diphosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
CoA	Coenzyme A
Tris	2-Amino-2-hydroxymethylpropane-1, 3 diol

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INTRODUCTION

The unique characteristic of the embryonic system is the development of a heterogeneous cell population from a homogeneous one. In order to define the changes which actually take place and in order to ultimately understand the mechanism by which differentiation takes place it is necessary to correlate morphological and functional changes with biochemical changes. The waxing and waning of enzyme activities, the changes in intracellular distribution of enzymes and the changes in isozyme patterns are all biochemical parameters which may be of significance. One group of chemical entities that is related to a particular morphogenetic event is the lipids. From present knowledge it is reasonable to assume that lipid formation is related to myelination, and this has been demonstrated (5). In the synthesis of fatty acids the reductive step requires NADPH and it has been suggested that extramitochondrial isocitrate-NADP oxidoreductase serves to generate this coenzyme (47, 48). It has been reported that isocitrate-2-H^3 incorporates tritrium into fatty acids to a greater extent than does glucose-6-phosphate-1-H^3 (39). Other citric acid cycle intermediates also stimulate fatty acid synthesis and the point of stimulation has been shown to be at the

level of conversion of acetyl-CoA to malonyl-CoA (1, 57, 80). Hülsmann (34, 35) reports that acetyl-CoA stimulates isocitrate oxidation in soluble preparations from rat heart sarcosomes, that acetyl-CoA can replace divalent metal ions in this citrate oxidizing system, that the stimulation is stoichiometric in the absence of divalent metal ions and that the stimulation is greatly reduced in enzyme prepared from biotin deficient rats. Other workers (54, 79) have concluded that stimulation of the carboxylation reaction of fatty acid synthesis by isocitrate and citrate is not by reduction of isocitrate and a subsequent transcarboxylation reaction between oxalosuccinate and acetyl CoA as suggested by Hülsmann. They suggest (55, 56, 77) that the tricarboxylic acid cycle intermediates act directly on the enzyme to alter its activity.

Since isocitrate-NADP oxidoreductase may be involved in furnishing NADPH for the reduction step of fatty acid synthesis and possibly in the furnishing of carbon dioxide for the carboxylation step of fatty acid synthesis it seems reasonable to attempt to correlate the activity of this enzyme with lipid synthesis and myelination. This enzyme is of further interest since it has a role in both anabolic and catabolic reactions of the cell: for example, in lipid synthesis and in the tricarboxylic cycle.

Although the presence of isocitrate-NADP oxidoreductase activity has been reported in the chick embryo a comprehensive

study was lacking. Cazorla and Barron (11, p. 75) demonstrated the presence of isocitrate-NADP oxidoreductase in cell free extracts of whole embryos. Mahler and coworkers (51, p. 776) studied the intracellular distribution of isocitrate oxidizing enzymes in homogenates of four-day-old embryos and livers and hearts of ten-day-old embryos. They reported the presence of isocitrate-NADP oxidoreductases in both mitochondrial and supernatant cell fractions of all three homogenates. They also reported the presence of a low level of isocitrate-NAD oxidoreductase activity in the ten-day-old liver supernatant. Newburgh and coworkers (64) have studied isocitrate-NADP oxidoreductase activity in explants of early chick embryos, and it was suggested that isocitrate-NADP oxidoreductase may function in the embryo to furnish NADPH for use in the reductive steps of synthetic pathways. It was therefore decided to measure the activity of isocitrate-NADP oxidoreductase in the developing chick embryo as a function of such parameters as nitrogen and nucleic acid concentration.

MATERIALS AND METHODS

Fertile Eggs and Embryos

The embryos were obtained from White Leghorn eggs purchased from Hanson Leghorn Farm, Corvallis, Oregon, and Hy-Line 950-A eggs purchased from Jenks' Hatchery, Tangent, Oregon. The eggs were incubated in a Jamesway Model 252B incubator with automatic turner. The eggs were turned every two hours and incubated at a wet bulb temperature of 86° F and a dry bulb temperature of 99° F.

Chemicals

NADP (sodium salt), DL-isocitric acid (trisodium salt) and Naja naja venom were obtained from the Sigma Chemical Company. Desoxyribonucleic acid, which was obtained from Nutritional Biochemicals Corporation, had been prepared from herring sperm by hot alkaline extraction. Diphenylamine was recrystallized from ethanol before use. All other chemicals were of reagent grade and were obtained from commercial sources.

Cell Fractionation

Cell fractionation was carried out by the method of Carey

and Greville (10), except that the homogenization medium consisted of 0.25 M sucrose containing EDTA (1 mM), pH 7.2, rather than 0.31 M sucrose. Organs were dissected from embryos, placed immediately into ice-cold homogenization medium and the pooled organs were later homogenized with a glass Dounce homogenizer. The homogenates were made so that the wet weight of the organs being homogenized was two to six percent of the volume of homogenizing media used. The homogenates were centrifuged at 600 x g for 20 minutes to remove nuclei and cell debris, and then centrifuged at 12,000 x g for ten minutes to obtain particulate (mitochondrial) and supernatant (soluble) fractions. The particulate fraction was resuspended in a suspension medium of 0.25 M sucrose containing EDTA (1 mM) and bovine serum albumin (0.1 percent), pH 7.2 and recentrifuged at 12,000 x g for ten minutes. The washed particulate fraction was then resuspended in fresh suspension medium. Centrifuging was carried out in a Servall type RC-2 automatic superspeed refrigerated centrifuge with type ss-34 rotor.

Determinations of Nitrogen in Homogenates and in Cell Fractions

Nitrogen was determined by an adaptation of the modified Kjeldahl method of Lang (43). Aliquots of samples whose nitrogen content was being determined were evaporated to dryness in an oven at 90° to 100° C. To the dried samples was added 0.2 ml of digestion mixture. The samples were then placed in a sand bath the

temperature of which was slowly raised to between 200° and 250° C. This heating was continued until the samples were colorless, a period of 12 to 24 hours. Occasionally it was necessary to cool the samples, add two drops of 30 percent hydrogen peroxide and slowly reheat the samples in order to obtain complete digestion.

After digestion was complete one of two methods was used for color development depending upon the amount of nitrogen present. The dilution method was used for samples containing 14 to 140 μg of nitrogen. The sample was diluted with 8.0 ml of water and a 3.0 ml aliquot was taken and diluted to 4.0 ml. To this was added 2.0 ml of Nessler reagent. The optical density of the solution was determined with a colorimeter after standing for ten minutes in the dark. The direct method was used for samples containing from 1.0 to 10.0 μg of nitrogen. To the digested sample was added 1.4 ml of water and then 5.0 ml of a solution containing two parts Nessler reagent to three parts ten percent sodium hydroxide. After standing in the dark for 30 minutes the optical density at 420 mμ was determined with a colorimeter.

The digestion mixture was prepared by combining 40 grams of potassium sulfate, 2.0 ml of selenium oxychloride and diluting to 250 ml with distilled water. To this was then added 250 ml concentrated sulfuric acid.

The Nessler reagent used was that of Koch and McMeekin (42)

which was prepared by dissolving 22.5 gm iodine in 20 ml of water containing 30 gm of potassium iodide. After solution was complete 30 gm of mercury was added and the mixture was stirred, while being kept cool by intermittent immersion of the flask in cold running tap water. After the supernatant liquid had lost all of the yellow color due to iodine it was decanted and a portion was tested with starch solution. If a positive starch test for iodine was obtained a few drops of an iodine solution of the same concentration as employed above was added until a faint excess of free iodine could be detected by the starch test. The resulting solution of potassium mercuric iodide was diluted to 200 ml mixed well and added to 975 ml of an accurately prepared ten percent (w/v) sodium hydroxide solution. This mixture was allowed to clear for several days before using.

The nitrogen values for the particulate fraction were obtained as the difference between the nitrogen contents of resuspended particles and the nitrogen of the suspending medium. This was necessary since this medium contained bovine serum albumin. A serine solution was used for a standard in the nitrogen determinations. Samples containing known amounts of serine were digested each time along with the samples being determined.

Extraction of Nucleic Acid from Homogenates

A modified Schneider (70) procedure was used for the

extraction of nucleic acids from samples and for obtaining protein residue which was used in determining protein nitrogen. This method usually consists of cold trichloroacetic acid precipitation of such compounds as lipids, protein and nucleic acids, washing of the precipitate with trichloroacetic acid and lipid solvents and finally hot extraction of the precipitate with perchloric acid to separate the nucleic acids from the protein.

In preliminary experiments 1.0 ml cold (approximately 3° C) ten percent trichloroacetic acid was added to 1.0 ml cold samples. The mixture was centrifuged in a refrigerated centrifuge and the supernatant was removed by aspiration. The precipitate was subsequently washed with 2.0 ml cold five percent trichloroacetic acid, 2.0 ml 95 percent ethanol and 2.0 ml 95 percent ethanol saturated with sodium acetate, the sample being centrifuged after each washing and the supernatant being removed by aspiration. The precipitate was then extracted in a water bath at 70° C for 15 minutes with 2.0 ml of 1:1 chloroform:ethanol. After centrifugation and aspiration of lipid-bearing supernatant the tube was inverted and allowed to drain thoroughly. The precipitate was then extracted once with a 0.50 ml portion and a second time with a 0.25 ml portion of 1.0 N perchloric acid in a water bath at 70° C for 20 minutes. The supernatants from both the first and second extractions were removed by means of a Pasteur pipet and pooled. Table I shows data

Table I. Amounts of nitrogen after various steps of the Schneider extraction procedure.

A) A homogenate of 11-day embryo hearts prepared in isotonic sodium chloride.

B) A homogenate of 11-day embryo hearts prepared as described in the text.

C) A solution of bovine serum albumin containing 0.66 mg of albumin per ml.

Values given are in micrograms of nitrogen per sample.

	A	B	C
Homogenate	60	65	93
First TCA precipitate	44	--	--
Second TCA precipitate	40	53	96
First ethanol precipitate	37	--	--
Second ethanol precipitate	39	--	--
$CHCl_3$-ethanol precipitate	35	41	54
First perchloric acid precipitate	27	--	--
Second perchloric acid precipitate	--	23	30

on amount of nitrogen remaining in the precipitate after various steps of the extraction procedure. Part of this loss is undoubtedly due to removal of acid soluble compounds such as amino acids, removal of phospholipids and finally removal of nucleic acids. The bulk of the nitrogen lost, however, is probably protein nitrogen. This loss would fall into two categories. The first would be acid soluble peptides and lipoproteins and proteins soluble in lipid solvents. The second would be mechanical loss, particularly in the removal of the supernatant by aspiration. Both of these could be reduced by reducing the number of extraction steps.

Because of the above data and because in their recent review on the determination of nucleic acids Hutchison and Munro (36, p. 772) point out that the intermediate extractions by lipid solvents are not necessary when methods of nucleic acid analysis are not dependent on phosphorus determination, it was decided to omit these steps in the procedure and the following procedure was used. To a sample containing from 10 to 80 μg of RNA and from 5 to 50 μg of DNA was added 1.0 ml of ice-cold ten percent (w/v) trichloroacetic acid. After centrifuging the supernatant was removed and the precipitate was washed with 2.0 ml of five percent (w/v) trichloroacetic acid. The precipitate was then extracted twice at 70° C for 20 minutes with 0.5 ml portions of N-perchloric acid. Table II shows some representative data on the amount of nitrogen found in various

Table II. Amounts of nitrogen after various steps of the modified extraction procedure.

A) A homogenate of 14-day embryo hearts prepared as described in the text.

B) A solution of egg albumin containing 0.60 mg of albumin per ml.

Values are given in micrograms of nitrogen per sample.

	A	B
Homogenate	200	87
First TCA precipitate	181	84
First TCA supernatant	22	--
First perchloric acid precipitate	148	57
First perchloric acid supernatant	14	--
Second perchloric acid precipitate	140	57
Second perchloric acid supernatant	14	--

fractions when this procedure is used.

Determination of RNA

The orcinol method (58) was used to determine RNA in aliquots of the perchloric acid extract from above. In this determination 1.5 ml of orcinol reagent was added to sample which had been diluted to 1.5 ml with 1 N perchloric acid. The mixture was then heated in a water bath at 100° C for ten minutes. After cooling the optical density was determined at 660 mμ with a Beckman model B spectrophotometer. The orcinol reagent was made up fresh just before using by dissolving 0.048 gm of ferric sulfate in 100 ml of concentrated hydrochloric acid and then adding 1.00 gm of orcinol. As ribose released by the perchloric acid hydrolysis of the extraction procedure is what was actually measured by this method, ribose dissolved in 1 N perchloric acid was used as a standard and 1.00 mg ribose was considered to be equivalent to 3.76 mg of RNA. The range of the method is from 10 to 80 μg of RNA.

Determination of DNA

The diphenylamine method (9) was used to determine DNA in aliquots of the perchloric acid extract from above. In this method 1.0 ml of diphenylamine reagent is added to the sample which is contained in 0.5 ml of 1 N perchloric acid. This mixture was allowed

to stand at 37° C for 16 to 20 hours in covered tubes. The optical density at 600 mμ was then determined with a Beckman Model B spectrophotometer. Diphenylamine reagent was made up fresh just before using by dissolving 0.300 gm of diphenylamine in 20 ml of stock acid mixture and then adding 0.1 ml of aqueous acetaldehyde solution. The stock acid mixture was made by mixing 400 ml of glacial acetic acid and 11 ml of concentrated sulfuric acid. The aqueous acetaldehyde solution was made by dissolving 10 ml of acetaldehyde in 500 ml of water. It was kept stored in a refrigerator. DNA which had been hydrolysed for 20 minutes at 70° C in 1 N perchloric acid was used as a standard. The range of the method is from 5 to 50 μg of DNA.

Determination of Protein

The Folin method (25, p. 627-650; 50) was used for the determination of protein in the attempts at enzyme solubilization and purification. Although the method is not ideal it was chosen because it is more sensitive than the biuret method, more accurate than spectrophotometric readings at 260 and 280 mμ and yields data more rapidly than the Kjeldahl nitrogen method.

To samples containing from 5 to 25 μg of protein per ml was added 5.0 ml of an alkaline copper sulfate solution. After standing at room temperature for ten minutes, 0.5 ml of Folin reagent was

added and the samples were mixed rapidly and immediately. After 30 minutes or more the optical density at 750 mμ was determined with a colorimeter. Although this method is better suited for the determination of less than 25 μg of protein, samples having from 25 to 100 μg protein can be estimated by this method by determining optical density at 500 mμ.

The alkaline copper sulfate solution was prepared daily by mixing 1.0 ml of a stock solution of 0.5 percent $CuSO_4 \cdot 5\ H_2O$ in 1.0 percent sodium potassium tartrate with 50 ml of a stock solution of 2.0 percent sodium carbonate in 0.1 N sodium hydroxide.

The Folin reagent was prepared as follows. To 100 gm of sodium tungstate ($Na_2WO_4 \cdot 2\ H_2O$) and 25 gm of sodium molybdate ($Na_2MoO_4 \cdot 2\ H_2O$) in 700 ml of water was added 50 ml of 85 percent phosphoric acid and 100 ml of concentrated hydrochloric acid. The resulting mixture was boiled gently under reflux for ten hours. At the end of the boiling period 150 gms of lithium sulfate, 50 ml of water and a few drops of liquid bromine were added and the mixture was boiled without condenser for about 15 minutes to remove the excess bromine. The resulting mixture was then cooled, diluted to 1000 ml with water and filtered.

Bovine serum albumin was used as a standard.

Assay of Isocitrate-NADP Oxidoreductase Activity

The reaction mixture consisted of enzyme, $MnCl_2$ (0.67 mM), DL-isocitrate (1.33 mM), NADP (0.050 mM) and tris, pH 7.8 (33 mM). The rate of reduction of NADP was measured at 25° C with a Beckman Model DU spectrophotometer with Gilford recording attachment by following the increase of optical density at 340 mμ with time. One enzyme unit brings about the reduction of 1.0 μmole of NADP per minute.

A rather broad pH optimum centered at pH 7.8 was found for both the particulate and the supernatant preparations. Most of the supernatant preparations and all of the particulate preparations were assayed after storage in a deep freeze. The fresh particulate fraction showed no activity when assayed in media containing sucrose (0.25 M). After freezing and thawing, particulate preparations showed maximum activity when assayed in hypo-osmotic media. Freezing and thawing had no effect on the activity of the supernatant fraction. The supernatant preparations retained full activity when stored in a deep freeze for over two weeks. The particulate preparations retained full activity for somewhat shorter periods of time.

Assay of Isocitrate-NAD Oxidoreductase Activity

The reaction mixture was essentially that of Chen and Plaut

(12). It consisted of enzyme, $MnCl_2$ (0.67 mM), DL-isocitrate (1.33 mM), NAD (0.53 mM), ADP (0.67 mM), and tris, pH 7.1 (33 mM). The rate of reduction of NAD was measured at 25° C with a Beckman Model DU spectrophotometer with Gilford recording attachment by following the increase of optical density at 340 mμ with time. One enzyme unit brings about the reduction of 1.0 μmoles of NAD per minute.

Solubilization of Particulate Enzyme

Sonication, treatment with digitonin suspensions or aqueous solutions of dioxane, acetone or ethanol did not satisfactorily solubilize the mitochondrial isocitrate-NADP oxidoreductase activity. It was possible to solubilize the activity by treatment of mitochondrial preparations with snake (Naja naja) venom. The method employed was to prepare a mitochondrial pellet as described above. This was then washed with mitochondrial suspension media and resedimented at 12,000 x g. It was then resuspended in cold glass distilled water and frozen. The resulting suspension had a protein concentration of from two to six mg per ml. To the frozen and then thawed mitochondrial suspension was added an equal volume of a cold solution containing 1.0 mg of snake venom per ml of glass distilled water. The resulting mixture which had a pH of from 6.0 to 6.4 was heated in a water bath at 37° C for from five to ten minutes.

During this time the actual temperature of the mixture the volume of which was from 100 to 200 ml increased to 20° to 25° C. The mixture was then cooled in an ice bucket and centrifuged in a refrigerated centrifuge at 30, 000 x g for 30 minutes. The supernatant contained most of the original particulate enzyme activity. Data on representative preparations from 18-day embryo liver are given in Table III. It was observed that preparations with more enzyme activity but lower specific activity could be obtained by treatment of the mitochondria at lower temperatures and at pH 7. 4 with one-tenth as much snake venom.

Table III. Solubilization of particulate enzyme.

See experimental details in text. Enzyme activity is expressed as change in O. D. units per minute.

	Experiment A		Experiment B	
	Suspension Before Venom Treatment	Supernatant After Venom Treatment	Suspension Before Venom Treatment	Supernatant After Venom Treatment
Time at 37° C	---	10 minutes	---	5 minutes
ΔO. D. min^{-1} ml^{-1} prep.	1. 84	. 624	. 980	. 360
Volume	100 ml	200 ml	100 ml	200 ml
Mg protein ml^{-1} prep.	5. 40	. 78	2. 15	. 58
ΔO. D. min^{-1} mg^{-1} protein	. 34	. 80	. 46	. 62

RESULTS

Changes in the Content of Nitrogen and Nucleic Acid in Organs of the Developing Embryo

The determinations of total nitrogen, RNA and DNA in hearts, livers and brains of chick embryos and young chickens are summarized in Table IV. The ratios of total nitrogen, particulate nitrogen and supernatant nitrogen to DNA for these same organs in embryos and young chickens are given in Table V. The ratios of RNA to DNA and RNA to nitrogen are given in Table VI.

The ratio of total nitrogen to DNA in the brain increases markedly during development. In this organ the ratio of supernatant nitrogen to DNA also increases, and at a rate approximately proportional to that observed for the ratio of total nitrogen to DNA. The ratio of particulate nitrogen to DNA, however, increases at a somewhat faster rate than that of total nitrogen to DNA between the eleventh day of incubation and the first day after hatching.

The ratio of total nitrogen to DNA in the heart remains constant except for a temporary increase at about the eleventh day of incubation. The ratio of particulate nitrogen to DNA remains constant or increases slightly during the period of the experiments. The ratio of soluble nitrogen to DNA decreases after the eleventh day.

Table IV. Weights of nitrogen, DNA and RNA per organ of chick embryos and young chickens.

The results are expressed as mg per organ. Values are the means ± S.E.M. where two to five determinations were made.

	Day of Incubation							
	4	7	11	14	15	18	1-day Chick	36-day Chick
Brain								
Nitrogen	.0871 ±.0051	.739 ±.065	2.49 ±.03	4.63 ±.13	5.74 ±.11	9.04 ±.30	14.4 ±.7	30.4
DNA	.0244 ±.0010	.207 ±.017	.322 ±.011	.612±.027	.660±.037	.897±.052	1.55 ±.22	2.97
RNA	.0385 ±.0011	.253 ±.020	.483 ±.066	.995±.061	1.21 ±.02	1.78 ±.03	2.66 ±.42	4.28
Heart								
Nitrogen	.0177 ±.0010	.0962±.0059	.589 ±.031	1.28 ±.04	1.53 ±.05	2.70 ±.08	5.46 ±.24	60.8
DNA	.00285±.0010	.0162±.0016	.0699±.0043	.229±.004	.302±.011	.471±.023	.963±.088	6.75
RNA	.00714±.00055	.0308±.0008	.130 ±.016	.291±.008	.354±.028	.430±.069	.788±.062	7.02
Liver								
Nitrogen	---	.178 ±.020	1.56 ±.11	4.02 ±.15	5.52 ±.18	9.57 ±.40	18.9 ±2.1	375.
DNA	---	.0295±.0029	.193 ±.006	.482±.013	.726±.026	1.12 ±.05	3.12 ± .04	43.3
RNA	---	.0660±.0053	.395 ±.015	1.05 ±.06	1.48 ±.15	2.05 ±.23	4.68 ± .04	83.6

Table V. Ratios of nitrogen to DNA, particulate nitrogen to DNA, and supernatant nitrogen to DNA, in organs of chick embryos and young chickens.

The results are expressed as mg of nitrogen, particulate nitrogen, and supernatant nitrogen per mg of DNA. Values in some instances differ slightly from those calculated from the values in Table IV. This is because results for some of the parameters were not available for every experiment and some ratios therefore are calculated from fewer experiments than the values in Table IV. Values are the means ± S. E. M. where two to five determinations were made.

	Day of Incubation							
	4	7	11	14	15	18	1-day Chick	36-day Chick
Brain								
Nitrogen:DNA	3.5±.10	3.6 ±.06	7.8 ±.22	7.6±.19	8.8 ±.33	10.4±.38	9.3 ±.75	10.2
Particulate N:DNA	---	.77±.07	.60	1.0±.13	.91±.01	2.3±.35	2.5 ±.66	--
Supernatant N:DNA	---	3.1	7.9	6.0	6.3 ±.46	6.9±.80	7.7	--
Heart								
Nitrogen:DNA	6.2±.20	5.7 ±.36	8.4 ±.16	5.6±.20	5.0 ±.14	5.9±.27	5.7 ±.30	9.0
Particulate N:DNA	---	.59±.06	.95	1.0±.28	.70±.09	1.2±.26	.82±.07	--
Supernatant N:DNA	---	4.2	6.2	2.8	3.0 ±.30	2.7±.16	2.1	--
Liver								
Nitrogen:DNA	---	5.9 ±.23	8.8 ±.56	8.4±.22	7.6 ±.13	8.7±.39	6.1 ±.75	8.7
Particulate N:DNA	---	1.0 ±.09	1.0	1.4±.06	1.2 ±.06	1.8±.31	.85±.15	--
Supernatant N:DNA	---	4.6	6.0	4.8	5.9 ±.50	5.8±.30	5.2	--

Table VI. Ratios of RNA to total nitrogen and DNA in organs of chick embryos and young chickens.

The results are expressed as mg of RNA per mg of nitrogen and DNA. Values in some instances differ slightly from those calculated from the values in Table IV. This is because results for some of the parameters were not available for every experiment and some ratios therefore are calculated from fewer experiments than the values in Table IV. Values are the means ± S. E. M. where two to five determinations were made.

	Day of Incubation							
	4	7	11	14	15	18	1-day Chick	36-day Chick
Brain								
RNA:Nitrogen	.46±.04	.35±.15	.20±.04	.21±.02	.22±.01	.20±.05	.18±.02	.14
RNA:DNA	1.6 ±.04	1.2 ±.04	1.5 ±.23	1.7 ±.06	1.9 ±.09	1.7 ±.13	1.7 ±.07	1.4
Heart								
RNA:Nitrogen	.56±.13	.35±.04	.17±.02	.23±.02	.23±.02	.18±.03	.14±.01	.12
RNA:DNA	2.6 ±.12	2.1 ±.19	1.7 ±.10	1.3 ±.04	1.2 ±.06	.80±.22	.83±.16	1.0
Liver								
RNA:Nitrogen	---	.40±.04	.19±.03	.26±.01	.29±.04	.22±.05	.25±.03	.22
RNA:DNA	---	2.3 ±.08	1.9 ±.04	2.2 ±.12	2.0 ±.17	1.6 ±.15	1.5 ±.07	1.9

This decrease is largely due to the increase in the fraction sedimenting at 600 x g. This additional material is probably heart muscle which would be mostly protein. In the liver a similar pattern is observed; the ratio of total nitrogen to DNA remains relatively constant but the ratio of particulate nitrogen to DNA increases slightly. In the liver the ratio of supernatant nitrogen to DNA remains relatively constant.

In the brain the ratio of RNA to DNA remains constant, but in the heart there is a decrease between the fourth day of incubation and the first day after hatching. A similar but less dramatic decrease is observed in the liver. The RNA to nitrogen ratio decreases during development in all three tissues.

The nitrogen content of the protein residue remaining after nucleic acid extraction was also determined. This value is referred to as "protein nitrogen." The ratios of protein nitrogen to total nitrogen and protein nitrogen to DNA are given in Table VII. The percent of the total nitrogen recovered as protein nitrogen after the nucleic acid extraction procedure ranges from 45 to 70 percent. The percent recovered as protein nitrogen tended to be somewhat higher in all three organs of older embryos and young chickens than in organs for younger embryos. The ratio of protein nitrogen to DNA follows about the same pattern during the course of development as the ratio of total nitrogen to DNA. In brain the ratio of protein nitrogen

Table VII. Ratios of protein nitrogen to total nitrogen and DNA in organs of chick embryos and young chickens.

The results are expressed as mg of protein nitrogen per mg nitrogen and DNA. Values in some instances differ slightly from those calculated from the value in Table IV. This is because results for some of the parameters were not available for every experiment and some ratios therefore are calculated from fewer experiments than the values in Table IV. Values are the means ± S. E. M. where two to five determinations were made.

	Day of Incubation							
	4	7	11	14	15	18	1-day Chick	36-day Chick
Brain								
Protein N:Total N	.61±.03	.50±.05	.52±.06	.64±.02	.58±.07	.69±.02	.64±.01	.58
Protein N:DNA	2.2 ±.18	2.2 ±.55	4.1 ±.56	4.8 ±.12	5.1 ±.70	7.1 ±.67	6.0 ±.50	6.0
Heart								
Protein N:Total N	.48±.02	.52±.01	.44±.03	.56±.03	.57±.03	.65±.03	.60±.04	.58
Protein N:DNA	2.9 ±.14	3.2 ±.26	3.8 ±.23	3.2 ±.09	2.9 ±.25	3.8 ±.42	3.5 ±.36	5.3
Liver								
Protein N:Total N	---	.57±.06	.46±.09	.65±.05	.66±.02	.70±.03	.64±.06	.56
Protein N:DNA	---	3.4 ±.48	4.0 ±.35	5.4 ±.18	5.0 ±.09	6.2 ±.69	3.9 ±.16	4.9

to DNA increases markedly during development. This ratio increases slightly in heart and liver tissue during development.

Changes in Isocitrate-NADP Oxidoreductase Activity During Development

Figures I, II, and III show enzyme activities based upon the DNA content of an equivalent quantity of original homogenate in brain, heart and livers respectively of chick embryos and young chickens. A general increase in activity was observed in the particulate fraction of the heart and the liver except for a peak on the fourteenth day of incubation. Low levels of supernatant activity in the heart and high levels of supernatant activity in the brain and the liver were observed. The enzyme activity in the supernatant fractions of both the brain and the liver increases rapidly during development and reaches a maximum during the last third of the embryonic period.

A comparison of the results in Table VIII with Figures I, II and III shows that similar changes are observed when enzyme activity is based on nitrogen from an equivalent quantity of original homogenate or on fresh weight of tissue. Differences in the magnitude of these changes are due to such factors as an increase in the ratio of total nitrogen to DNA in brain and a decrease in the water content of organs during development. The specific activities of the supernatant fractions also show the same patterns as activities based on

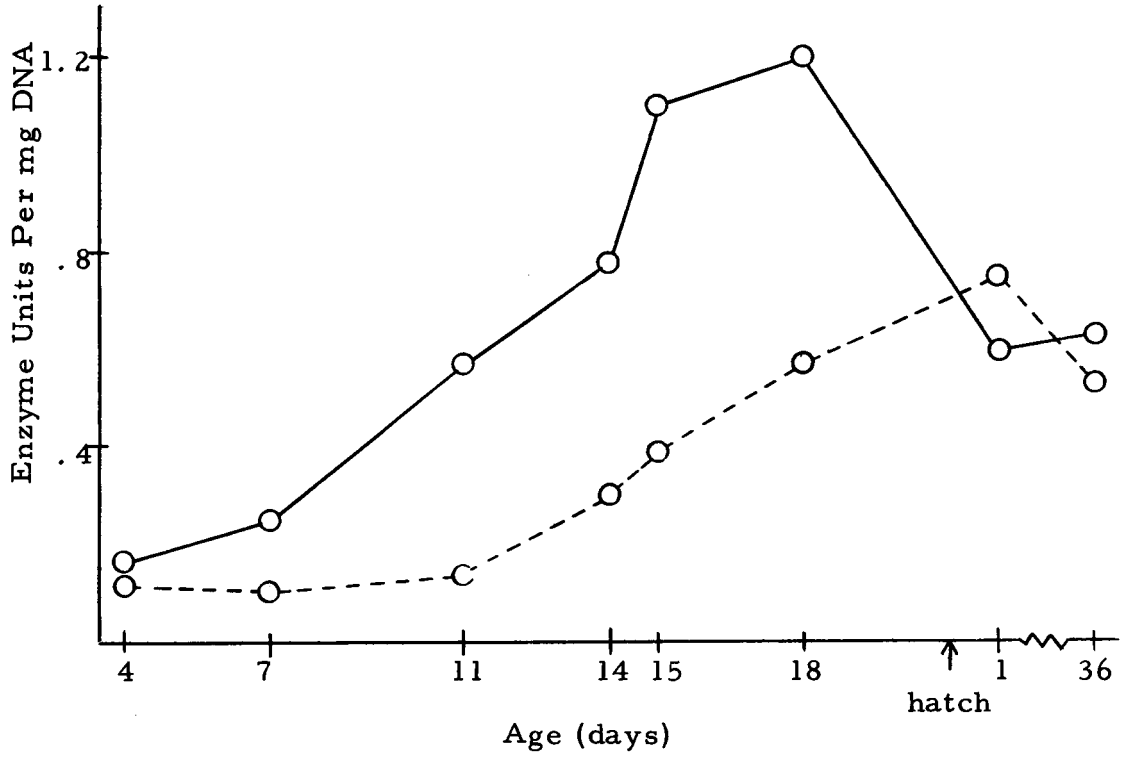

Figure I. Intracellular distribution of isocitrate-NADP oxidoreductase in developing brain.

Enzyme activity in mitochondrial (broken line) and soluble (solid line) cell fractions per mg DNA in an equivalent quantity of original homogenate is shown as a function of age. One enzyme unit brings about the reduction of 1.0 μmole of NADP per minute.

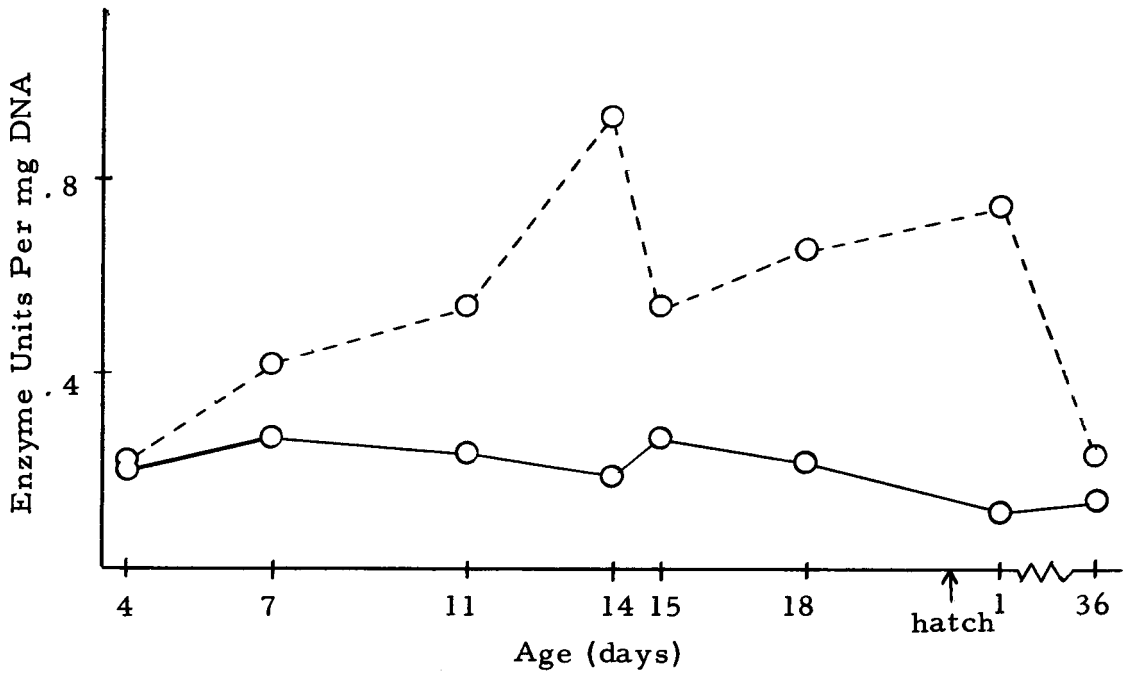

Figure II. Intracellular distribution of isocitrate-NADP oxidoreductase in developing heart.

Enzyme activity in mitochondrial (broken line) and soluble (solid line) cell fractions per mg DNA in an equivalent quantity of original homogenate is shown as a function of age. One enzyme unit brings about the reduction of 1.0 μmole of NADP per minute.

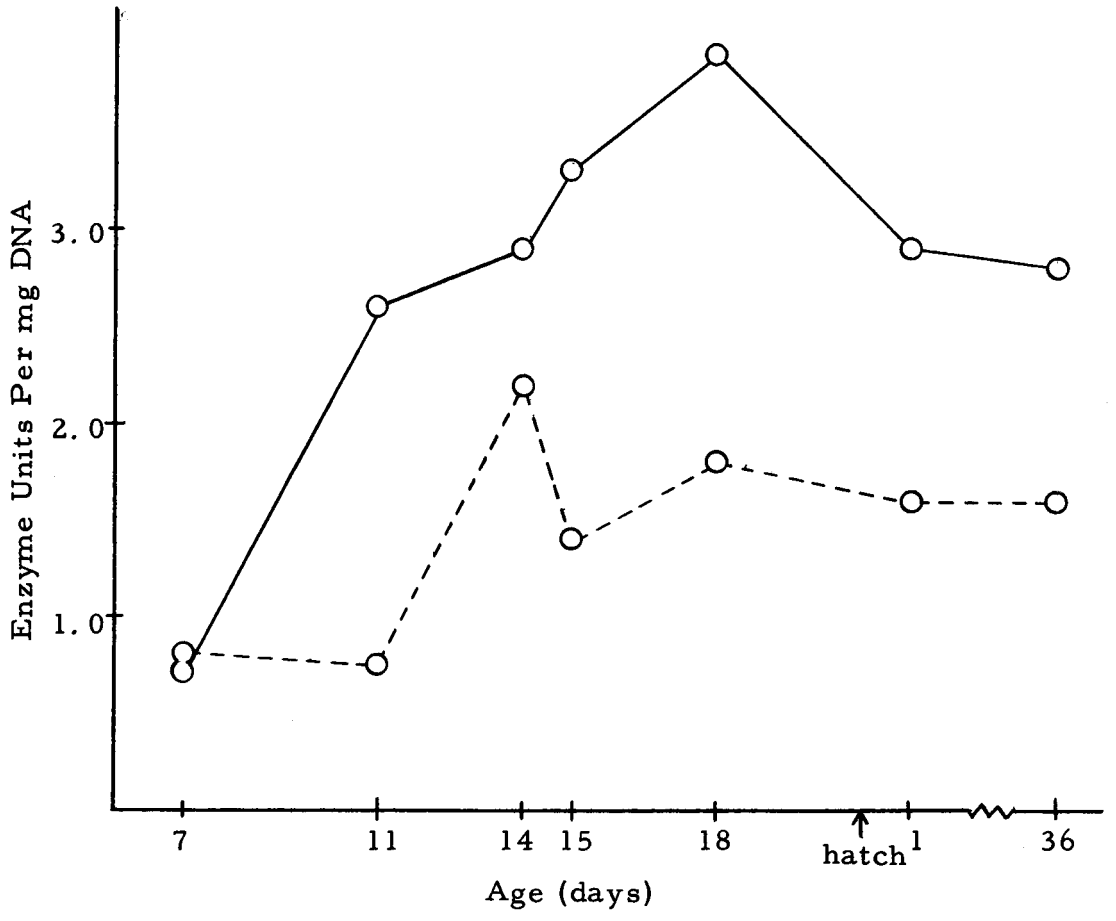

Figure III. Intracellular distribution of isocitrate-NADP oxidoreductase in developing liver.

Enzyme activity in mitochondrial (broken line) and soluble (solid line) cell fractions per mg DNA in an equivalent quantity of original homogenate is shown as a function of age. One enzyme unit brings about the reduction of 1.0 μmole of NADP per minute.

Table VIII. Isocitrate-NADP oxidoreductase activity in mitochondrial and soluble cell fractions from organs of chick embryos and young chickens.

One enzyme unit brings about the reduction of 1.0 μmole of NADP per minute. Values are the means±S. E. M. where two to five determinations were made.

	Day of Incubation							
	4	7	11	14	15	18	1-day Chick	36-day Chick
Brain								
Supernatant								
U/mg supernatant N	---	.084	.10	.12	.18 ±.07	.14 ±.03	.084	---
U/mg homogenate N	.042±.004	.069±.007	.069±.007	.099±.004	.13 ±.01	.12 ±.03	.063±.002	.061
U/gm wet weight	.21	.37 ±.01	.65 ±.17	.81 ±.01	1.2 ±.1	1.3 ±.1	.93 ±.17	.89
Particulate								
U/mg particulate N	---	.13 ±.02	.28	.30 ±.04	.45 ±.09	.22 ±.02	.30 ±.02	---
U/mg homogenate N	.030±.015	.028±.005	.018±.003	.039±.003	.045±.008	.057±.004	.077±.017	.052
U/gm wet weight	.21	.15 ±.03	.19 ±.02	.33 ±.03	.40 ±.06	.65 ±.06	1.2 ±.4	.75
Heart								
Supernatant								
U/mg supernatant N	---	.066	.051	.069	.11 ±.01	.087±.018	.069	---
U/mg homogenate N	.033	.044±.002	.029±.002	.033±.001	.055±.008	.041±.002	.022±.005	.016
U/gm wet weight	---	.27 ±.01	.30 ±.06	.31 ±.04	.72 ±.04	.70 ±.06	.38 ±.13	.26
Particulate								
U/mg particulate N	---	.71 ±.05	.78	.87 ±.27	.78 ±.04	.80 ±.02	.90 ±.06	---
U/mg homogenate N	.036	.065±.008	.064±.009	.16 ±.03	.11 ±.01	.12 ±.01	.13 ±.02	.026
U/gm wet weight	---	.41 ±.07	.72 ±.13	1.7 ±.4	1.5 ±.3	2.0 ±.2	2.2 ±.1	.45
Liver								
Supernatant								
U/mg supernatant N	---	.17	.36	.51	.59 ±.08	.71 ±.11	.69	---
U/mg homogenate N	---	.11 ±.01	.31 ±.02	.35 ±.03	.44 ±.03	.48 ±.02	.48 ±.06	.32
U/gm wet weight	---	1.3 ±.2	4.7 ±1.3	4.9 ±.2	6.8 ±.6	9.5 ±.7	10.2 ±2.8	9.26
Particulate								
U/mg particulate N	---	.81 ±.18	1.3	1.6 ±.4	1.2 ±.1	1.2 ±.2	1.9 ±.3	---
U/mg homogenate N	---	.12 ±.02	.12 ±.05	.27 ±.06	.19 ±.02	.23 ±.01	.27 ±.03	.19
U/gm wet weight	---	1.3 ±.2	1.7 ±.3	4.4 ±1.2	2.9 ±.3	4.2 ±.3	5.5 +.1	5.4

homogenate DNA, homogenate nitrogen or wet weight. The specific activities of the particulate fraction of the brain and the heart increase during development, but this increase is much smaller than that of activities based on other parameters. The pattern and magnitude of changes of the specific activity of the particulate fraction of the liver are very similar to those observed for activity based on homogenate DNA or homogenate nitrogen.

Isocitrate-NAD Oxidoreductase Activity During Development

Table IX gives data on enzyme activity in the 600 x g supernatant fraction per mg of homogenate nitrogen of chick embryo hearts, livers and brains. The activity of isocitrate-NAD oxidoreductase was five to ten fold greater in heart from 11 and 15-day embryos than in brain or liver from embryos of the same age. The activity in all preparations was much lower than that of the NADP dependent reaction in the same preparations.

In Table X are given data on the intracellular distribution of NAD dependent activity in 18-day embryos and one-day chicks. The percent of the NADP activity in the same preparations which this NAD activity represents is also given. Thus in both cell fractions from liver the activity in the presence of NAD is less than one percent of the activity in the presence of NADP. Only in two of the fractions tested, brain mitochondria and heart supernatant over

Table IX. Isocitrate-NAD oxidoreductase activity in the 600 x g supernatant of organs from chick embryos and young chickens.

Enzyme activity is expressed in enzyme units per mg of nitrogen in an equivalent quantity of homogenate. One enzyme unit brings about the reduction of 1.0 μmole of NAD per minute.

	Day of Incubation			One-day Chick
	7	11	15	
Brain	.0011	.0016	.0030	.0082
Heart	---	.013	.015	---
Liver	---	.0014	.0031	---

Table X. Isocitrate-NAD oxidoreductase activity in mitochondrial and soluble cell fractions from organs of chick embryos and young chickens.

Enzyme activity is expressed in enzyme units per mg of nitrogen in an equivalent quantity of homogenate. One enzyme unit brings about the reduction of 1.0 μmole of NAD per minute. NAD-stimulated activity is also given as a percent of NADP-stimulated activity in these same preparations.

	18-day Embryo		1-day Chick	
	NAD-stimulated Activity	Percent of NADP Activity	NAD-stimulated Activity	Percent of NADP Activity
Brain supernatant	.0054	3.5	<.0010	< 1.5
Brain particulate	.0072	9.3	.0049	5.2
Heart supernatant	.0040	9.6	.0030	11.1
Heart particulate	.0025	4.0	.0046	4.1
Liver supernatant	.0025	.52	.0037	.69
Liver particulate	---	---	<.0010	< .50

mitochondria, do activities in the presence of NAD reach values as high as ten percent of the activity in the presence of NADP.

Effects of Various Metal Ions on Isocitrate-NADP Oxidoreductase Activity

It was found that magnesium ions can substitute for manganese ions in the reaction, giving activities of from 55 to 90 percent of the activity of that in the presence of manganese ions. Table XI gives data on activity in the presence of magnesium ions expressed as percent activity in the presence of 0.67 mM $MgCl_2$ of that of the same preparation in the presence of 0.67 mM $MnCl_2$. Activity in the mitochondrial preparations was generally about 60 percent as high in the presence of magnesium ions as manganese ions while activity in the soluble fractions was from 70 to 80 percent as high in the presence of magnesium ions as in the presence of manganese ions.

Both calcium and zinc ions were found to inhibit isocitrate-NADP oxidoreductase activity. Inhibition by zinc ions was greater than that by the same concentration of calcium ions. Inhibition of mitochondrial activity was generally greater than inhibition of the enzyme activity in the soluble fraction. Table XII gives activity of isocitrate-NADP oxidoreductase in the presence of 0.67 mM $ZnCl_2$ or 0.67 mM $CaCl_2$ in addition to 0.67 mM $MnCl_2$ as a percent of activity of the same preparation in the presence of the manganese

Table XI. Isocitrate-NADP oxidoreductase activity in mitochondrial and soluble cell fractions in the presence of magnesium ions expressed as percent of the activity in the presence of manganese ions.

Values given are activities in the presence of 0.67 mM $MgCl_2$ expressed as percent of the activity in the presence of 0.67 mM $MnCl_2$. Where determinations on more than one enzyme preparation was made, values of each preparation are given in parenthesis.

	7-day Embryo	18-day Embryo
Brain supernatant	73 (72, 75)	76
Brain particulate	57 (54, 61)	71 (71, 72)
Heart supernatant	79 (72, 85)	75
Heart particulate	62 (65, 59)	63 (66, 60)
Liver supernatant	79 (72, 87)	83
Liver particulate	63 (64, 62)	61

Table XII. Inhibition of isocitrate-NADP oxidoreductase activity in mitochondrial and soluble cell fractions by zinc and calcium ions.

Values given are activity in the presence of 0.67 mM $ZnCl_2$ or 0.67 mM $CaCl_2$ in addition to 0.67 mM $MnCl_2$ as a percent of activity of the same preparation in presence of 0.67 mM $MnCl_2$ alone.

	7-day Embryo		18-day Embryo	
	$ZnCl_2$ Added	$CaCl_2$ Added	$ZnCl_2$ Added	$CaCl_2$ Added
Brain supernatant	20	--	19	--
Brain particulate	31	91	13	96
Heart supernatant	75	--	26	--
Heart particulate	--	--	8.7	91
Liver supernatant	17	82	15	83
Liver particulate	--	--	7.1	--

chloride alone.

Heat Inactivation of Isocitrate-NADP Oxidoreductase from Different Cell Fractions

Heat inactivation of three different enzyme preparations from 18-day chick embryo livers was compared. The isolation of the mitochondrial fraction and the preparation of the solubilized mitochondrial enzyme have been previously described. The third preparation was the soluble fraction obtained as the 12, 000 x g supernatant as described previously which was centrifuged again at 30, 000 x g for 45 minutes to give a supernatant of the same absolute activity and slightly higher specific activity.

After these three preparations had been diluted so that each had approximately the same enzyme activity per unit volume, a volume of cold 0. 05 M cacodylate buffer, pH 6. 4 equal to the volume of the enzyme preparation was added to each. The resulting three mixtures are referred to below as "100 percent mitochondria," "100 percent solubilized mitochondria" and "100 percent soluble." Appropriate mixtures of aliquots of the above were made to give 50:50 mixtures of each of the above pairs which are referred to below as "50 percent mitochondria:50 percent solubilized mitochondria," "50 percent mitochondria:50 percent soluble" and "50 percent solubilized mitochondria:50 percent soluble."

From these six enzyme solutions 0. 5 ml aliquots were taken and these were heated in a water bath at 46° C for intervals of from 0 to 80 minutes. Immediately after the samples were removed from the water bath they were cooled in an ice bucket. They were assayed for isocitrate-NADP oxidoreductase activity as soon as possible after cooling. The data on enzyme activity for the unmixed samples are recorded in Table XIII and the data for the mixed samples are recorded in Table XIV. Along with the experimentally determined activity for the mixed samples is given the value expected by calculating from the data on the unmixed enzymes. Heat denaturation in the three different cell fractions took place at different rates. The values determined in enzyme mixtures agree well with the expected values indicating that the differences in rates of heat denaturation in the different cell fractions is due to properties of the enzymes rather than to differences in the media.

In Figures IV, V and VI log percent of original activity is plotted against duration of heat treatment. It may be seen from this that the mitochondrial fraction consists of two components, a rapidly inactivated component and a heat stable component. By extrapolating to zero time it may be determined that the heat stable component makes up about one-third of the enzyme activity present in the mitochondrial fraction. On the other hand the heat stable component apparently makes up 90 percent of the solubilized mitochondrial

Table XIII. Heat inactivation of isocitrate-NADP oxidoreductase activity from different cell fractions.

Experimental details are described in the text. Enzyme activity is expressed as change in optical density per minute per ml of preparation.

Time at 46° C	100 Percent Mitochondria		100 Percent Solubilized Mitochondria		100 Percent Soluble	
	Enzyme Activity	Percent Original Activity	Enzyme Activity	Percent Original Activity	Enzyme Activity	Percent Original Activity
0 minutes	.230	100	.260	100	.304	100
5	.106	46	--	--	.256	84
20	.070	30	.218	84	.136	45
40	.066	29	.206	79	.068	22
60	.062	27	--	--	--	--
80	.060	26	.184	71	.032	11

Table XIV. Heat inactivation of isocitrate-NADP oxidoreductase activity from mixed cell fractions.

Experimental details are described in the text. Enzyme activity is expressed as change in optical density per minute per ml of preparation. Expected activity is that activity predicted by calculating from data on unmixed enzymes in Table XIII.

Time at 46° C (minutes)	Enzyme Activity		Percent Original Activity	
	Found	Expected	Found	Expected
50 percent mitochondria: 50 percent solubilized mitochondria				
0	.244	.245	100	100
5	.180	--	74	--
20	.134	.144	55	59
40	.138	.136	57	56
60	.147	--	60	--
80	.126	.122	52	50
50 percent mitochondria: 50 percent soluble				
0	.284	.267	100	100
5	.188	.181	66	68
20	.128	.103	45	39
40	.110	.067	39	25
80	.035	.046	12	17
50 percent solubilized mitochondria: 50 percent soluble				
0	.270	.282	100	100
20	.234	.237	87	84
40	.183	.137	68	49
80	.114	.108	42	38

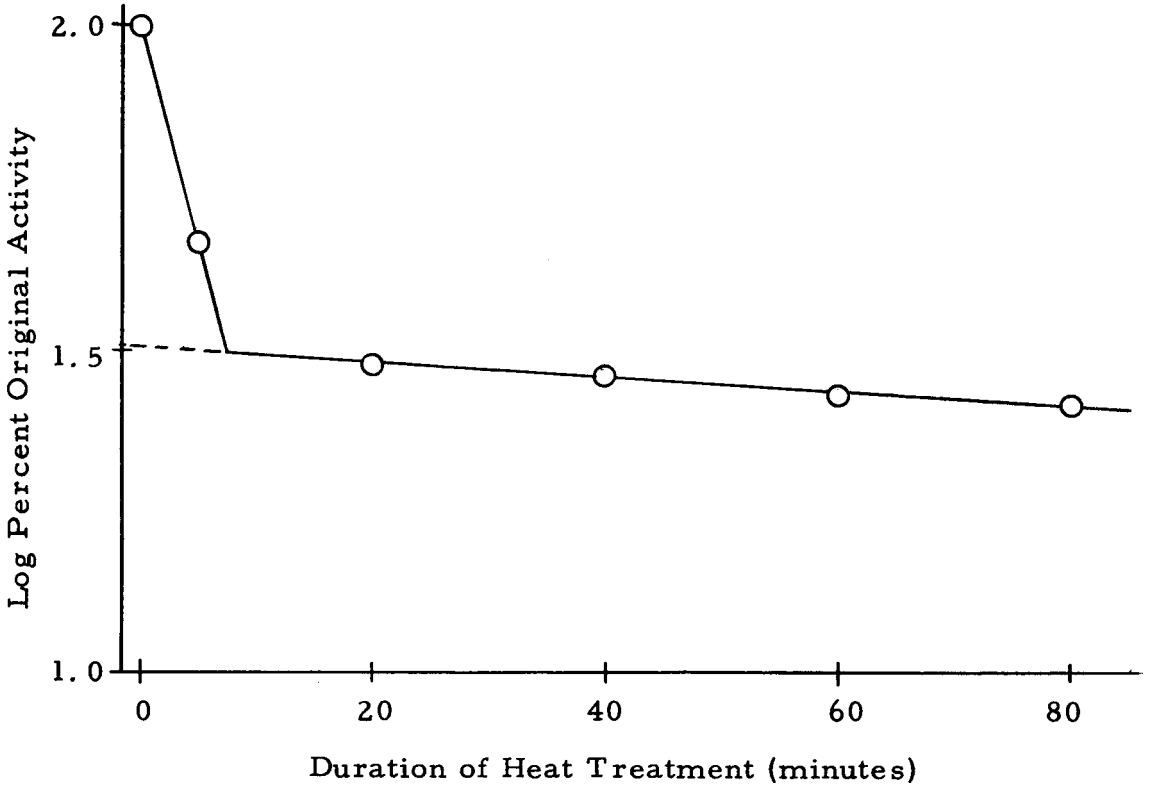

Figure IV. Heat inactivation of mitochondrial enzyme.

The log percent of original activity is shown as a function of duration of treatment of enzyme at 46° C.

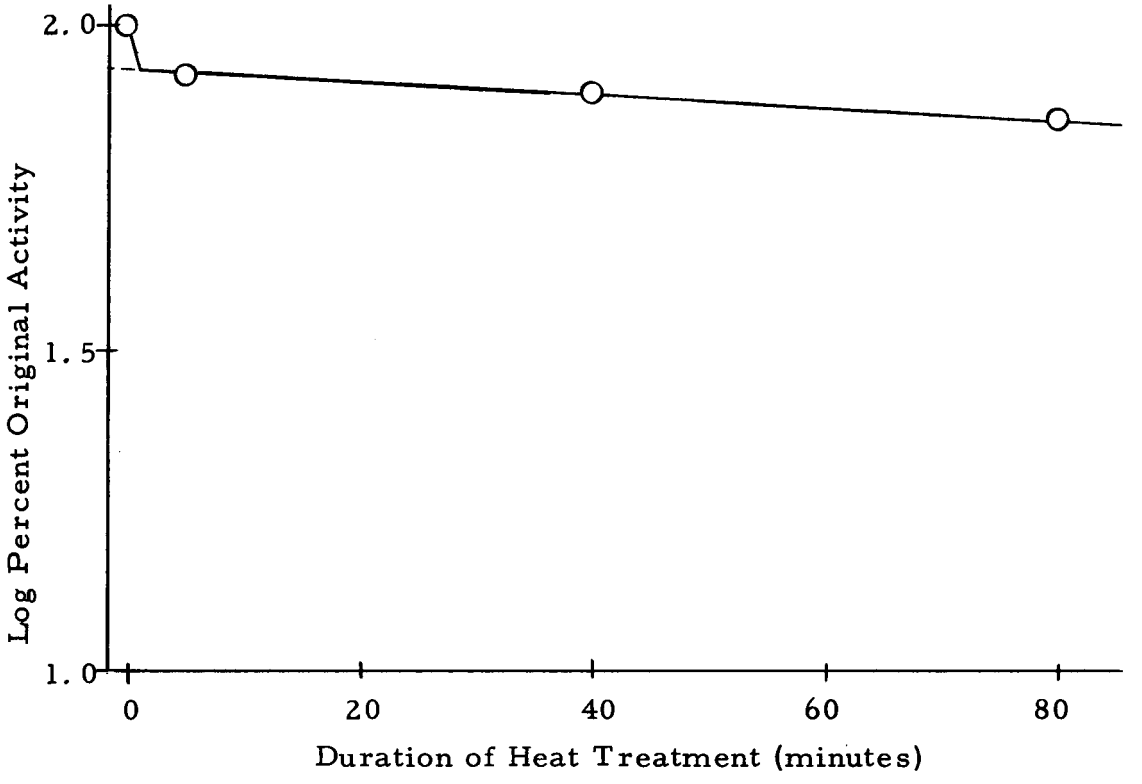

Figure V. Heat inactivation of solubilized mitochondrial enzyme.

The log percent of original activity is shown as a function of duration of treatment of enzyme at 46° C.

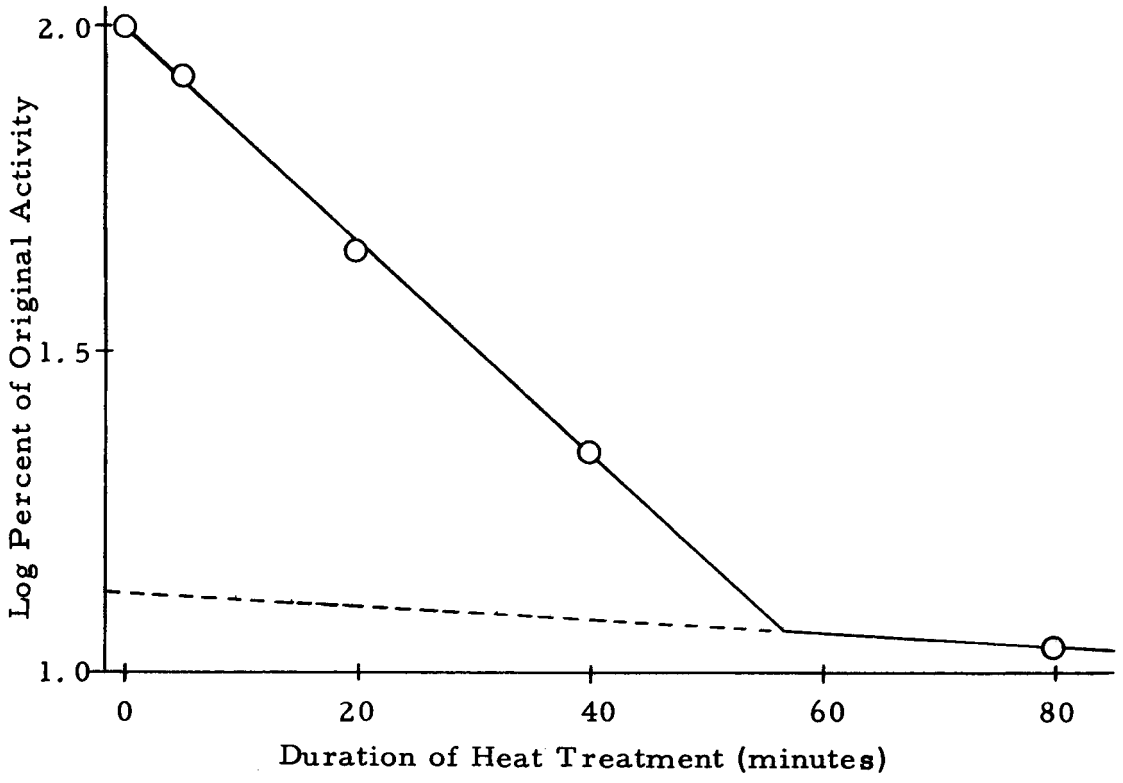

Figure VI. Heat inactivation of soluble enzyme.

The log percent of original activity is shown as a function of duration of treatment of enzyme at 46° C.

enzyme.

The soluble fraction contains a major component with a heat lability intermediate between the two mitochondrial components and a minor component whose heat lability is probably similar to that of the stable mitochondrial component.

DISCUSSION

Changes in the Amounts of Nitrogen and Nucleic Acids in Organs of Developing Chick Embryos

Several workers have determined nitrogen and nucleic acids in chick embryos and chick embryo organs. Klein (41) determined the percent nitrogen in heart ventricles of chick embryos of from four days incubation to hatching. He determined the protein content of the ten percent trichloroacetic acid precipitate by means of the biuret method, then estimated nitrogen by dividing the protein content by 6.25 and expressed nitrogen content as percent of wet weight. Szepsenwol and coworkers (74) determined the concentration of total nitrogen of hearts, livers and brains of embryos from eight days incubation to hatching as percent of wet weight by means of a micro-Kjeldahl procedure. Leslie and Davidson (44, p. 413-428) determined the protein nitrogen content of hearts, livers and brains from embryos of eight days incubation to hatching. They determined nitrogen in the sodium hydroxide digestion mixture from the Schmidt and Thannhauser (69) extraction method for nucleic acids. This mixture contains DNA, protein and ribonucleotides from hydrolyzed RNA, and from the nitrogen value determined was subtracted 1.69 times the nucleic acid phosphorus value to correct for nucleic acid nitrogen.

It is somewhat difficult to compare the results reported in this thesis with those of the above workers because of differences in method, ages studied or the way in which the data are reported. The values reported by Leslie and Davidson are about one-half of those reported here for total nitrogen and in fairly good agreement with the values reported here as protein nitrogen. The percent nitrogen as given by Klein and Szepsenwol and coworkers has been estimated from their graphs and by using the organ weights given by Romanoff (67, p. 1149) the nitrogen content of the organs has been calculated. The calculated values along with values from Table IV are given in Table XV. The correlation between the values is good when one considers all of the possible sources of error in the assumptions on which the above calculations were based.

Szepsenwol and coworkers (73) and Leslie and Davidson (44, p. 413-428) have determined RNA phosphorus and DNA phosphorus by the method of Schmidt and Thannhauser (69) in hearts, brains and livers of chick embryos from between eight days of incubation and hatching. By assuming that RNA and DNA are 9. 0 percent phosphorus and by using the organ weights given by Romanoff (67, p. 1149) the RNA and DNA content of the organs has been calculated. These calculated values along with values taken from Table IV are compared in the accompanying tables.

Table XVI gives the data on DNA. The values reported in this

Table XV. Nitrogen content of chick embryo organs as determined by different workers.

A) Data taken from Table IV of this thesis.

B) Data taken from Klein (41) recalculated as indicated in the text.

C) Data from Szepsenwol and coworkers (74) recalculated as indicated in the text. Values are expressed as mg nitrogen per organ.

	Heart			Brain		Liver	
Age (days)	A	B	C	A	C	A	C
4	.018	.011	--	.087	--	--	--
7	.096	.11	--	.74	--	.18	--
11	.59	.42	.42	2.5	3.0	1.6	1.5
14	1.3	1.1	1.1	4.6	5.4	4.0	4.9
15	1.5	1.1	1.2	5.7	6.5	5.5	6.1
18	2.7	2.5	2.7	9.0	11	9.6	12

Table XVI. DNA content of chick embryo organs as determined by different workers.

A) Data taken from Table IV of this thesis.

B) Data from Szepsenwol and coworkers (73) recalculated as indicated in the text.

C) Data from Leslie and Davidson (44, p. 413-428) recalculated as indicated in the text. Values are expressed as mg DNA per organ.

	Heart			Brain			Liver		
Age (days)	A	B	C	A	B	C	A	B	C
11	.07	.054	.028	.32	.30	.26 (11.5 da)	.19	.18	.11 (11.5 da)
14	.23	.14	.13 (14.5 da)	.61	.51	.42 (14.5 da)	.48	.54	.29 (13.5 da)
15	.30	.14	--	.66	.62	--	.73	.66	.57
18	.47	.28	.32 (17.5 da)	.90	.94	.59 (17.5 da)	1.1	1.4	.89 (17.5 da)

thesis are generally about the same or slightly higher than those of the other workers. The major sources of error in using the Schneider extraction procedure are incomplete extraction of DNA because of the use of too mild extraction conditions or destruction of DNA due to using too concentrated acid or heating too hot or too long during the extraction (36, p. 798). Apparently the extraction procedure used here was neither too vigorous nor too mild.

Table XVII gives the data on RNA. The values reported here are generally the same or considerably lower than those reported by the other workers. These differences are probably because of the different methods used to determine RNA. There is evidence that the RNA fraction obtained by the Schmidt and Thannhauser (69) procedure often contains considerable amounts of organic phosphorus compounds other than ribonucleotides (18; 36, p. 783; 46). This of course leads to high estimates of RNA when it is determined as RNA phosphorus.

If one accepts three premises the changes in relative concentrations of nitrogen, RNA and DNA are in agreement with what would be expected from the embryological and cytological literature. The amount of nitrogen in a tissue is related to mass; the amount of RNA is related to rate of tissue growth; and the amount of DNA is related to the number of cells present.

Figures VII, VIII and IX show the data from Table IV on the

Table XVII. RNA content of chick embryo organs as determined by different workers.

A) Data taken from Table IV of this thesis.

B) Data from Szepsenwol and coworkers (73) recalculated as indicated in the text.

C) Data from Leslie and Davidson (44, p. 413-428) recalculated as indicated in the text. Values are expressed as mg RNA per organ.

	Heart			Brain			Liver		
Age (days)	A	B	C	A	B	C	A	B	C
11	.13	.12	.078	.48	.76	.72 (11.5 da)	.40	.66	.54 (11.5 da)
14	.29	.31	.36 (14.5 da)	1.0	1.5	1.4 (14.5 da)	1.1	2.0	1,4 (13.5 da)
15	.35	.33	--	1.2	1.7	--	1.5	2.4	2.1
18	.43	.78	.79 (17.5 da)	1.8	2.9	2.2 (17.5 da)	2.1	4.8	4.1 (17.5 da)

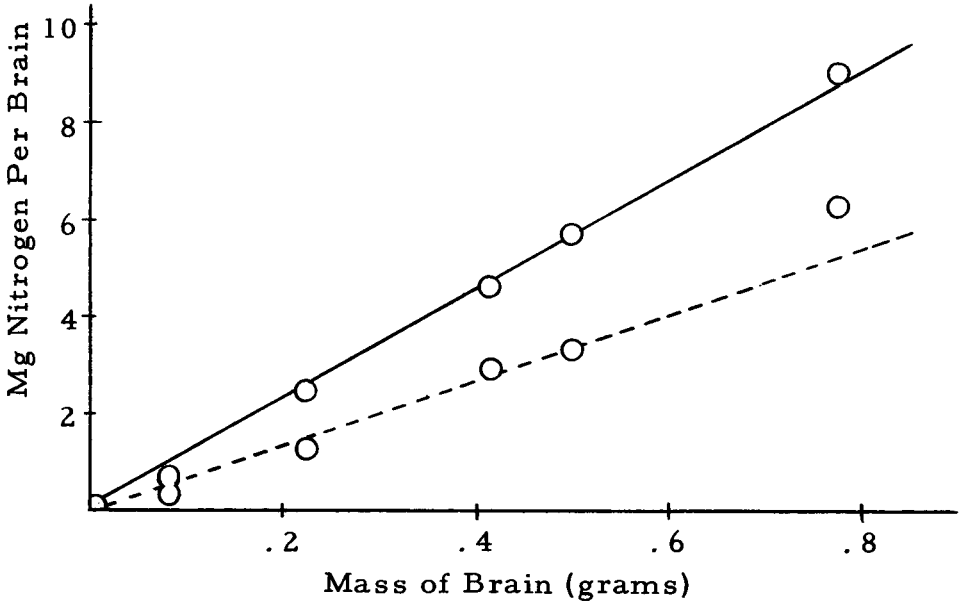

Figure VII. Nitrogen content of embryonic brain as a function of organ weight.

The total nitrogen (solid line) and protein nitrogen (broken line) content of brain is shown as a function of organ mass of four, seven, eleven, fourteen, fifteen and eighteen day embryos.

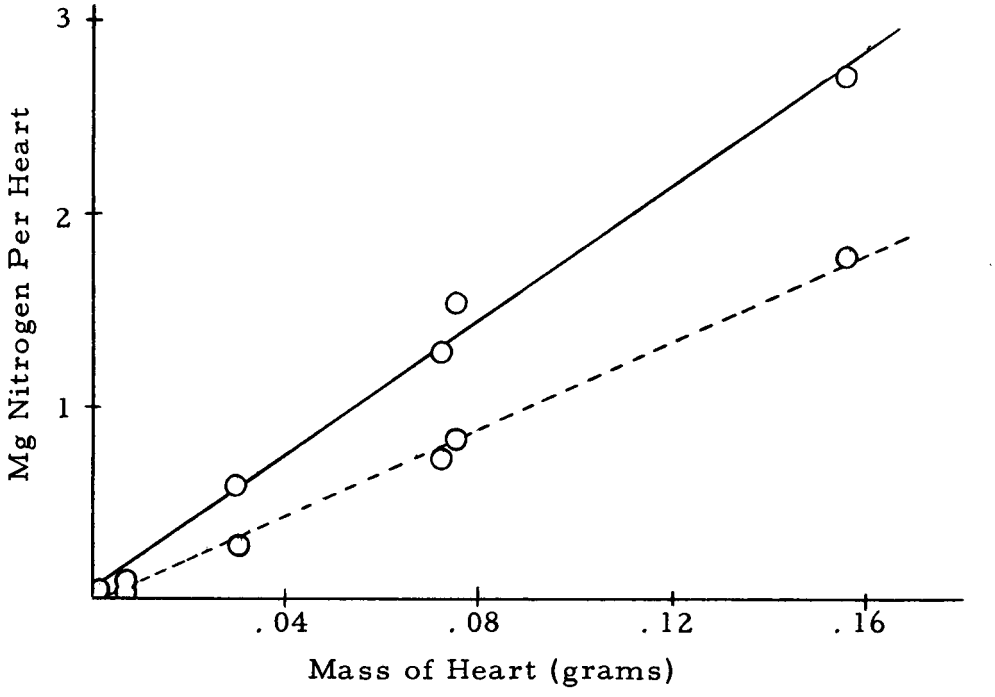

Figure VIII. Nitrogen content of embryonic heart as a function of organ weight.

The total nitrogen (solid line) and protein nitrogen (broken line) content of heart is shown as a function of organ mass of four, seven, eleven, fourteen, fifteen and eighteen day embryos.

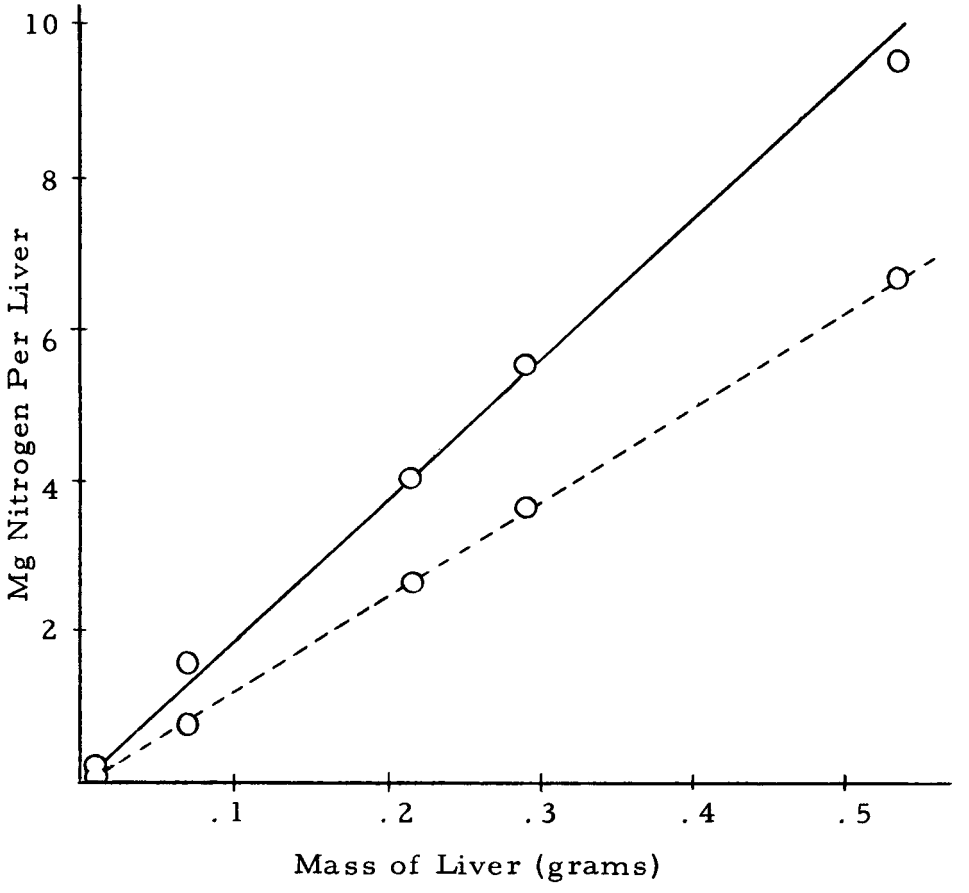

Figure IX. Nitrogen content of embryonic liver as a function of organ weight.

The total nitrogen (solid line) and protein nitrogen (broken line) content of liver is shown as a function of organ mass of seven, eleven, fourteen, fifteen and eighteen day embryos.

amount of nitrogen per brain, heart and liver respectively as function of the mass of the organ at the same age as given by Romanoff (67, p. 1149). This demonstrates that nitrogen content is proportional to mass. In Figures X, XI and XII the rate of growth in grams increase per day per gram of tissue and the concentration of RNA in mg of RNA per mg of nitrogen are shown as a function of age of incubation. The rate of growth was calculated from organ weights given by Romanoff (67, p. 1149) using the following formula:

$$\text{rate} = \frac{m_{n+1} - m_{n-1}}{2m_n} \quad \text{gm da}^{-1}\ \text{gm}^{-1} \text{ of tissue}$$

where m_n = organ weight at day n

m_{n+1} = organ weight at day n+1

m_{n-1} = organ weight at day n-1.

The ratios of RNA to nitrogen are taken from Table VI. It is seen that at times of high RNA concentration the rate of organ growth is high and at times of low concentration the rate is low. It has been demonstrated that in chick embryo hearts, livers and brains the amount of DNA per cell is constant during development (17).

The increase in the ratio of nitrogen to DNA in the brain indicates an increase in average cell size with increasing time of incubation. The growth of the brain therefore is due to both growth and proliferation of cellular units. The constancy of the ratio of RNA to DNA during development is taken to indicate a relatively constant

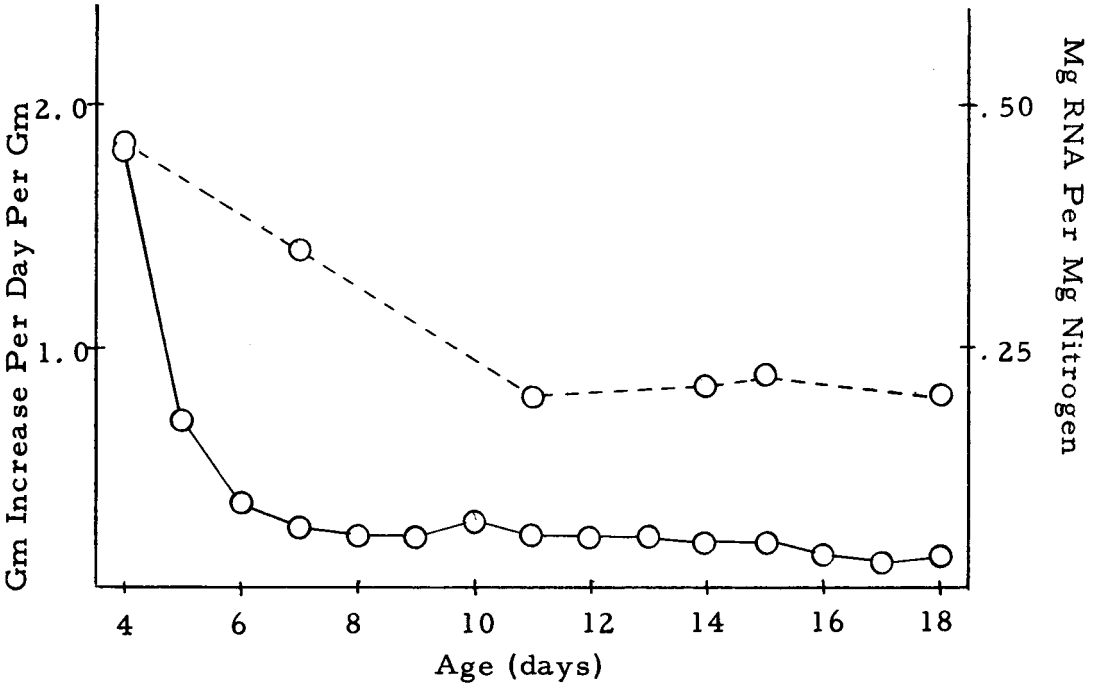

Figure X. Relationship between RNA concentration and growth rate in developing brain.

Rate of growth (solid line) calculated as indicated in the text and RNA to nitrogen ratio (broken line) are shown as a function of age of embryos.

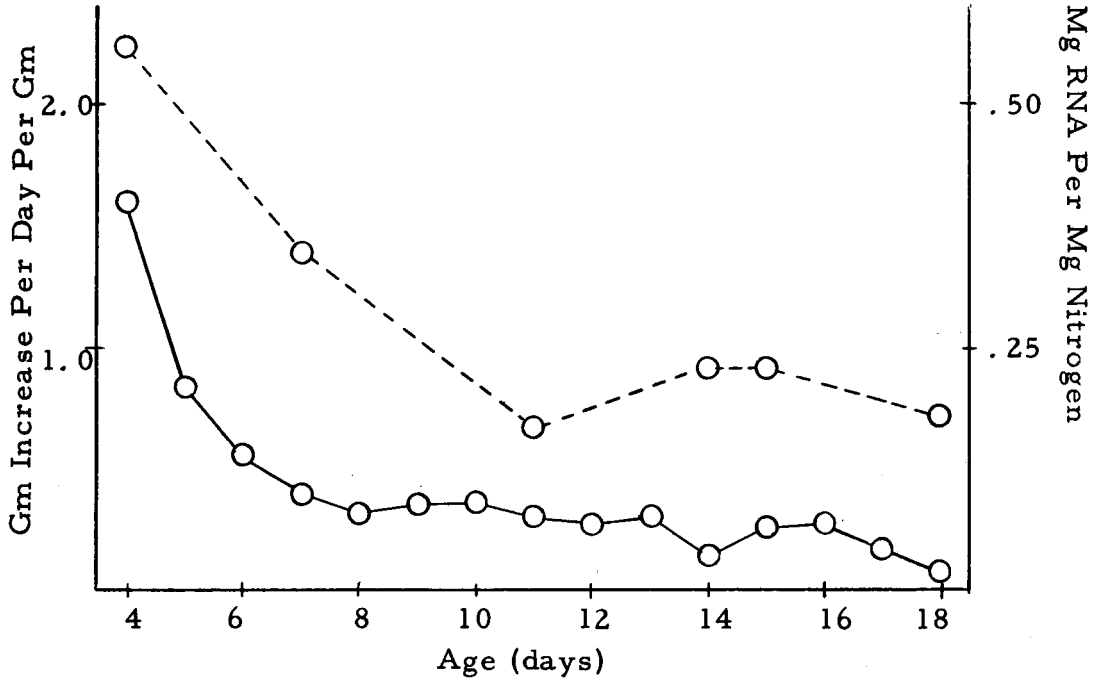

Figure XI. Relationship between RNA concentration and growth rate in developing heart.

Rate of growth (solid line) calculated as indicated in the text and RNA to nitrogen ratio (broken line) are shown as a function of age of embryos.

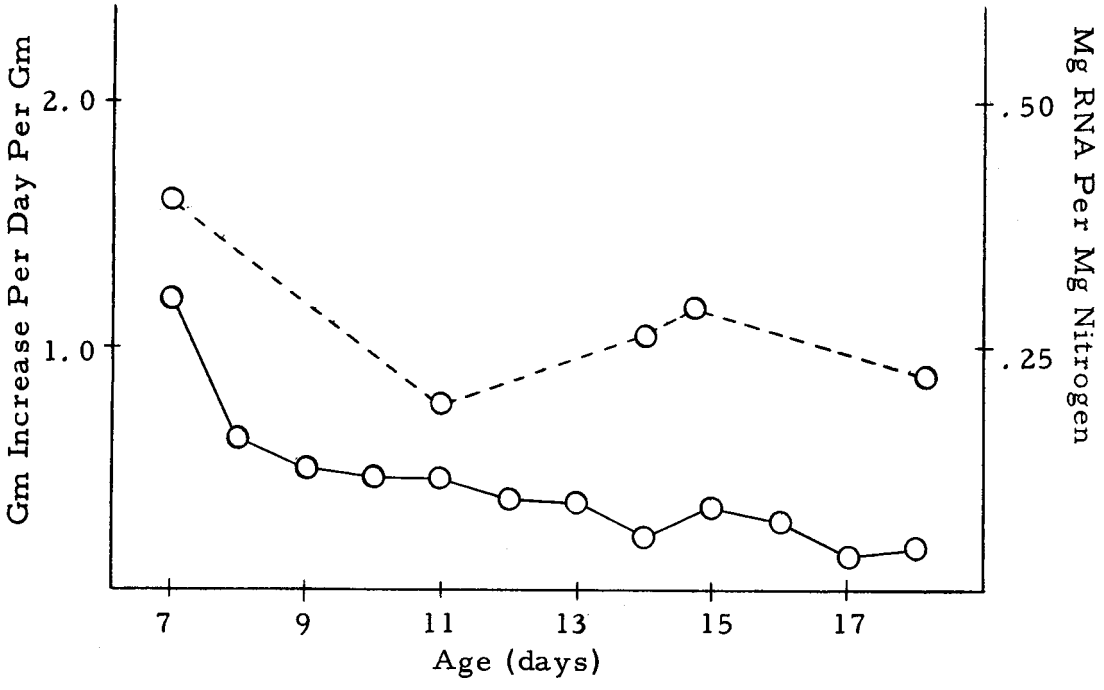

Figure XII. Relationship between RNA concentration and growth rate in developing liver.

Rate of growth (solid line) calculated as indicated in the text and RNA to nitrogen ratio (broken line) are shown as a function of age of embryos.

rate of protein synthesis per cell. During the development of the brain the rate of growth and the mitotic index decrease (37; 38, p. 171-184). Because the mitotic index decreases and the cell size increases during this period, the rate of protein synthesis per cellular unit remains constant even though the rate of synthesis decreases when expressed per unit mass of tissue, per unit mass of protein, or per unit mass of nitrogen.

The ratio of nitrogen to DNA in the heart and the liver indicates that cell size remains relatively constant in these organs and that growth is the result of cell proliferation. It has long been known that growth rate closely parallels the mitotic index of the chick heart (65, 66). In both of these organs the rate of growth and the rate of cell proliferation are known to decrease during development (6, p. 407-436) whereas cell size remains nearly constant. The decrease in the ratio of RNA to DNA is therefore considered to reflect a decrease in the amount of protein synthesized per cell in a given time.

Changes in Enzyme Activities in Organs of Developing Chick Embryos

Mahler and coworkers (51, p. 776) ran their enzyme assays at 37° C whereas these assays were run at 25° C. If one assumes a two-fold increase of reaction rate for every ten degree increase in

temperature then reasonable agreement exists between the data reported in this thesis and that of Mahler's group. The function of isocitrate-NADP oxidoreductase in the supernatant fraction may be to provide NADPH for use in fatty acid synthesis. The increase in this activity in the brain can be correlated with the period during which myelination occurs. It has been shown that there is a rise in the concentration of phosphatides of whole embryo at this time, and it is thought that this is also related to myelin formation (5). It is also interesting to note that an increase in isocitrate-NADP oxidoreductase activity occurs in Rana pipiens embryos at the time of neurulation (81, p. 496-509).

The increase in enzyme activity in liver can be correlated with the accumulation of fat in this tissue. Lipoid globules are known to appear in liver cells between the sixth and eighth day of incubation, and these increase in size and number during embryonic development (40, p. 170-173). The quantity of fat, as percent of wet weight of liver, is known to increase with development after the seventh day of incubation (20). The low level of supernatant activity of the heart may be due to a low level of reductive synthesis in this organ.

The increase of particulate isocitrate-NADP oxidoreductase activity can be correlated with the general rise in the activity of enzymes involved in oxidative metabolism which occurs during development. Increases in the activities of cytochrome c-oxygen

oxidoreductase and succinate-cytochrome c oxidoreductase (16) and malate-NAD oxidoreductase (72) have been found during the development of these three organs. Malate-NAD oxidoreductase has been reported to exhibit peaks of activity in the developing heart and liver at about 14 days of incubation, similar to those now observed for isocitrate-NADP oxidoreductase.

The significance of this peak and the subsequent drop in activity is uncertain. The large standard error of the mean for values of isocitrate-NADP oxidoreductase activity at 14 days of incubation makes one doubt that a peak of activity really exists. On the other hand the large variability may itself be significant. The chick embryo heart accelerates its pulse rate during development to a maximum frequency which some workers (7, 15) believe to occur between the thirteenth and sixteenth days of incubation and others believe to occur much earlier at eight days (68) or five days (2, p. 157-170). Once the maximum rate is attained it is agreed that a deceleration occurs. This might mean a lessened need for oxidative metabolism. It has also been observed that both the heart and the liver show an unusually small increment of growth between the fourteenth and fifteenth days of incubation (67, p. 1149). This low rate of growth might result in a lessened need for capacity for oxidative metabolism.

An additional function of mitochondrial enzyme may be that

proposed for the soluble enzyme. That is the furnishing of NADPH for the reduction step of fatty acid synthesis and possibly the furnishing of carbon dioxide for the carboxylation step of fatty acid synthesis. Mitochondrial fatty acid synthesis has been demonstrated in mammalian liver (30, 31) and in rabbit heart and pigeon liver (14). It is suggested that the function of the mitochondrial fatty acid synthesizing system is the elongation of acyl-CoA units from the supernatant.

It has been demonstrated that fatty acid synthesis does occur in chick embryos (8, 29) however a comprehensive study has not been made. There is no direct evidence available that lipid synthesis, as measured for example by the incorporation of labeled acetate into fatty acids, is greater in one organ or at one period of development.

The low levels of isocitrate-NAD oxidoreductase activity seem almost insignificant in comparison to the NADP dependent activity and this leads one to conjecture that this is NAD activity shown by an NADP enzyme. The demonstration of detectable isocitrate-NAD oxidoreductase in some soluble preparations, which is confirmed by the findings of Mahler and coworkers (51, p. 776) is most surprising. In spite of the fact that the demonstration of extramitochondrial NAD dependent activity is unusual, the explanation is probably not leakage from the mitochondria. Mitochondrial preparations which show no or very little oxidation of isocitrate in

isotonic media by a very active NADP enzyme would not be expected to have lost a measurable quantity of a much less active enzyme (84). The presence of NAD dependent activity in soluble fractions would make the chick embryo almost unique for the rule in the past seems to have been, "Isocitrate-NADP oxidoreductase in either the mitochondrial or soluble fraction; isocitrate-NAD oxidoreductase in only the mitochondrial fraction."

Neonatal rat brain mitochondria are reported to have activity in the presence of either NAD or NADP, while soluble preparations have only NADP stimulated activity (62; 63, p. 328-339). Mitochondria from adult rat liver, kidney and brain are reported to have both activities, while the soluble fraction has only NADP dependent activity (21, 22, 78). The same is reported to be true in Ehrlich ascites tumor cells (32, 33) and ripe fruit of papaya (75). NADP dependent activity has been reported to be present in both mitochondrial and soluble preparations from rabbit cerebral cortex and liver (71). Delbrück (19) reports the presence of isocitrate-NADP oxidoreductase activity in both mitochondrial and cytoplasmatic preparations from bovine cornea epithelium and a much lower level of NAD stimulated activity in the mitochondria with some NAD stimulated activity possible in the soluble fraction.

In most but not all of the above references cited the levels of NAD stimulated activity in mitochondria are reported to be much

lower than the levels of NADP stimulated activity. Chen and Plaut (12, 13) report that ADP stabilizes and enhances the activity of isocitrate-NAD oxidoreductase from bovine heart mitochondria. By assaying in the presence of ADP and using enzyme preparations extracted from mitochondria in the presence of reduced glutathione and ADP Goebell and Klingenberg (26, 27) have demonstrated NAD stimulated activity in Locusta flight muscle, rat skeletal muscle, heart, kidney, brain and liver and pigeon heart which is in near constant proportion to the respiratory chain. Extraction under these conditions might yield higher activities of isocitrate-NAD oxidoreductase from chick embryo tissues.

Comparison of Mitochondrial and Soluble Isocitrate-NADP Oxidoreductase Activity

The differences between the effects of metal ions on different enzyme preparations might be due to real differences between the active proteins. It is also possible that other undefined differences between the preparations were the cause of the differences. It is likely, however, that the differences observed in the heat inactivation study are due to real differences between the soluble and particulate enzyme molecules. The differences between the mitochondria and solubilized mitochondria are probably due to the denaturation of the labile isozyme during the solubilization procedure. If the

differences in rate of heat inactivation between the enzyme preparations were due to factors such as the presence in one preparation or another of protecting or labilizing agents then enzyme mixtures would give slower or faster rates of heat inactivation than expected from a consideration of the rates in unmixed preparations.

It is not surprising to find different forms, or isozymes, of isocitrate-NADP oxidoreductase activity. By means of starch gel electrophoresis Markert and Møller (52) have determined that pig heart isocitrate-NADP oxidoreductase exists as three isozymes and Bell and Baron (4) have detected four isozymes in tissues of the rat. Lowenstein and Smith (49) demonstrated immunological differences between intramitochondrial and extramitochondrial isocitrate-NADP oxidoreductase. This finding has recently been confirmed by Bell and Baron (3) in rat heart and liver by means of starch gel electrophoresis. Differences between isozymes of soluble and mitochondrial preparations are not limited to enzymes which oxidize isocitrate. Electrophoretic patterns of soluble and mitochondrial preparations of pig heart and of horse heart malate:NAD oxidoreductase have been shown to be different (76).

As the proportion of mitochondrial to soluble isocitrate-NADP oxidoreductase changes during development and as they are physically different, it follows that there is an ontogeny of isozyme patterns which depends ultimately on changes in the activities of the genes

which code the structure of the different isozymes. These shifting patterns of isozymes during development have been demonstrated for lactate:NAD oxidoreductase in the chick embryo (45, p. 75-89; 83), a developing snail (28) and embryos of several mammalian organisms (23; 24, p. 313-328; 53, p. 363-381; 82; 83), and for malate:NAD oxidoreductase in developing sea urchins (59, 60, 61) and embryonic tissues of avian and mammalian organisms (82, 83). The means by which gene activities are controlled during development is the central problem of chemical embryology.

SUMMARY

One-half to two-thirds of isocitrate-NADP oxidoreductase activity of chick embryo livers and brains is located in the supernatant fraction obtained by centrifugation at 12, 000 x g. In the heart most of the activity is located in the particulate (mitochondrial) fraction. Soluble activity increases in liver and brain at times of lipid accumulation. The enzyme is suggested to function in fatty acid synthesis by furnishing reduced NADP and possibly carbon dioxide for fatty acid synthesis.

Particulate isocitrate-NADP oxidoreductase increases during development in all three organs studied. This increase is correlated with a general increase in enzymes of oxidative metabolism. Possibly the particulate enzyme also functions in fatty acid synthesis as the soluble enzyme is thought to do.

Heat inactivation studies have indicated that the particulate and soluble isocitrate-NADP oxidoreductases do not have the same isozymic make-up. It follows from this that their structures are coded and their syntheses are controlled at different gene loci.

Isocitrate-NAD oxidoreductase activity was also demonstrated in some preparations. This activity was observed to be ten percent or less of the NADP dependent activity. This activity was present in some soluble preparations.

The amounts of RNA, DNA and nitrogen per organ were determined. The changes in relative concentrations of these compounds indicate that there is a decrease in the rate of protein synthesis in all three tissues during development. Brain cells, however, increase in size and maintain a constant rate of synthesis per cell. Heart and liver cells maintain a constant size during development.

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