AN ABSTRACT OF THE THESIS OF

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Carnitine, acetylcarnitine, and long-chain acylcarnitine concentrations were determined for heart, brain, and liver of chick embryos during various stages of development. The total carnitine concentration was approximately the same in all three organs and showed only small variations during development. Acetylcarnitine was not detected in any organ until the seventeenth day of incubation and represented about 20% of the total carnitine on the day of hatch, in each organ. Long-chain acylcarnitine concentrations generally represented from five to ten percent of the total carnitine in each case. In the heart, the carnitine used for the synthesis of acetylcarnitine appeared to come from long-chain acylcarnitine. In the brain and liver, this carnitine apparently was derived from free carnitine.

Levels of carnitine acetyltransferase activity were measured in hearts, brains, livers and yolk sacs of chick embryos at various

stages of development. The levels of activity of this enzyme corresponded to the increase in acetylcarnitine concentrations with development in the heart and liver. Only very low levels of transferase activity were detected in the brain. The increase in acetylcarnitine concentration and the parallel increase in carnitine acetyltransferase activity in organs correlated with the increase of fatty acid oxidation in the embryo during the last week of development. The relatively high level of carnitine acetyltransferase activity in the yolk sac appeared to be localized in the yolk sac-membrane. The possibility that this yolk sac-enzyme may function in the transfer of fatty acyl groups from the yolk into the embryo is presented.

Perchloric acid extracts of chick embryonic tissues contained a substance which interferes with the assays for carnitine and acetylcarnitine. The interference was attributed to an inhibitor of the carnitine acetyltransferase reaction used in the assays. The low molecular weight inhibitor was not a protein and was heat stable, soluble in aqueous solvents but insoluble in chloroform-methanol. In addition, the inhibitory phenomenon appeared to be enhanced by treatment with dilute base. By careful adjustment of the tissue extract concentration in the assay mixture, carnitine and acetylcarnitine were accurately determined in the presence of the inhibitor.

Carnitine and Derivatives in Embryonic Chick Tissues

by

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for her confidence and encouragement

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LIST OF ABBREVIATIONS AND DEFINITIONS USED IN THIS THESIS

Abbreviations

CoA	Coenzyme A
DTNB (Ellman's Reagent)	5,5'-dithiobis-2-nitrobenzoic acid
EDTA	ethylene diamine tetraacetic acid
NAD, NADH	the oxidized and reduced forms of nicotinamideadenine dinucleotide
TCA	Trichloroacetic acid
Tris	2-amino-2-hydroxymethylpropane-1,3, diol

Definitions

Enzyme unit	amount of enzyme catalyzing the formation of one μmole of product per minute at 35°

CARNITINE AND DERIVATIVES IN EMBRYONIC CHICK TISSUES

INTRODUCTION

Chemical Embryology and the Chick Embryo

One of the major problems in chemical embryology is to find relationships between events at a molecular level and the structural components in which these molecular events find expression. Therefore, a common aim of embryologists is to describe as fully as possible the complex and intricately coupled reactions and interactions, at all levels, in the development of the egg into the fully differentiated adult. Chemical studies of development contribute to this goal by providing one of the parameters of description. The chick embryo provides an excellent system for such studies because of its availability, ease of handling, and the multitude of information that has been recorded concerning this system.

The characteristic metabolic pattern of cells of various tissues, at any stage of differentiation, may be analyzed in terms of the substances present in those tissues at any given time. A wide variety of substances has been measured in various parts of developing chick embryos in order to provide a biochemical description of development (37, 52, 64). Because of the truly important role that enzymes play in the metabolic scheme, they have been extensively investigated in

developmental studies (37). The reactants and products of enzymatic activity also have been studied both for their own importance and as indicators of in vivo enzymatic activity. Moog (51), in her review article, has shown that correlations may be made between enzyme levels and the functional state of the embryo.

Needham (52, p. 986-999) collected an impressive body of data that indicated that during development the chick embryo uses different primary energy sources in the order: carbohydrate, protein, fat. The importance of carbohydrate as an energy source for the early chick embryo has been more recently substantiated by Spratt (69, 70). Most of the protein absorbed by the embryo during development is used for structural material. Protein utilization for catabolic purposes begins to be significant about the fifth or sixth day and reaches a maximum on the eighth or ninth day of embryogenesis (6). Approximately 50% of the total lipid present in the egg is oxidized (52, p. 996; 64, p. 238), and from this the embryo derives 90% of its caloric yield (52, p. 997). Needham has summarized data on the absorption of the primary energy sources from the yolk into the embryo. The absorption curve for fat shows a peak on the tenth day of incubation, followed by a trough, and then another peak about the eighteenth day (52, p. 925). Similar results have been reported by Budowski, Bottino and Reiser (15), who found that during the last week of the incubation period, relatively large amounts of lipids are incorporated into the

embryo while the yolk lipid decreased accordingly. The data compiled by Needham also shows that in terms of percentage composition of the embryo (relative to dry weight), fat content increases gradually throughout development until approximately the fourteenth day. At this point the curve shows an inflection upwards and a peak occurs on the day of hatch. Similar data for carbohydrate and protein show peaks on the fifth and eleventh day, respectively. More recently, and at the organ level, Dumm and Levy (20) showed that a striking increase in the lipid content of the liver occurred during the latter part of the incubation.

Carnitine and its Relationship to Intermediary Metabolism

Carnitine (γ-trimethylammonium β-hydroxybutyrate) was discovered in 1905 and its chemical structure was established in 1927 (25). Its ubiquitous presence in the tissues of plants, animals, and microorganisms has been reviewed by Fraenkel and Friedman (25). Discovery of its function as a growth factor in insects in 1952 (16) was responsible for the new flourish of activity in carnitine research that is presently under way. The role of carnitine in metabolism remained relatively obscure until the investigations of Friedman and Fraenkel (28) and of Fritz (32) implicated carnitine in fat metabolism. The role of carnitine in fatty acid metabolism has recently been reviewed by Fritz (30).

Fritz (32) identified carnitine as the stimulatory component, present in liver slices and homogenates, that caused an increase in the rate of oxidation of palmitic acid. Further studies showed that carnitine catalyzes an increase in long chain fatty acid oxidation by favoring the formation of acylcarnitine, which is mediated by the enzyme, carnitine palmityltransferase (11, 35, 55). The reaction involved can be written as follows:

$$\text{palmityl-CoA} + \text{carnitine} \rightleftharpoons \text{palmitylcarnitine} + \text{CoA}$$

At approximately the same time, Friedman and Fraenkel (28) demonstrated a reversible acetylation of carnitine by acetyl-CoA and from this concluded that the ester bond of acetylcarnitine was a high energy bond comparable to the thioester bond of acetyl-CoA. Since then, carnitine acetyltransferase which catalyzes the reaction:

$$\text{carnitine} + \text{acetyl-CoA} \rightleftharpoons \text{acetylcarnitine} + \text{CoA}$$

has been purified (34) and obtained in crystalline form (17). The two enzymes involved in the acylation of carnitine share many of the same general properties except that carnitine palmityltransferase exhibits a different fatty acid chain length specificity than that of carnitine acetyltransferase (34). Although hexanyl-CoA shows limited activity as a substrate in the short chain acyltransferase reaction, palmityl-CoA shows no activity as a substrate.

The work from the laboratories of Fritz (30), Bremer and Norum

(10, 56, 57), and Bressler (12, 13) has supported the hypothesis that carnitine acts as a carrier of activated fatty acyl groups across the mitochondrial membrane. Because of the impermeability of the mitochondrial membrane to acyl-CoA derivatives (57, 68), carnitine esters of fatty acids are formed to effect the movement of activated fatty acyl groups across the barrier. The rate of translocation of fatty acyl groups across the mitochondrial membrane barrier is thought to be the rate-controlling reaction of long-chain fatty acid oxidation (66). More recent publications by many investigators (3, 65, 73, 74) have elevated the importance of carnitine in intermediary metabolism even further by illustrating the effect of carnitine on gluconeogenesis. This is not surprising since it is well known that increased rates of fatty acid oxidation enhance gluconeogenesis and/or depress glycolysis. These close biochemical links between the complex pathways of glucose and fatty acid metabolism prompted Fritz to present a hypothesis concerning the role of carnitine in the control of fatty acid and carbohydrate metabolism (29). Wittels and Blum (75) detected carnitine and carnitine palmityltransferase in the protozoan, *Euglena gracilis*, cultured in either acetate or palmitate as a sole carbon source. They propose that this system may be ideal for studying the role of carnitine and carnitine palmityltransferase in regulating carbohydrate-lipid interrelationships.

As discussed by Fritz (29), the role of carnitine

palmityltransferase in fatty acid and glucose metabolism is becoming clear. Unfortunately, a definite role for the short-chain acyltransferase is not established. Early investigations of Norum and Bremer (57) showed the formation of acetylcarnitine from pyruvate in mitochondria and suggested that this "active acetate" is moved out of the mitochondria as acetylcarnitine. In the extramitochondrial compartment of the cell the acetylcarnitine could then be used to regenerate acetyl-CoA. This acetyl-CoA would then be available for fatty acid and cholesterol synthesis, which takes place in the cytosol. Other investigations by Bremer (10) and Bressler and Katz (12, 13) supported this hypothesis. However, there also is a large amount of evidence that the enzyme is not involved to any great extent in the transfer of acetyl groups from mitochondria to sites of fatty acid synthesis. Evidence against this hypothesis centers around the following observations: (1) citrate appears to be the main carrier of "active acetate" units from the mitochondria to the site of fatty acid synthesis (1, 63, 68), and (2) carnitine acetyltransferase activity appears to be lowest in tissues which have the highest rates of fatty acid synthesis and vice versa (2, 45, 47).

More recently, Fritz has suggested that carnitine acetyltransferase acts to catalyze the transfer of acetyl groups within mitochondrial compartments (29). There is also some evidence that the carnitine acetyltransferase system buffers the tissue content of

acetyl-CoA against rapid changes (19, 61). Both of these latter functions postulated for the short chain transferase system were suggested earlier by Lowenstein (44).

Involvement of Carnitine in Functions Apparently Unrelated to Mitochondrial Transfer

Carnitine is unable to substitute for dietary choline as a growth factor in several choline-requiring organisms (26). In two species of the order Diptera, however, carnitine can replace choline. Hodgson, Cheldelin, and Newburgh (38) reported that carnitine can completely replace choline in *Phormia regina* and Fraenkel *et al.* (26) reported that *Drosophila melanogaster* could grow almost as well on carnitine as on choline. Bieber *et al.* (4, 5) have shown that phospholipids from *Phormia regina* larvae raised on carnitine instead of choline contain β-methylcholine as a constituent of phosphatides rather than choline.

Although carnitine has been found to be a growth factor in insects, the evidence for a similar function in higher organisms has not been satisfactory. Strack (72) has claimed carnitine as an essential food factor for the growth of human infants. Khairallah and Wolf (42) have suggested that in rats carnitine has a methionine-sparing action and may thus be considered a food factor required in marginal diets. In addition to their nutritional aspects, carnitine and its derivatives may be related to nervous system functions. In his

review, Fritz (30) discusses the cholinergic properties of carnitine, acetylcarnitine and other structurally related compounds. More recently, reports from the laboratory of Hosein (39, 40) have suggested that some of the acetylcholine-like activity in subcellular particles from rat, rabbit and calf brain is attributable to acetylcarnitine and acetylcarnityl-CoA. Hosein and Smoly (41) have presented evidence that acetylcarnityl-CoA is used to biosynthesize acetylcarnityl choline in rat brain extracts. It is suggested that this compound is responsible for the acetylcholine-like activity.

Analytical Methods for Carnitine and its Derivatives

The difficulties of determining carnitine in biological materials have been discussed by Friedman (27), Mehlman and Wolf (49), Mehlmen and Therriault (48), and Marquis and Fritz (46). Many of the early values reported for the distribution of carnitine, reviewed by Fraenkel and Friedman, were based on the bioassay procedure of Fraenkel (25). It is clear from Fraenkel's data that precision is no greater than ± 50% under optimal conditions, and that several factors can operate to disturb the accuracy of the assay (23). Strictly speaking, the assay measured vitamin B_T activity. The possibility exists that carnitine may not be the only naturally occurring substance with B_T activity. More recently published data are based upon a variation of Friedman's colorimetric technique, in which bromophenol

blue is complexed with an ethyl ester of carnitine or a carnitine derivative after removal of interfering compounds (27, 50). Unfortunately, in some cases these measurements do not exclude compounds that are closely related structurally to carnitine (27). The identification and purification of carnitine acetyltransferase has led to the development of enzymatic analyses for carnitine and its derivatives which are more specific, more precise, and less laborious than previous methods (10, 46, 59). Unfortunately, as shown in this thesis, and as reported by others (19), carnitine-containing extracts of biological tissue sometimes contain substances which inhibit the enzyme and create problems in the analysis.

Carnitine and its Relationship to the Chick Embryo

The pioneering studies of Fraenkel, using his bioassay procedure, first demonstrated the dramatic increase in carnitine within a matter of days during development of the chick embryo (22). On the basis of these results, Mehlman and Wolf (50) decided to use this system for the study of carnitine biosynthesis. These authors investigated the increase in carnitine with time of incubation in whole egg, chick embryo and yolk sac using a chemical assay. They reported approximately a nine-fold increase in carnitine per whole egg from the tenth to the sixteenth day of incubation. They also reported that the yolk sac contained 40-50% of the carnitine in the

whole egg. During the course of their extraction procedures it became apparent that whole eggs contained some form of carnitine released by trichloroacetic acid after 72 hours but not in two hours. This "bound carnitine" comprised 20-45% of the carnitine in the yolk sac but the embryo alone was found to contain very little carnitine in this form. In a later publication (49), these authors identified the "bound carnitine" as phosphatidylcarnitine and concluded that this compound is characteristic of embryonic tissue. Subsequent investigations by Soderberg, Therriault and Wolf (67), and by Fritz (31) indicated that the compound previously identified as phosphatidylcarnitine did not contain phosphorous and finally identified it as a long-chain O-acylcarnitine.

In summary, this study of the levels of carnitine and its derivatives contained in the tissues of developing chick embryos was then undertaken for the following reasons: (1) Previous studies on the distribution of carnitine in the chick embryo, although interesting, were controversial and incomplete. The reports of Fraenkel and of Mehlman and Wolf were concerned primarily with the carnitine content in the whole egg and the entire embryo. A more detailed study of the distribution of carnitine in this system was therefore, apparent. (2) The development of new methods for the determination of carnitine made it possible to assay for carnitine and its derivatives with greater precision, and with less elaborate procedures then was

previously possible. (3) The recent accumulation of knowledge about carnitine and its role in intermediary metabolism, particularly fatty acid oxidation, suggested that the chick embryo, which derives most of its energy from lipid metabolism, would be an ideal system for the study of the relationships between carnitine and its derivatives *in vivo*. (4) The possibility exists that carnitine may play a role in the chick embryo which is unique to developing systems.

The data presented in this study not only lend support to the involvement of carnitine in fatty acid oxidation, but also emphasize its role in the transfer of acyl groups across biological membranes.

MATERIALS AND METHODS

Fertile Eggs and Chicks

Fertile eggs (Indian River Hybro breed) were purchased from Jenk's Hatchery, Tangent, Oregon, and were incubated in a Jamesway Model 252 B incubator equipped with an 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. If required, chicks were hatched in the incubator, removed and placed in a Leahy brooder at a temperature of approximately 25° C. The chicks were maintained *ad libitum* on a diet of Purina mash and water.

Chemicals and Enzymes

DL-Carnitine hydrochloride, 5,5'-dith*obis*-2-nitrobenzoic acid, L-malic acid, acetyl coenzyme A, coenzyme A, nicotinamide-adenine dinucleotide, were obtained from Calbiochem. Calcium phosphate gel was obtained from Sigma. DL-acetylcarnitine was synthesized as described by Bremer (10). The product was twice recrystallized from butanol and its melting point was 186-187° C. All other chemicals were of reagent grade and were obtained from commercial sources. Pronase (B grade), malic dehydrogenase (A grade), and citrate-condensing enzyme (B grade) were obtained from Calbiochem.

Protein Determination

The spectrophotometric method described by Layne (43) was used to estimate protein in solution. Solutions were appropriately diluted and their optical densities were measured at both 280 and 260 mμ. The ratio of optical density at 280 mμ to optical density at 260 mμ was used to calculate the protein concentration in the sample using information provided in the reference.

Preparation of Tissues for Carnitine, Acetylcarnitine, and Long-Chain Acylcarnitine Analysis

Tissue extracts were prepared using a modification of the method described by Pearson and Tubbs (62). A flow diagram that summarizes the modified procedure appears in Figure 1. Organs to be analyzed were dissected from embryos, or young chicks, and frozen immediately in liquid nitrogen. The frozen tissues were then homogenized in ice-cold 5% $HClO_4$ (one to two ml per gram wet weight of tissue). The fraction insoluble in dilute perchloric acid was collected by centrifugation at 10,000 x g in a Servall centrifuge at 0-5° C for five minutes. This fraction was then homogenized in 5% perchloric acid and again collected by centrifugation at 10,000 x g. The resulting precipitate was homogenized in five ml of ice-cold, glass distilled water. The supernatant obtained after centrifuging the aqueous homogenate at 10,000 x g for five minutes was combined with

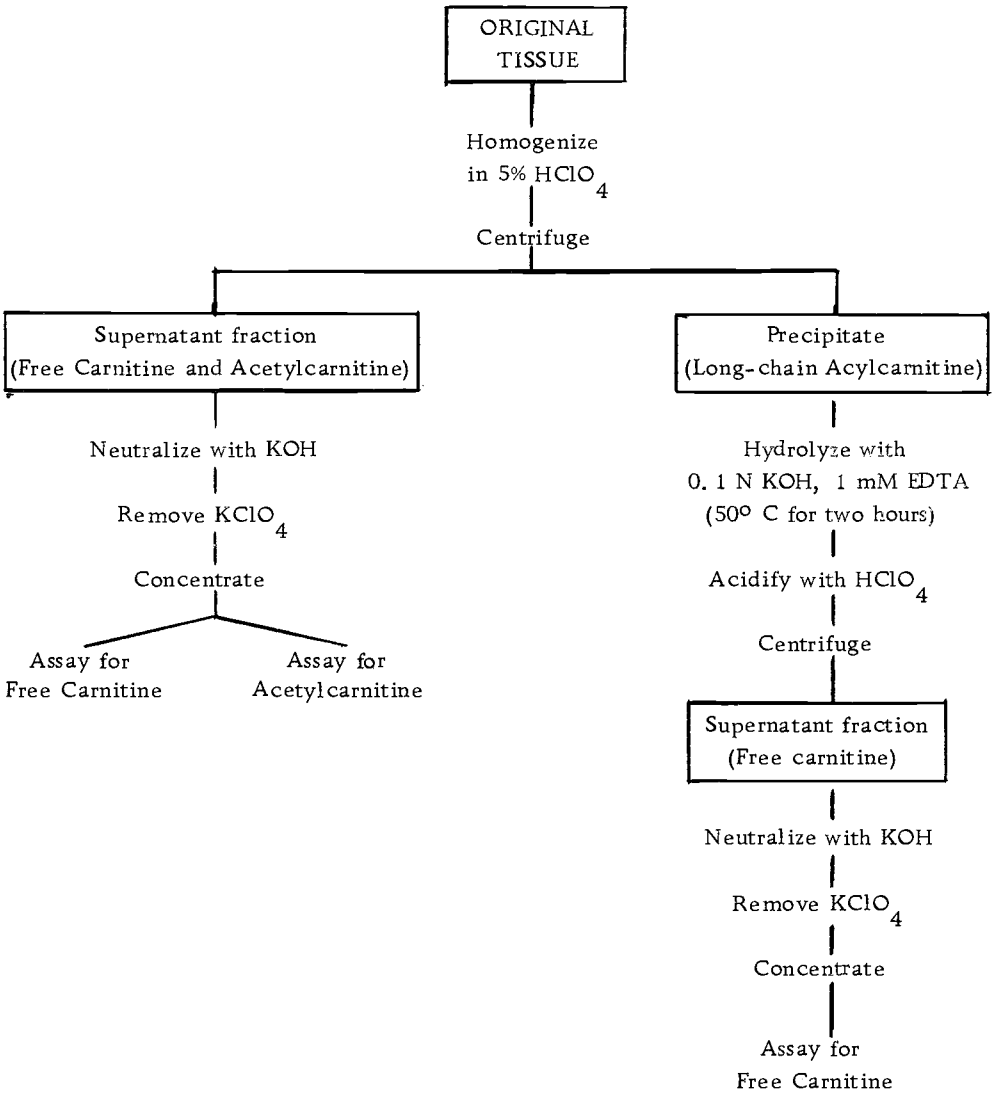


Figure 1. Flow diagram representing procedure used for the preparation of tissues for free carnitine, acetylcarnitine, and long-chain acylcarnitine determinations.

the two supernatants obtained after dilute perchloric acid homogenizations. The combined supernatants were brought to pH 7.0 by the addition of 5N KOH and the $KClO_4$ that formed was allowed to precipitate for 30 minutes while the extract was maintained at near 0° in an ice bath. After subsequent removal of the $KClO_4$ precipitate by centrifugation at 10,000 x g for three minutes, the supernatant was concentrated in vacuo to a volume of two to three ml in a rotary evaporator. The concentrated extracts were cooled in an ice bath for 30 minutes and any $KClO_4$ that precipitated was quickly removed by centrifugation in a clinical centrifuge. The volume of the extract was then measured and 0.05 ml aliquots were assayed for free carnitine and 0.10 ml aliquots were assayed for acetylcarnitine.

The washed dilute perchloric acid precipitate, isolated above by centrifugation, was resuspended in 10-12 ml distilled water by homogenization. An aliquot of this fraction (usually 1/5 of the total volume) was dryed to a constant weight at 65° C in tared containers. The remainder of this fraction was heated at 50° C for two hours in 0.01N KOH, containing one mmole EDTA per liter, in order to hydrolyze long-chain acylcarnitines. After cooling the hydrolysate and acidifying with three to five drops of 72% (w/v) perchloric acid, the precipitate was collected by centrifugation at 5000 x g for five minutes. This precipitate then was washed once with three ml distilled water and the wash combined with the above supernatant.

Neutralization of this supernatant with KOH yielded a precipitate of $KClO_4$ which was removed by centrifugation. The supernatant was then concentrated in a rotary evaporator to two ml and 0.10 ml aliquots were assayed for free carnitine.

Preparation of Partially Purified Carnitine Acetyltransferase

Carnitine acetyltransferase was partially purified using a modification of the procedure described by Chase, Pearson and Tubbs (17). Approximately 600 g of frozen pigeon breast muscle was thawed, and homogenized for one minute in a Waring blendor with three volumes of cold (-5° C) 20% ethanol containing 0.4 M KCl. The homogenate was centrifuged at 12,000 x g for 45 minutes, filtered through cheese cloth and dialyzed against two changes (12 liters per change) of 2 mM phosphate buffer (pH 7.5) containing 0.5 mM EDTA. All procedures, unless otherwise noted, were carried out at 2° C, in an ice bath or in a cold room.

Solid ammonium sulfate was added to the dialyzed extract to give 50% saturation (312 g/liter) and the precipitate was discarded. Further ammonium sulfate was added (82 g/liter) to 62.5% saturation and the precipitated protein was collected by centrifugation at 18,000 x g for 30 minutes. The protein pellet was then dissolved in 0.1 M phosphate buffer (pH 7.5) and dialyzed as before against two changes of the dialysing solution (five liters per change).

The dialysate was maintained at 0° C and cold acetone (-15° C) was added slowly, with stirring, to give a final solution containing 32% acetone (v/v), the temperature being lowered progressively to -5° C in an ice-salt bath. The resulting precipitate, collected by centrifugation at 18,000 x g for 30 minutes, was dissolved in 0.05 M phosphate buffer (pH 7.5) and dialyzed as before against two changes of dialyzing solution (two liters per change). Next, the protein content of the dialysate was estimated and three mg of calcium phosphate gel was added for each mg of protein in the dialysate. After collecting the gel by centrifugation at 5000 x g for five minutes, the gel was washed three times with a total volume of 150-200 ml of 0.1 M phosphate buffer (pH 7.5). The washes were discarded and the enzyme was eluted from the gel using three elutions of 0.4 M phosphate buffer (pH 7.5) containing 10% ammonium sulfate (about 100 ml total volume). This purified preparation had a specific activity of one to four units per mg protein, and was stable for at least three months when stored at 0° C.

Although, as mentioned above, Chase et al. discards the precipitate formed upon dialyzing the original ethanol-KCl extract; some of our preparations yielded precipitates containing up to 40% of the original enzyme activity. In these cases, most of this activity was recovered by extracting the precipitate with 0.1 M phosphate buffer (pH 7.5). After dialysis against two changes of the dialyzing solution

(three liters per change), a partially purified enzyme preparation was then obtained from the extract by following the procedure described above, starting with the first ammonium sulfate precipitation.

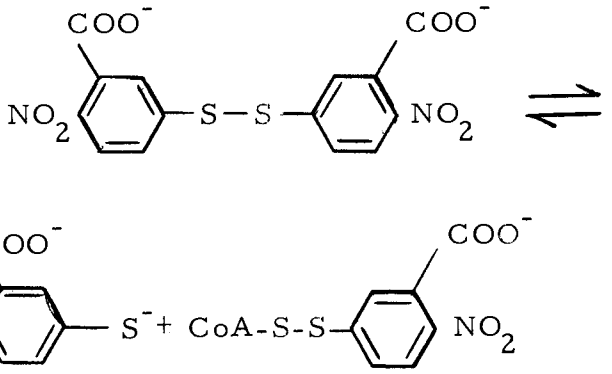
The procedure described above for the preparation of partially purified carnitine acetyltransferase was modified further to obtain a best preparation of higher specific activity (six units/mg protein). Since the acetone fractionation procedure, described above, gave quite variable results (yields of enzyme activity for this step alone varied from 50 to 11%), we decided to eliminate this step altogether. The dialyzed solution obtained after fractionation with 62.5% saturated ammonium sulfate was analyzed for protein and was treated with calcium phosphate gel as described above. The enzyme solution eluted from the gel with 0.4 M phosphate was dialyzed against two liters of 2 mM phosphate (pH 7.5). This dialysate was made 20% (v/v) in ethanol by the slow addition of absolute ethanol (-15) and was maintained at -5° C for one hour in an ice-salt bath. The precipitate obtained by centrifuging the above solution at 18,000 x *g* for 30 minutes was discarded. The purified preparation, present as the supernatant, was stable for at least three months when stored at 0° C.

Enzymatic Method for the Analysis of Free Carnitine

Free carnitine was assayed by the procedure described by Marquis and Fritz (46). The essential reactions in this coupled

system are:

(1) Acetyl-CoA + carnitine $\rightleftharpoons$ acetylcarnitine + CoASH

(2) CoASH + 

Chromogenic

Reaction 1 is catalyzed by the enzyme, carnitine acetyltransferase. Reaction 2 employs the reaction described by Ellman (21) whereby the combination of DTNB with sulfhydryl groups form the thiophenolate ion which has a maximum absorbtion at 412 mμ ($E_{max} = 13.6 \times 10^3$ M^{-1} cm^{-1}).

Aliquots of concentrated tissue extracts were first heated at 90° C for five minutes at pH 8.5 to oxidize any sulfhydryl groups which might be present in the $HClO_4$ extracts. This was done to minimize the "tissue blank" which resulted from interaction between sulfhydryl groups contained in the extracts and DTNB. "Tissue blank" cuvettes contained 100 μmoles Tris-HCl at pH 7.5 (at 35° C), 0.05 μmole acetyl-CoA, 0.1 μmole DTNB (freshly prepared), 5 μmoles EDTA, aliquots of tissue extract, and water to a final volume of 1.0 ml.

Test cuvettes contained the same components plus carnitine acetyltransferase. Since some absorbance change occurred when only DTNB and enzyme were present, it was necessary to determine an "enzyme blank" in a separate cuvette. This "blank" was constant for a given amount of carnitine acetyltransferase and was subtracted from all final readings. The amount of enzyme added per assay was usually about 0.1 unit. Changes that occurred after incubation (10-30 minutes at 37° C) were measured at 412 mμ on a Bausch and Lomb Spectronic 600 spectrophotometer.

Standard curves were obtained by determining absorbance changes at 412 mμ after known amounts of DL-carnitine were added to the system. In each set of experiments, a carnitine standard was employed to insure that reagents and enzyme were adequate. Amounts of carnitine present in aliquots of tissue extract were estimated by comparison with the standard curve. Amounts of carnitine are expressed in terms of μmoles carnitine per gram dry weight of initial tissue.

Enzymatic Method for the Analysis of Acetylcarnitine

Acetylcarnitine was estimated by following the production of NADH in the coupled enzyme system described by Marquis and Fritz (47). This method employs the citrate condensing enzyme assay system of Ochoa (58) to measure the formation of acetyl-CoA from

acetylcarnitine during the acetyltransferase reaction. The essential reactions in this coupled enzyme assay system are:

(1) Acetylcarnitine + CoASH $\rightleftharpoons$ Acetyl-CoA + carnitine

(2) Acetyl-CoA + oxalacetate $\rightleftharpoons$ citrate

(3) NAD + malate $\rightleftharpoons$ oxalacetate + NADH

Reaction 1 is catalyzed by carnitine acetyltransferase, reaction 2 by citrate-condensing enzyme, and reaction 3 by malate dehydrogenase. Fritz et al. has shown that a linear relationship exists between acetylcarnitine present and total NADH produced and that one mole of NADH is produced per mole of acetyl-CoA generated from acetylcarnitine (34). NADH formation was determined by following the increase in absorbance at 340 mμ using a Bausch and Lomb Spectronic 600 spectrophotometer.

Cuvettes contained, in a final volume of 1.0 ml, 100 μmoles of Tris-HCl (pH 7.8 at 35° C), 2.5 μmoles of NAD, 0.17 mg of CoA, 5 μmoles of L-malic acid, 5 μmoles of EDTA, 0.07 unit of malate dehydrogenase, 0.07 unit of citrate-condensing enzyme, 1 μmole NaCN, aliquots of tissue extract, and 0.1 unit of carnitine acetyltransferase. Since tissue extracts contained endogenous acetyl-CoA precursors, "tissue blanks" were prepared containing all components except carnitine acetyltransferase. This blank was subtracted from readings made on identical test solutions. Since carnitine

acetyltransferase preparations, in the absence of tissue extracts, made no contribution to the absorption observed at 340 mμ, an "enzyme blank" was not required in these assays. Preincubation of the tissue extract with all the components of the system except carnitine acetyltransferase, for ten minutes at 37° C, was necessary to permit equilibration of the malate dehydrogenase reaction. After addition of the transferase to the samples, the samples and blanks were incubated at 35° C for one hour before the absorption at 340 mμ was determined.

Preparation of Tissues For Assays of Carnitine Acetyltransferase Activity

The method of Marquis and Fritz (47) was used to measure enzyme levels of carnitine acetyltransferase in embryonic and young chick tissues. One to two grams of wet tissue were dissected and placed directly into homogenizers containing approximately five ml of ice-cold 0.1 M K_2HPO_4 (pH 8.0), containing 0.01 M EDTA and 0.1% deoxycholic acid. After homogenization in motor-driven, ground glass homogenizers, the extracts were allowed to stand 15 minutes, and then were centrifuged for 15 minutes at 15,000 x g. The homogenization and centrifugation procedures were repeated on the precipitate and the supernatants were pooled to be assayed for carnitine acetyltransferase activity. Protein in the supernatant was

estimated at this time.

In the case of yolk sacs, the extraction procedure was carried out in two different ways. In preliminary experiments the yolk sac, complete with yolk, was extracted in large glass homogenizers with 30-40 ml of the extraction solution as outlined above. Assays for enzyme activity were performed on the cloudy supernatant obtained by centrifuging the extract at 30,000 x g for 30 minutes. In later experiments, the yolk was removed from the yolk sac by puncturing the sac in a seive and washing away the yolk with chick Ringer's solution. The yolk sac was removed from the seive and was then extracted using the procedure described above for other tissues. In some experiments, the sac-free yolk was extracted with an equal volume of extraction solution using the procedure described above. Aliquots of the centrifuged yolk extract were assayed for enzyme activity.

Since the method of Marquis and Fritz gave inconclusive results for the extraction of carnitine acetyltransferase from brain, distilled water extracts were prepared from this tissue and were found to contain transferase activity. The glass-distilled water extracts were prepared in the same manner as the phosphate buffer-deoxycholic acid-EDTA extracts.

Carnitine Acetyltransferase Assay System

Assays for carnitine acetyltransferase activity were carried out by measuring initial rates of the coupled assay reaction described previously for the measurement of acetylcarnitine. The reaction mixture was essentially the same as was employed in those assays except that 30 μmoles of DL-acetylcarnitine were added as substrate, and the reaction was started by the addition of an aliquot of tissue extract, rather than by the addition of the purified enzyme. The increase in absorbance at 340 mμ was followed with a Beckman DU spectrophotometer equipped with a Gilford optical density converter and a Leeds and Northrup recorder. The components of the reaction mixture were preincubated for ten minutes at 35° before addition of the tissue extract and the temperature was maintained at 35° throughout the rate determinations. Results are reported both on the basis of protein present in the tissue extract and on the basis of dry weight of tissue remaining after the enzyme extraction was completed. Protein was determined spectrophotometrically by the method of Layne (43) and the residual tissue precipitates were dryed to constant weight at 65° C.

EXPERIMENTAL AND RESULTS

Inhibition of Carnitine Acetyltransferase by Perchloric Acid Extracts of Embryonic Chick Tissues

General Characteristics of the Inhibitory Phenomenon

Using the method of Marquis and Fritz (46) for the determination of standard amounts of free carnitine, a standard curve was obtained and is shown in Figure 2. A linear relationship exists between carnitine concentration and the optical density readings. In their studies on rat tissues, these authors report that a linear relationship also exists between aliquot size from a given tissue extract and absorbancy change. That this was not the case with extracts from embryonic chick organs is shown in Figure 3. The pattern of decreasing absorbancy change with increasing aliquot size was typical under the conditions used in these experiments. Since the total extract volume used in each set of assays was always the same, one possible explanation for the observed results is the presence of a compound in the tissue extracts that interferes with the analytical procedure used. This is suggested by the fact that increasing the sample size would result in increasing the concentration of any inhibiting substance.

To further explore this possibility, another experiment was performed. A standard amount of free carnitine was added to the

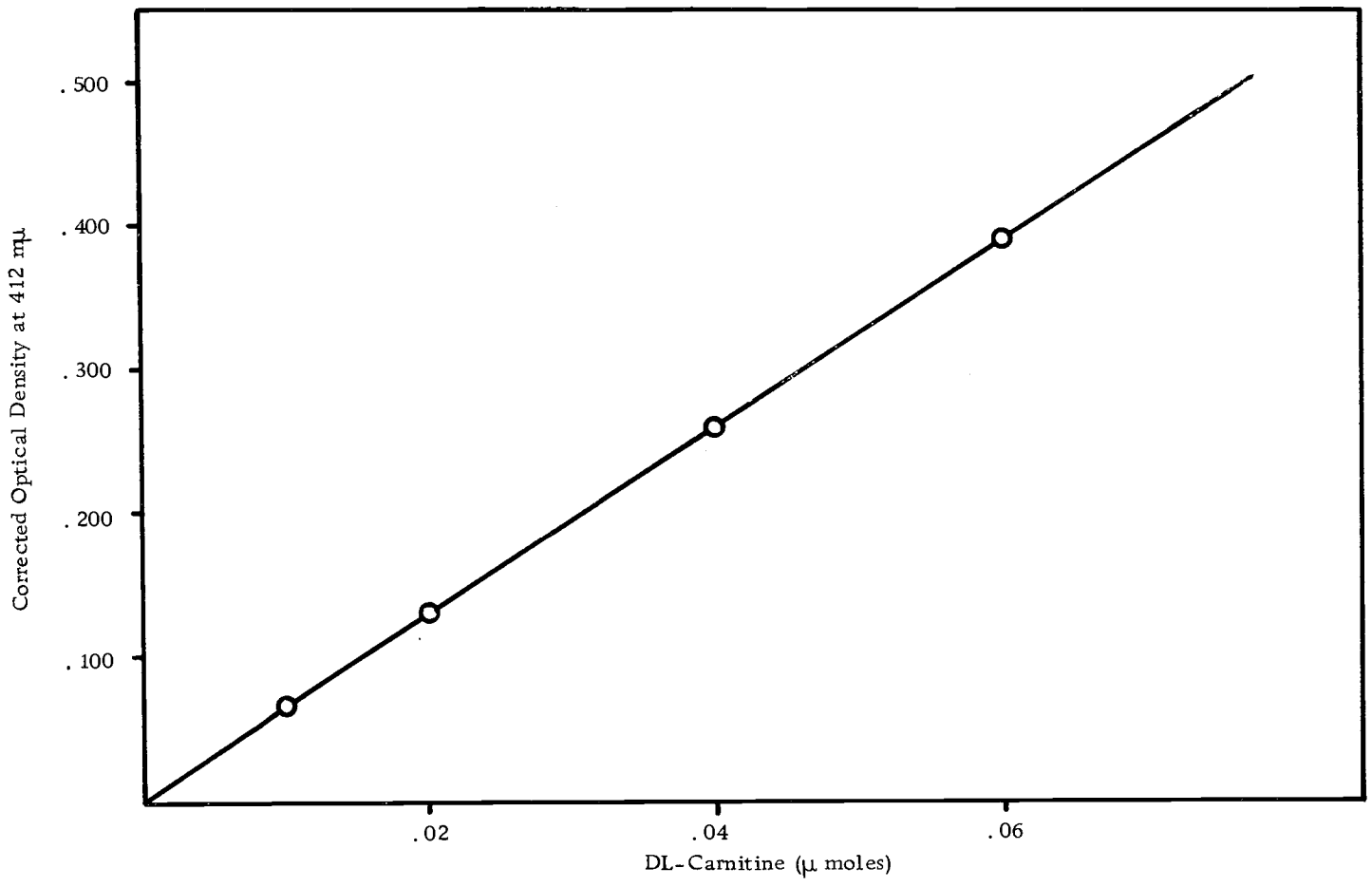


Figure 2. Standard curve for the determination of carnitine.

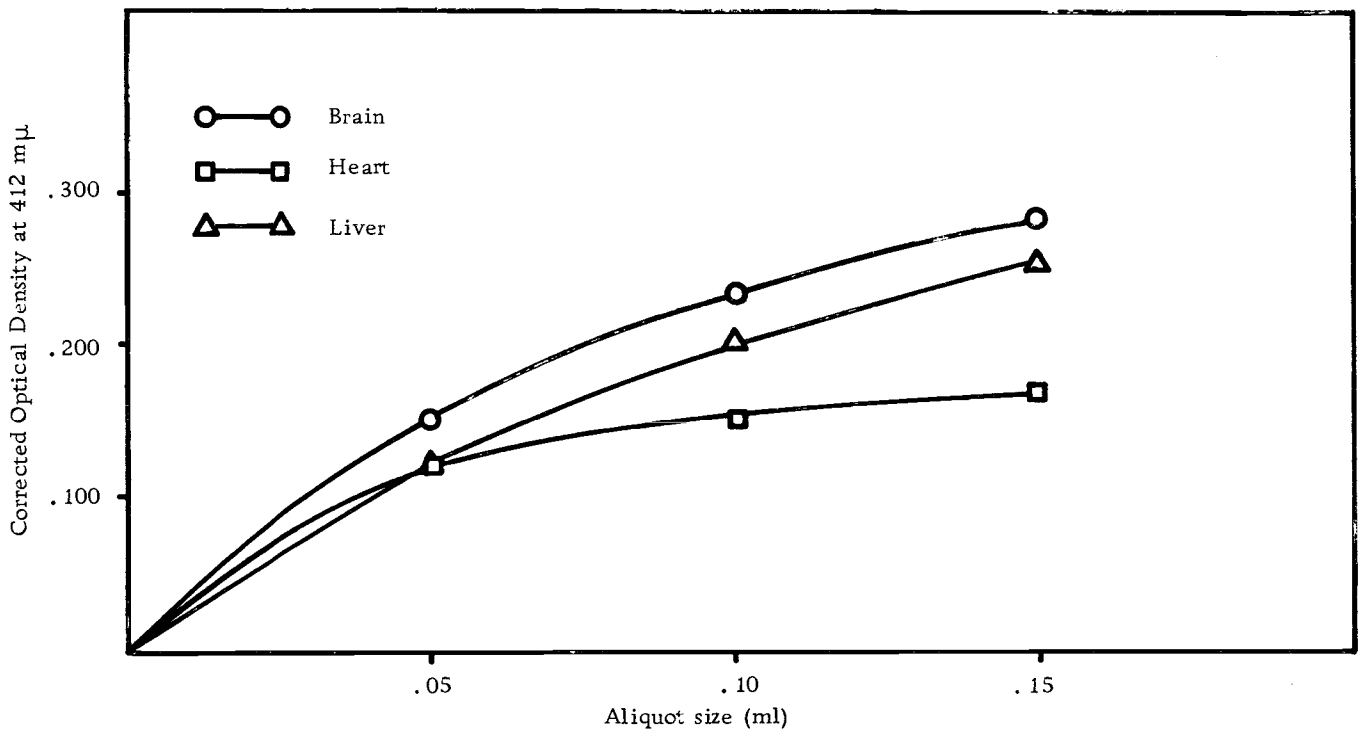


Figure 3. Inhibition of carnitine assay by perchloric acid tissue extracts. Pattern of inhibition observed in carnitine assays when different size aliquots are assayed. The total volumes of concentrated 13 day extracts were: Heart, 1.0 ml; Brain, 1.4 ml; and Liver, 1.1 ml.

tissue during homogenization. An aliquot of the resulting extract was then assayed for carnitine. The remaining extract was concentrated *in vacuo* from ten ml to one ml and an aliquot was assayed for carnitine. The remaining concentrated extract was then diluted with glass-distilled water to ten ml and assayed. The results are shown in Table 1, and were obtained from assays performed on 0.1 ml aliquots of the respective solutions.

Table 1. Effect of Concentration Upon the Analysis for Free Carnitine in Embryonic Chick Tissue Extracts

		Carnitine found (μmoles)		
Source of extract	Carnitine added (μmoles)	Original extract	Concentrated extract	Rediluted extract
Heart	0.60	1.21	0.89	1.13
Brain	0.60	1.17	0.53	1.0
Liver	0.50	0.76	0.56	0.70

Although assays of the original extracts were performed near the lower limit of detection for the assay method, the results clearly show decreased amounts of carnitine in the concentrated extracts. In fact, the amount of carnitine observed in the concentrated brain extract was not enough to account for the amount of carnitine added to the sample.

Hydrolysis Experiments

The experiments described thus far were concerned only with the measurement of free carnitine in dilute perchloric acid extracts. In addition to free carnitine, perchloric acid extracts of other tissues have been shown to contain short-chain fatty acid esters of carnitine, such as acetylcarnitine, which can be readily hydrolyzed by treatment with dilute base (61, 62). Pearson and Tubbs (62) have shown that, in rat hearts, base hydrolysis releases up to 150% more free carnitine than is present in the unhydrolyzed extract. Experiments using this technique on perchloric acid extracts from embryonic chick tissues gave the results shown in Table 2. Tissue extracts were prepared according to the procedure outlined in the methods section, but were not concentrated under vacuum. The extracts were halved and one half was made 0.1 N in KOH and 1 mM in EDTA by the addition of a concentrated stock hydrolysis solution. The hydrolysis was carried out at room temperature for one hour and was terminated by neutralization of the samples with perchloric acid. At this time, all of the samples were concentrated under vacuum to a final volume of approximately one ml. The $KClO_4$ precipitate present in the samples, formed after cooling the samples in an ice bath for 15 minutes, was removed by centrifugation and the volumes of supernatants were adjusted to 1.0 ml. These samples were heated at 90° C for five minutes,

cooled and then aliquots were assayed for free carnitine.

Table 2. Effect of Base Hydrolysis on the Determination of Free Carnitine in Perchloric Acid Extracts of 10 day Embryonic Chick Tissues

Source of extract	μmoles of carnitine found	
	Before hydrolysis	After hydrolysis
Heart	0.400	0.206
Brain	0.576	0.374
Liver	0.965	0.338

The results shown in Table 2 are typical of many experiments performed on extracts from embryonic tissues ranging in developmental age from 10-21 days of incubation. Again, the individual assays for free carnitine in each extract showed the characteristic decrease in change in absorbancy when increasing aliquot sizes were assayed. In no case was there an increase in carnitine due to hydrolysis, under the conditions employed.

An experiment was performed in order to be sure that acetylcarnitine was not hydrolyzed by the heat treatment performed on the samples before assay. A standard amount of acetylcarnitine (0.3 μmole per ml) was heated at 90° C for five minutes. Free carnitine assays performed on 0.1 ml aliquots of this solution, after cooling, did not show any carnitine released by the heat treatment.

Effect of General Hydrolysis Procedure on the Determination of Free Carnitine

In order to determine whether carnitine was destroyed or altered under the conditions employed in the hydrolysis procedure, solutions containing known amounts of carnitine were exposed to the various conditions encountered in the procedure. These conditions included base treatment, concentration under vacuum, and heating to 90° C for five minutes. The results obtained after assaying the various solutions for carnitine are summarized in Table 3.

The results shown in Table 3 represent average results from duplicate analyses. Assays were also performed on aliquots of different sizes and no significant differences were observed. These results suggest that the hydrolysis conditions employed do not effect the assay for carnitine in the absence of embryonic tissue. In both the cases showing less than 100% recovery, evaporation was carried out to dryness and the residue was redissolved in water. The small loss observed might be explained by failure to redissolve all of the residue from the evaporation flask. Generally, in concentrating solutions, samples were not evaporated to dryness.

Carnitine Recovery from Perchloric Acid Extracts

The possibility that perchloric acid treatment, perhaps by the presence of perchlorate ion remaining in the extracts after

Table 3. Effect of Hydrolysis Conditions on the Determination of Carnitine

Conditions	μmoles carnitine added	μmoles carnitine recovered
Ten ml aqueous solution[a]	10.0	10.0
Ten ml aqueous solution, concentrated[b]	2.0	2.2
Ten ml aqueous solution, concentrated, heated[c]	2.0	2.0
Ten ml 0.1 N KOH solution, concentrated, heated[d]	2.0	1.8
Ten ml 0.1 N KOH, 5 mM EDTA solution, concentrated[e]	2.0	1.8
Ten ml 0.1 N KOH, 5 mM EDTA solution, concentrated, heated[f]	2.0	2.1

[a]Carnitine was dissolved in ten ml of glass distilled water and aliquots were assayed.

[b]Sample was prepared as in (a) and was then concentrated to dryness. The residue was dissolved in one ml and aliquots were assayed.

[c]The sample was prepared as in (b) but the concentrated extract was heated at 90° C for five minutes before assay.

[d]Carnitine was dissolved in 0.1 N KOH and was allowed to stand at room temperature for one hour. After neutralization with perchloric acid, the sample was concentrated to one ml and assayed.

[e]Sample was prepared as in (d) except that the base contained 5 mM EDTA and the sample was not heated.

[f]The sample was prepared as in (e) except that it was heated at 90° C for five minutes before assay.

neutralization, interferes with the assay for the carnitine was also investigated. Standard amounts of carnitine were added to ten ml of perchloric acid and the extraction procedure used for tissue samples was carried out on this "mock sample." Free carnitine assays were performed on aliquots of the extract concentrated to one ml. The results of this experiment are shown in Table 4.

Table 4. Analysis of Carnitine Standards Subjected to Perchloric Acid Extraction Conditions

Aliquot size ml	Carnitine added μmoles	Carnitine recovered μmoles	Recovery %
0.05	1.20	1.04	86
0.10	1.20	1.15	96

These results show a 96% recovery in the most concentrated assay. This seems to eliminate the possibility that substances formed from carnitine by perchloric acid treatment, or that residual perchlorate ions in the extract, are possible inhibitors of the assay procedure.

Test of Method on Rat Tissues

Since the methods employed in this study were first used to measure carnitine and derivatives in rat tissues, in other laboratories (46, 47, 62), experiments on rats were performed in order to compare our results with those reported in the literature. Two adult

rats were killed by a sharp blow on the head and the hearts were removed immediately and placed in ice-cold 0.1 N Tris buffer (pH 7.5). Unconcentrated perchloric acid extracts were prepared from these tissues as described previously and each extract was divided in two. Enough of a KOH-EDTA solution was added to half of the extracts to make them 0.1 N in KOH and 1 mM in EDTA. Hydrolysis was carried out for one hour, as described previously, and all the extracts were concentrated to a final volume of one to two ml. Aliquots of these concentrated extracts were then assayed for carnitine and the results are shown in Table 5.

Table 5. Tissue Levels of Perchloric Acid-Soluble Carnitines in Adult Rats

Rat	Carnitine present before hydrolysis μmole/g dry wt	Carnitine present after hydrolysis μmole/g dry wt	Carnitine released by hydrolysis μmole/g dry wt
1	2.27	4.60	1.33
2	3.37	3.87	0.50

The carnitine concentrations shown in Table 5 are similar to the values reported by Marquis and Fritz (47) and to those reported by Pearson and Tubbs (62). Although the relationship between aliquot size from a given tissue extract and absorbancy change was not investigated, an increase in free carnitine was obtained by dilute base hydrolysis. The large discrepancy between the amounts of

carnitine released by hydrolysis in the two different samples can be explained by the fact that the rat hearts in our study were not frozen immediately upon removal from the animal. Pearson and Tubbs (61) report increases in acetylcarnitine levels in rat heart that is not frozen immediately. Even though the tissues were not frozen before perchloric acid extraction, it is true that sample (1) remained in buffer approximately fifteen minutes longer than sample (2) before extraction.

Reaction Site of the Inhibitory Phenomenon

As described in the methods, the essential reactions in the coupled assay system used to determine carnitine in these studies are as follows:

$$\text{(1)}\quad \text{Acetyl-CoA} + \text{carnitine} \underset{\text{acetyltransferase}}{\overset{\text{carnitine}}{\rightleftharpoons}} \text{acetylcarnitine} + \text{CoASH}$$

$$\text{(2)}\quad \text{CoASH} + \text{DTNB} \rightleftharpoons \underset{\text{(chromogenic)}}{\text{thiophenolate ion}} + \text{product}$$

Experiments described previously indicated that carnitine-containing perchloric acid extracts of embryonic chick tissues interfere, in some way, with this assay system. Experiments were performed in order to determine the effect of tissue extracts upon each of the reactions shown above.

Reaction (2) involves the combination of DTNB with sulhydryl groups to form the thiophenolate ion which absorbs at 412 mμ.

Experiments were performed in order to determine whether perchloric acid tissue extracts interfered with the formation of the chromogenic species in the Ellman reaction. Known amounts of free CoASH were added to tubes containing Ellman reagent under the usual reaction conditions (100 μmoles Tris-HCl buffer, pH 7.5 at 35°; 0.1 μmole DTNB). Amounts of tissue extract known to interfere with the carnitine assay system were added to duplicate samples containing Ellman's reagent and standard amounts of CoASH, and the absorbance at 412 mμ was measured in all the samples. The results of this experiment are shown in Table 6.

Table 6. Effect of Chick Embryo Tissue Extracts Upon Color Development in the Ellman Reaction

ml CoASH standard added	ml of tissue extract added	Optical density at 412 mμ (corrected for blank)
0	0	0
0.01	0	0.130
0.02	0	0.260
0.03	0	0.395
0	0.10	0
0.01	0.10	0.135
0.02	0.10	0.260
0.03	0.10	0.410

Since the absorbance values obtained after the subtraction of "tissue blanks," are the same as the absorbance values obtained in the absence

of tissue extract, one must conclude that the formation of the thiophenolate ion is not inhibited by tissue extracts.

Effect of Tissue Extracts on the Rate of the Enzymatic Reaction

Since tissue extracts did not interfere directly with the formation of the thiophenolate ion described by reaction (2), the effect of tissue extract on reaction (1) was investigated. This was accomplished by noting the effect of added extracts on the rate of the enzymatic reaction. Measured amounts of tissue extract were added to cuvettes containing the reaction mixture, and the rate of increase in absorption at 412 mμ, after the addition of carnitine acetyltransferase, was measured on a recording spectrophotometer. Cuvettes contained, in a final volume of one ml, the following components: tissue extract; none, 0.05, 0.10, or 0.15 ml; acetyl-CoA, 0.06 μmole; carnitine, 0.40 μmole; tris-HCl, pH 7.5 at 35° C, 100 μmoles; EDTA, 5 μmoles; DTNB, 0.1 μmole; carnitine acetyltransferase, 0.1 unit. The reactions were started by the addition of the enzyme and the temperature was maintained at 35° C. Since the enzyme is inactivated slowly by Ellman's reagent (33), linear rates were obtained only during the first 30 seconds to one minute after the addition of the enzyme. Therefore, the rates used to obtain the results summarized in Table 7 were those measured in this time interval. This table shows a comparison of the rates obtained in the

presence and absence of tissue extracts. The rate obtained in the absence of added tissue extract was arbitrarily assigned a value of 100% and the rate obtained in the presence of added extract is expressed relative to this control.

Table 7. Effect of Added Perchloric Acid Tissue Extracts on the Rate of the Enzymatic Reaction

Source of extract	Volume of original extract	Relative rates obtained in the presence of different volumes of tissue extract: ml of extract added to the assay medium		
		0.05	0.10	0.15
Rat Heart 1	2.0	100	114	138
Rat Heart 2	2.0	134	130	
15 day Brain	1.5	100	82	62
15 day Brain after hydrolysis	1.5	82	76	58
15 day Heart	1.5	91	86	63
15 day Heart after hydrolysis	1.5	79	70	67
15 day Liver	1.5	100	86	69
15 day Liver after hydrolysis	2.0	88	87	80

The values reported in Table 7 were obtained with perchloric acid extracts prepared as described previously. Hydrolyzed and unhydrolyzed samples, for each tissue, were derived from the identical tissue sample by splitting the original dilute perchloric

acid extract before concentrating it *in vacuo*. Total extract volumes are included in the table because, as shown before, the amount of interferance by tissue extracts is concentration dependent.

The results of this experiment indicate that an inhibitor is present in the perchloric acid embryonic chick tissue extracts that inhibits the rate of the enzymatic reaction used to measure carnitine. Relative rates greater than 100%, obtained upon the addition of rat tissue extract, may result from endogenous carnitine present in the original extract.

Effect of Extract Dialysis Upon Inhibitory Properties of the Extracts

In order to characterize the inhibitor, with regard to size, some of the extracts were dialyzed and then tested for inhibitory properties after dialysis. Some of the extracts which showed maximal inhibition of the enzymatic reaction (Table 7) were dialyzed slowly in a dialysis cell of 0.75 ml volume. The cell was constructed in a way that allowed the dialysate to flow from a large reservoir continuously over the dialysis membrane. Each extract was dialyzed for two hours against two or three liters of glass-distilled water. The membrane used in the dialysis cell was a piece of dialysis tubing of pore size small enough to retain RNAse molecules. Aliquots of the dialyzed extract were added to cuvettes containing the standard

reaction mixture and rates were again determined as described earlier. Table 8 gives the relative rates obtained upon the addition of various volumes of extract both before and after dialysis.

Table 8. Effect of Dialysis Upon the Capacity of Tissue Extracts to Inhibit the Enzymatic Reaction used to Measure Carnitine

	Relative rates obtained in the presence of added volumes of tissue extracts		
	ml of extract added to assay medium		
Source of extract	0.05	0.10	0.15
hydrolyzed 15 d Brain (before dialysis)	85	50	50
hydrolyzed 15 d Brain (after dialysis)	100	103	100
15 day Liver (before dialysis)	91	84	83
15 day Liver (after dialysis)	100	96	95

The results shown in Table 8 indicate that the inhibitor present in the extracts is small enough to be removed by dialysis under the conditions employed here.

Pronase Treatment of Extracts

In an effort to further characterize the nature of the inhibiting substance present in perchloric acid tissue extracts, the effect of pronase treatment on the inhibitory properties of the extracts was

investigated. Nomoto suggests that treatment with this proteolytic enzyme, under specified conditions, will digest almost any protein virtually to free amino acids (53). One set of duplicate extracts, obtained by dividing the original dilute perchloric acid extract in half, was treated with 0.1 mg of purified pronase (approximately 4 E.U.) for 24 hours at 37° C. Following this incubation, all the samples were heated at 90° C for five minutes in order to inactivate the pronase. After concentrating the samples under vacuum, aliquots were assayed for carnitine in the usual manner. Table 9 shows the results of this experiment.

Table 9. Effect of Pronase Treatment upon Inhibitory Properties of Perchloric Acid Tissue Extracts

		Optical density reading at 412 mμ					
		Expected[a]		Found			
Source of extract	Aliquot size (ml)	not treated	pronase treated	not treated	% relative inhibition	pronase treated	% relative inhibition
Heart	0.05	0.070	0.080	0.070	0	0.080	0
	0.10	0.140	0.160	0.125	10	0.125	22
	0.15	0.210	0.240	0.170	19	0.160	33
Brain	0.05	0.060	0.055	0.060	0	0.055	0
	0.10	0.120	0.110	0.110	10	0.105	10
	0.15	0.180	0.165	0.145	20	0.135	18
Liver	0.05	0.220	0.205	0.220	0	0.205	0
	0.10	0.440	0.410	0.370	20	0.340	28
	0.15	0.660	0.615	0.450	32	0.410	33

[a]On the basis that no inhibition occurs with 0.05 ml of the extract

The results shown in Table 9 suggest that pronase treatment of tissue extracts, under the conditions employed here, does not relieve the

inhibition of the enzymatic assay system observed with these extracts. This evidence, together with data obtained in the dialysis experiments, suggest that the inhibitor present in the extracts is not a large molecular weight compound or a dialyzable peptide.

Summary of Properties of the Inhibitor

In summary, studies on the nature of the inhibitor have yielded the following facts about its properties:

(1) The inhibitor appears to be of low molecular weight. Dialysis experiments showed that the capacity of the extract to inhibit the assay for carnitine could be removed by dialysis against distilled water. The apparent low molecular weight of the inhibitor was further suggested by passing the extracts over a Bio-Rad P-2 column, which excludes globular molecules larger than approximately 2500 in molecular weight. Assay of the distilled water elute from the column showed that the inhibitor was present in approximately the same fraction as carnitine, which has a molecular weight of 198.

(2) It is heat stable. Concentrated tissue extracts were routinely heated to 90° C for five minutes before aliquots were removed to be assayed for carnitine. This procedure had no effect on the inhibition observed in the results of the assay. Assays of aliquots of the concentrated, unheated samples displayed the same inhibitory phenomenon; and, in addition, displayed higher "tissue blanks" than

were observed in the case of the heated extracts.

(3) Apparently, the inhibitor is not a protein. The dialysis experiments and heat treatments discussed above suggest that it is not a large macromolecule. In addition, pronase treatment of the extracts, under conditions known to degrade many proteins to free amino acids (53), had no effect on the ability of the extract to inhibit the enzymatic assay.

(4) The inhibitor is insoluble in chloroform-methanol (3:2 v/v), but soluble in aqueous solutions. When aliquots of chloroform-methanol tissue extracts were assayed, the typical "perchloric acid extract inhibition" was not observed. Unfortunately, the organic extraction procedure was not efficient in extracting carnitine added to the tissues before extraction. Details of these experiments are given in the following section. In addition to the results obtained with dilute perchloric acid extracts, preliminary experiments showed that the same type of inhibition occurred when aliquots of distilled water, 5% TCA, and chick Ringer's extracts were assayed.

Another aspect of the inhibitory nature of the perchloric acid extracts which warrants further mention is the apparent enhancement of the inhibitory phenomenon by dilute-base treatment. Attempts to measure short-chain acylcarnitine concentrations by determining carnitine released by base hydrolysis were never successful. The early suspicion that short-chain acylcarnitines were not present in

the extracts was unfounded since subsequent studies using a direct assay for acetylcarnitine showed the presence of the carnitine ester in extracts that gave negative results by the hydrolysis procedure. Furthermore, studies on the effect of added extract on the rate of the enzymatic reaction (Table 7) showed that inhibition of the reaction was greater with hydrolyzed extracts than with extracts not previously exposed to base.

One consequence of this apparent enhancement of the inhibition by base treatment is that the presence of short-chain acylcarnitines other than acetylcarnitine was not substantiated. Bohmer and Bremer (8), who used a radiometric method of analysis, have recently reported the presence of propionylcarnitine in rat liver and kidney. Pearson and Tubbs (61) report in their study of carnitine and its derivatives in rat tissues that in many cases, the total perchloric acid-soluble carnitine appeared to exceed by an appreciable margin the sum of free carnitine and acetylcarnitine. They report that the original form of the extra carnitine is unknown.

Selection of Experimental Conditions and Extraction Procedures

Extraction of Embryonic Chick Tissues Using Organic Solvents

The data presented so far suggest that a substance present in perchloric acid extracts interferes with the assay used for determining

carnitine in our studies. Since other investigators have described extraction procedures using non-aqueous solvents (7, 9, 48), one of these procedures was tested with embryonic chick tissues in an attempt to find a method more suitable for our purposes than the perchloric acid extraction procedure. The method of Bohmer, Norum and Bremer (9), used to study the distribution of carnitine and its derivatives in animal tissues, was modified for use with chick embryonic tissue. These authors were interested in the relative distribution of various carnitine compounds in rats under different nutritional states and a radiometric assay involving labeled carnitine was suitable for their purpose. Modification of their procedure was required in order to place the extracted carnitines in an environment suitable for the enzymatic assay used in this study.

Approximately two to three grams of tissues from 12 day embryos were dissected and immediately frozen in ten ml of chloroform-methanol (3:2 v/v) that was precooled in an acetone-dry ice bath. The frozen tissues were homogenized directly in the cold solvent in motor-driven, ground glass homogenizers. The residue was removed by filtration and was washed twice with five ml volumes of the extraction solvent. The washes and original extract were pooled and were evaporated to dryness under vacuum. The residue remaining after evaporation was suspended in eight ml of water and the long-chain acylcarnitines were extracted by shaking the aqueous suspension

vigorously with four ml of n-butanol. The two layers were separated by centrifuging the mixture for ten minutes at 5,000 x g and then carefully pipetting the upper layer into a separate test tube. The aqueous phase, supposedly containing free carnitine and acetylcarnitine, was divided and one half of the extract was treated with base, as previously described, in order to hydrolyze acetylcarnitine present in the extract. Both the base-treated and untreated extracts were concentrated to a final volume of one ml, under vacuum, and aliquots were assayed for free carnitine. The results of this experiment are shown in Table 10.

Table 10. Carnitine Levels in Chloroform-Methanol Extracts of 12 day Embryonic Chick Tissues

Source of extract	Aliquot size (ml)	Carnitine present before hydrolysis (μmoles/g dry wt)	Carnitine present after hydrolysis (μmoles/g dry wt)
Heart	0.05	1.19	0.90
	0.10	1.19	0.90
Brain	0.05	1.85	0.97
	0.10	2.11	1.04

Even though the results shown in Table 9 indicate, in the case of heart and brain, the absence of the type of inhibition found with the perchloric acid extracts, total amounts of free carnitine were lower than those found using perchloric acid extraction. The apparent loss of carnitine in the base-treated samples is puzzling since, as in the case of the samples not treated with base, an increase in aliquot size

did not result in a decrease in the total amount of carnitine found.

Recovery of Added Carnitine Using the Chloroform-Methanol Extraction Procedure

Since the amount of free carnitine obtained by perchloric acid extraction of embryonic tissues was greater than the amount of carnitine found using organic solvent extractions, an experiment was performed to determine the efficiency of the organic solvent extraction procedure. Standard amounts of carnitine were added to duplicate tissue samples and the chloroform-methanol extraction procedure outlined above was performed on the samples. Carnitine analyses were carried out in the usual manner on aliquots of the final concentrated extracts. The results of this experiment should indicate the efficiency of the procedure in accounting for total free carnitine present in the original sample. The results of this experiment are shown in Table 11.

Table 11. Recovery of Added Carnitine Using the Chloroform-Methanol Extraction Procedure

Source of extract	Carnitine found (no additions) (μmoles)	Carnitine found (0.10 μmole carnitine added) (μmoles)	Carnitine expected (μmoles)
Heart	0.100	0.134	0.200
Brain	0.276	0.220	0.376

The results shown above suggest that the chloroform-methanol extraction procedure was not efficient in extracting all the free carnitine present in the embryonic tissue samples. This is not in agreement with the results of Bohmer, Norum, and Bremer (9). In their experiments on rats they injected (Methyl ^{3}H) butyrobetaine which was rapidly converted into carnitine and presumably gave uniform labelling of all the carnitine compounds. They report that after the extraction procedure, which was used in our experiments, there was less than 1% of the total radioactivity remaining in the residue. The possibility exists that all the carnitine present in the chick tissues was extracted but that the resulting extracts contained enough dissolved organic solvents to interfere with the enzymatic method of analysis. In any case, the procedure, as performed by us, could not be used as a method of choice for the analysis of carnitine in embryonic chick tissues.

Recovery of Added Carnitine in Perchloric Acid Extracts Using Small Aliquots in the Assay

Data already presented indicate that dilute reaction conditions (0.05 ml per assay from a 1.5 to 2.0 ml sample) had little, if any, effect on the initial rates of the enzymatic reaction (Table 7). A carnitine recovery experiment was performed using the perchloric acid extraction procedure coupled with the use of small (0.05 ml)

aliquots in the assay procedure. This was done to determine if this was an acceptable method for the analysis of carnitine in these tissues. Standard amounts of carnitine were added to duplicate tissue samples, before extraction with perchloric acid, and the recovery of added carnitine was determined using the dilute reaction conditions in the assay of the concentrated extracts. The results of this experiment are shown in Table 12, and represent the average of duplicate analysis.

Table 12. Recovery of Added Carnitine in Perchloric Acid Extractions Using Dilute Assay Conditions

Source of extract	Carnitine found (no additions) (μmoles)	Carnitine found (0.30 μmole added) (μmoles)	Carnitine expected (μmoles)	Recovery %
Heart	0.448	0.768	0.748	105
Brain	0.669	1.00	0.969	104
Liver	1.155	1.38	1.45	95

The results summarized in Table 12 show that the perchloric acid extraction procedure works well on these tissues if small aliquots of the concentrated extracts are used in the assay for carnitine. These results suggest that the concentration of the inhibitor in the assay solution, under these conditions, is low enough not to interfere with the enzymatic reaction. Since this was true, the use of dilute assay conditions, with respect to extract concentration, was employed for the determination of free carnitine in chick embryos. The detailed

procedure for preparing the extracts, and performing the analyses appear in the methods section.

Selection of the Acetylcarnitine Assay

As described previously, preliminary experiments involving base hydrolysis of neutralized perchloric acid tissue extracts failed to show the release of carnitine due to the hydrolysis of carnitine esters. In fact, the usual pattern observed in these experiments showed less free carnitine present in the extracts after hydrolysis than was present before hydrolysis. One possible explanation for this behavior could be the enhancement, by base treatment, of the inhibitory phenomenon that is observed when these extracts are assayed for free carnitine. Since one of the primary objectives of this study was to detect and quantitate free carnitine to acetylcarnitine ratios as a function of development, a more direct approach for the determination of acetylcarnitine was investigated.

Marquis and Fritz (47) have described a method for the determination of acetylcarnitine which couples the acetyl-CoA formed in the carnitine acetyltransferase reaction with the citrate-condensing enzyme assay system of Ochoa (58). In this system NAD is reduced, on a mole for mole basis, with acetylcarnitine which is used to generate the acetyl-CoA. Aqueous solutions containing known amounts of acetylcarnitine were first assayed with this method. That a linear

relationship exists between NADH formed, measured by the increase in absorbance at 340 mμ, and acetylcarnitine present in the assay solution is shown in Figure 4.

Aliquots of perchloric acid extracts of newly-hatched chick tissue were also assayed using this coupled enzyme system. The same type of inhibitory phenomenon described previously in the assay procedure for free carnitine was again observed in these assays. This inhibition could be overcome by dilution of the original one ml extract to a final volume of two to three ml. The results of this representative experiment performed on the concentrated extracts, diluted as described above, is shown in Table 13.

Table 13. Acetylcarnitine Levels Present in Perchloric Acid Extracts of Newly-Hatched Chick Tissues

Source of extract	μmoles of acetylcarnitine found using various volumes of extract in the assay procedure		
	0.05 ml aliquot	0.10 ml aliquot	0.15 ml aliquot
Heart	0.70	0.75	0.64
Brain	0.40	0.45	0.25
Liver	1.50	1.50	0.75

The results shown in Table 13 point out that the values obtained in the acetylcarnitine determinations, carried out as described above, are linear with aliquot size up to 0.10 ml of extract per assay. Therefore, the aliquot sizes of tissue extract assayed for acetylcarnitine in

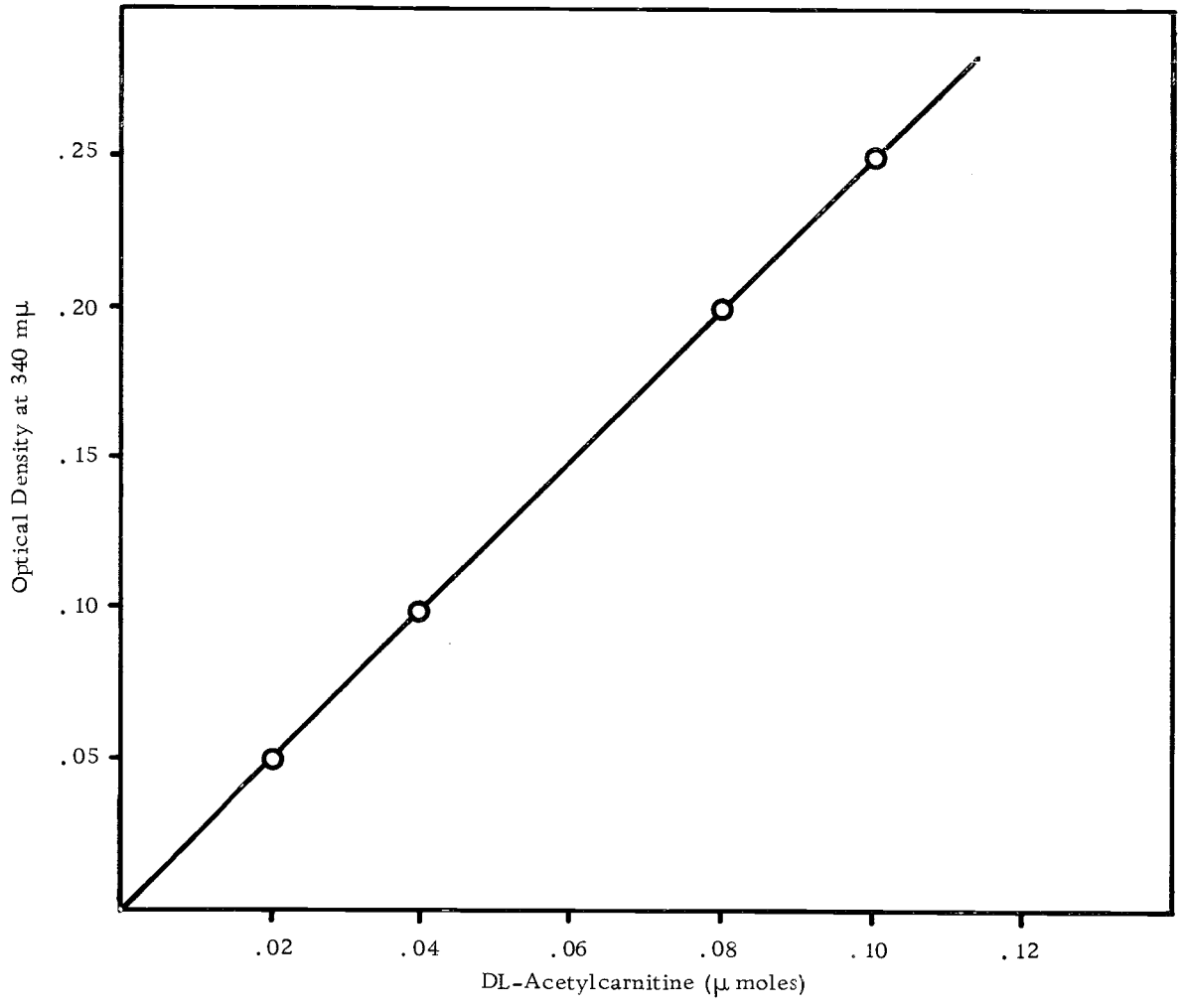


Figure 4. Standard curve for the determination of acetylcarnitine.

these studies were 0.10 ml, or less, per assay and the tissue extracts were prepared using approximately the same amount of tissue per sample. The concentrated extracts were always adjusted to a total volume of three ml.

Recovery of Acetylcarnitine Under Perchloric Acid Extraction Conditions

The possibility of acid hydrolysis of acetylcarnitine in dilute perchloric acid solutions has been pointed out by some investigators (48). In order to explore this possibility, a measured amount of acetylcarnitine was added to perchloric acid homogenizing media and was carried through the procedure routinely used for acetylcarnitine analysis in tissue samples. Table 14 shows the results obtained after assay of aliquots of the extract.

Table 14. Recovery of Acetylcarnitine Under Perchloric Acid Extraction Conditions

Acetylcarnitine added	Acetylcarnitine found using 0.10 ml aliquot	Acetylcarnitine found using 0.20 ml aliquot
1.0 μmole	1.14 μmole	1.09 μmole

The results shown in Table 14 indicate, that in this experiment, there was no hydrolysis of acetylcarnitine under the extraction conditions employed, and that the perchloric acid extract of standard acetylcarnitine did not interfere with the assay used in these determinations.

Long-Chain Acylcarnitine Determinations

The determination of long-chain acylcarnitine by the method outlined in the procedure is also complicated by the presence of an inhibitor in the concentrated extracts obtained from chick tissues. Aliquots of the concentrated, perchloric acid-insoluble, base hydrolysates exhibited the same inhibitory properties already described, when assayed for free carnitine. Unfortunately, the use of relatively dilute assay conditions, with respect to extract concentration, is not ideal for these determinations. The amount of free carnitine released by the hydrolysis technique, which represents the amount of long-chain acylcarnitines present in the tissue, is small compared to the amount of free carnitine present in the tissue. Assay conditions comparable to those used in the determination of free carnitine are too dilute, with respect to carnitine released by hydrolysis, to measure this carnitine accurately. The results reported in this study were obtained by measuring carnitine in 0.10 ml aliquots of the hydrolyzed extracts concentrated to a final volume of two ml. Rate experiments, similar to those reported previously, were performed on assay solutions containing this amount of tissue extract. The results obtained in these experiments showed from zero to fifteen percent relative inhibition with some extracts. In any case, even though the determinations were sometimes performed under conditions known to interfere with the

assay system, the results obtained are in approximate agreement with results obtained by other investigators with other animal tissues.

Embryonic Tissue Levels of Free Carnitine, Acetylcarnitine, and Long-Chain Acylcarnitine

Free Carnitine Levels in the Developing Chick Embryo

The results obtained in the determination of free carnitine are summarized in Table 15. These results show that the level of free carnitine in the heart remains constant over the period of development studied. In the brain, the free carnitine level remains relatively constant from 10 to 17 days of incubation. However, by the time of hatching, at 21 day incubation, the level has dropped to about 60% of the level present at 17 days (from 5.20 to 2.98 μmoles/g dry wt). Free carnitine in the liver shows a gradual decrease with development, from 5.84 μmoles/g dry wt on the tenth day to 3.87 on the day of hatch. The standard deviation in these determinations was relatively constant (approximately 15% of the average value) and ranged from 10% to 18% of the average value.

Acetylcarnitine Levels in the Developing Chick Embryo

Table 16 shows the results obtained in the determination of acetylcarnitine levels during the development of embryonic chicks from ten days of incubation to the day of hatch. The most interesting

Table 15. Tissue Levels of Free Carnitine Present in Developing Chick Embryos

Tissue	Incubation time (days)	Number of experiments	Free carnitine (average value) (μmoles/g dry wt)	Standard deviation
Heart	10	5	4.78	0.88
	14	6	4.05	0.66
	17	5	4.74	0.75
	21	7	4.12	0.51
Brain	10	5	4.18	0.85
	14	6	5.16	0.78
	17	5	5.20	0.86
	21	4	2.98	0.42
Liver	10	6	5.84	0.55
	14	5	4.55	0.65
	17	6	4.52	0.77
	21	5	3.87	0.51

Table 16. Tissue Levels of Acetylcarnitine Present in Developing Chick Embryos

Tissue	Incubation time (days)	Number of experiments	Acetylcarnitine (average value) (μ mole/g dry wt)	Standard deviation	Range of determinations (μ mole/g dry wt)
Heart	10	4	0	0	
	14	4	0	0	
	17	4	1.23	0.54	0.88 to 3.54
	21	4	2.93	1.03	0.68 to 4.06
Brain	10	3	0	0	
	14	3	0	0	
	17	3	1.72	1.61	0.53 to 3.56
	21	4	1.66	0.79	0.43 to 4.40
Liver	10	3	0	0	
	14	3	0	0	
	17	6	1.79	0.61	0.78 to 2.45
	21	4	1.73	0.63	0.65 to 2.45

observation from these results was that acetylcarnitine was not detected in any tissue prior to the seventeenth day of incubation. A factor which may be reflected in the relatively large standard deviations observed in these analyses, when compared to the standard deviations observed in the free carnitine determinations, is that the assay for carnitine is about three times as sensitive as the acetylcarnitine assay. In addition, acetylcarnitine determinations are complicated by the fact that the concentration of this compound changes rapidly as a function of elapsed time between excision of the tissue and freezing (61).

Long-Chain Acylcarnitine Levels Present in the Developing Chick Embryo

The results obtained for the determination of long-chain acylcarnitine are shown in Table 17. Brain and liver acylcarnitine levels during the period of development studied are quite similar and show a gradual decrease from the level found on the tenth day of incubation to the level found on the day of hatching. In the heart, there is a relatively high level of long-chain acylcarnitine on the tenth day (2.30 μmoles/g dry wt). The pattern shows a sharp decrease in this level by the fourteenth day (0.60 μmole/g dry wt) and remains approximately constant after this time until hatching. The standard deviations were, in general, more than those found in the determination of free

carnitine. This is not surprising in light of the fact that the absolute amounts of acylcarnitines are only about 10 to 20% of the amounts of free carnitine in the tissues. In addition, as was mentioned before, some of acylcarnitine assays were performed under assay conditions that may have been influenced by the presence of an inhibitor.

Table 17. Tissue Levels of Long-Chain Acylcarnitine Present in Developing Chick Embryos

Tissue	Incubation time (days)	Number of experiments	Acylcarnitine (average value) (μmole/g dry wt)	Standard deviation
Heart	10	5	2.30	0.58
	14	7	0.60	0.11
	17	4	0.76	0.03
	21	5	0.34	0.09
Brain	10	5	0.95	0.09
	14	6	0.65	0.09
	17	6	0.73	0.14
	21	6	0.30	0.11
Liver	10	6	0.70	0.07
	14	7	0.66	0.13
	17	6	0.36	0.10
	21	6	0.20	0.03

Patterns Observed in the Analysis of Carnitine Compounds as a Function of Developmental Age

Figure 5 shows the results of the free carnitine, acetylcarnitine, and long-chain acylcarnitine determinations plotted against time of development, for heart tissue. The pattern observed suggests that carnitine esterified with long-chain fatty acids at early ages, may be

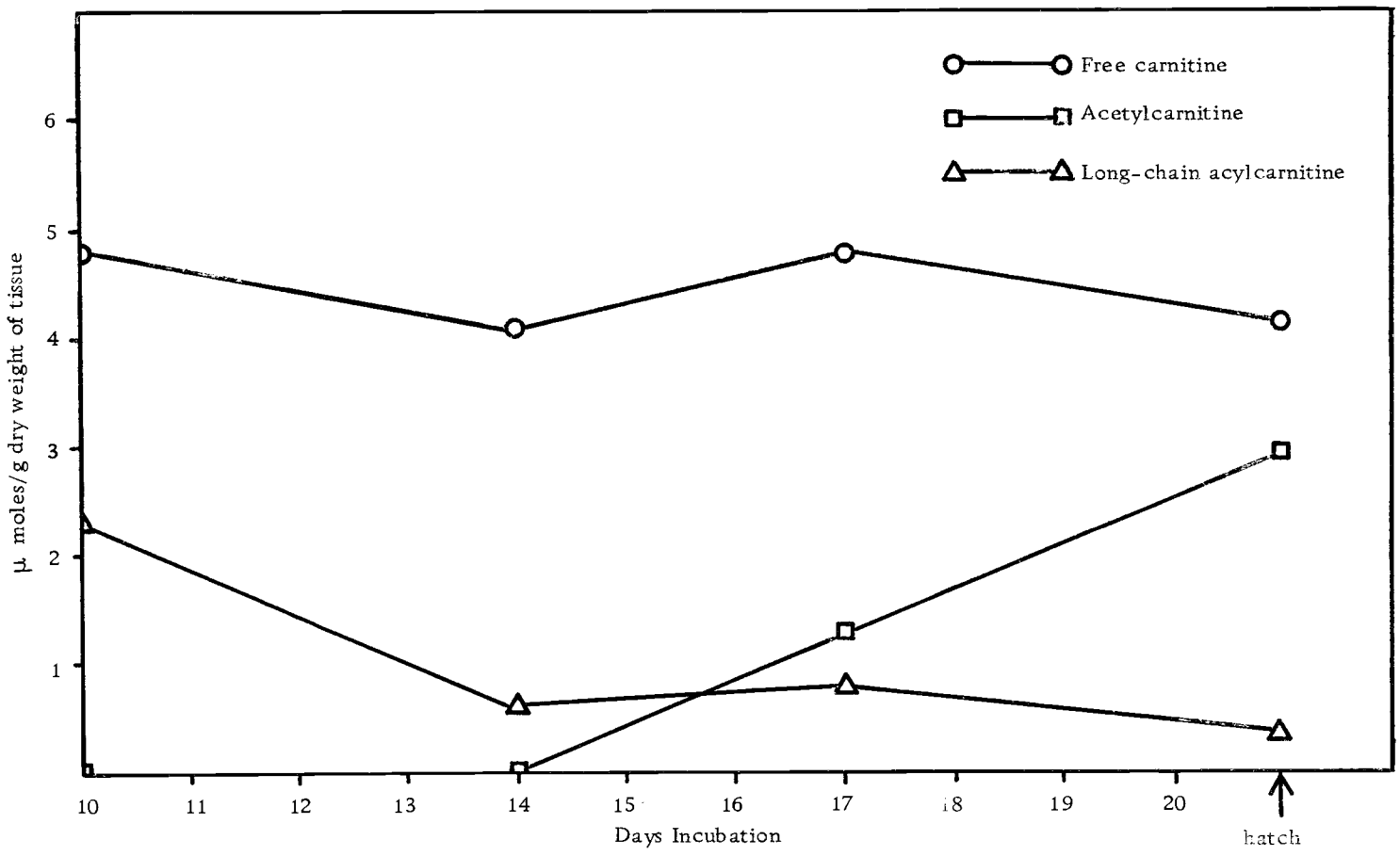


Figure 5. Carnitine patterns in heart during development.

exchanged to form increasing amounts of acetylcarnitine as the embryo develops. The patterns obtained in the same manner as described above for heart, are shown in Figures 6 and 7 for brain and liver respectively. The patterns observed here are similar for both tissues in that the drop in the free carnitine level observed at the hatching day may be approximately accounted for by the increase in the acetylcarnitine level observed at this time.

Total Carnitine Levels in the Developing Chick Embryo

The data shown in Table 18 represent the total amounts of carnitine present in tissues of various ages obtained by adding the values reported for free carnitine, acetylcarnitine and long-chain acylcarnitine.

Table 18. Tissue Levels of Total Carnitine Present in Developing Chick Embryos

Incubation time (days)	Total carnitine (μmoles/g dry wt of tissue) present in: Heart	Brain	Liver
10	7.08 ± 1.46	5.13 ± 0.92	6.54 ± 0.62
14	4.53 ± 0.77	5.81 ± 0.87	5.10 ± 0.78
17	6.73 ± 1.32	7.65 ± 2.51	6.67 ± 1.48
21	7.39 ± 1.63	4.94 ± 1.13	5.80 ± 1.17

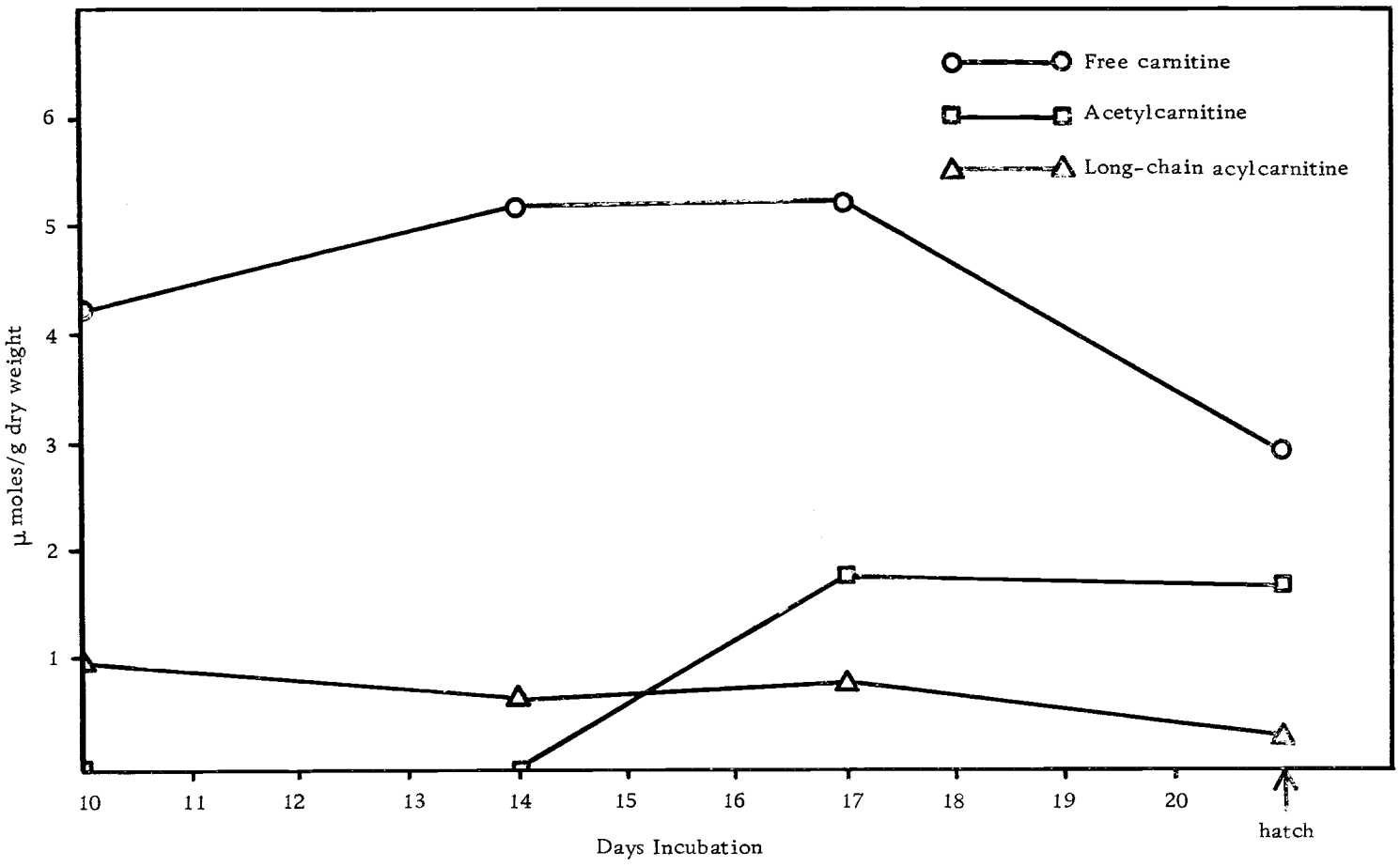


Figure 6. Carnitine patterns in brain during development.

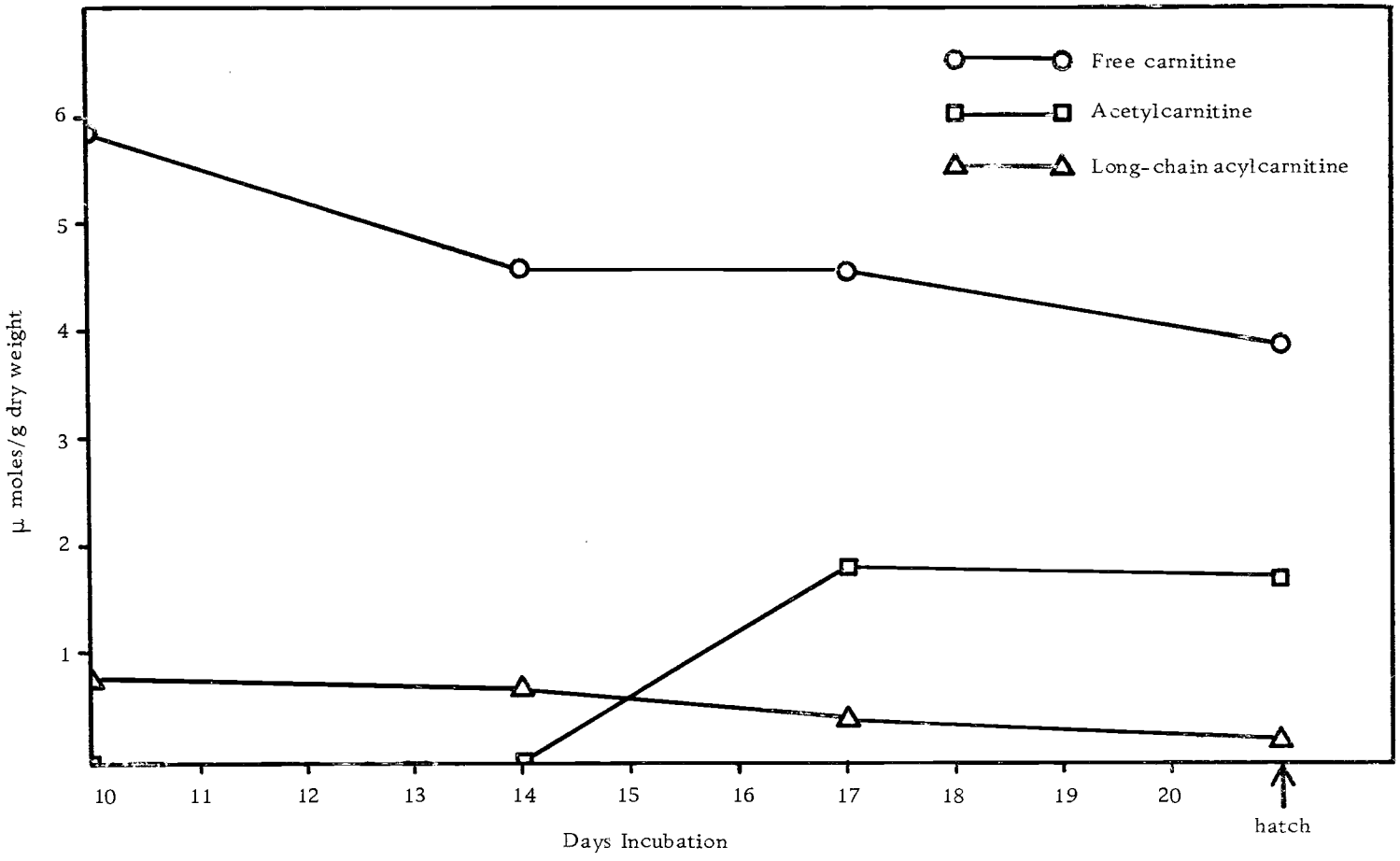


Figure 7. Carnitine patterns in liver during development.

The results shown above reinforce the observations made on the data shown in Figures 5, 6, and 7. That is, there appears to be only small significant differences in the total carnitine present in any given tissue at any of the developmental ages studied. Another interesting observation is that the total amount of carnitine is approximately the same in all the tissues studied.

Carnitine Acetyltransferase in the Developing Chick

Levels of Carnitine Acetyltransferase Activity During Development

The results already presented, suggest that acetylcarnitine does not appear in embryonic tissues until some time after the fourteenth day of development. For this reason, the levels of carnitine acetyltransferase, the enzyme that mediates the acetylation of carnitine, was measured in hearts, brains, livers, and yolk sacs during development. The transferase activity was measured spectrophotometrically in a coupled enzyme system in which one mole of NADH is produced for each mole of acetyl-CoA generated from the substrate, acetylcarnitine. The rate of NADH formation was followed by measuring the change in absorbance at 340 mμ. Table 19 gives the results of these experiments expressed on the basis of two different parameters. The (A) portion of this table gives the activities (ΔOD_{340}/min) on the basis of protein present in the soluble extract. Table 19 B gives the

activities (enzyme units/min) based on dry weights of tissue remaining after the soluble extract has been removed.

Table 19. Carnitine Acetyltransferase Levels in Developing Chick Tissues[a]

Tissue	Developmental age: Days incubation before hatching 3	7	10	14	17	Hatch day	Days after hatch 4
A. Transferase levels as ΔOD_{340}/min/mg protein							
Heart			0.021	0.033	0.020	0.118	0.088
Brain			0.010	0.019	0	0.033	0.048
Liver			0.052	0.047	0.060	0.143	0.033
Yolk sac	0.005	0.153	0.073	0.053	0		
B. Transferase levels in units/g dry weight of tissue							
Heart			4.20	2.53	4.12	15.7	12.3
Brain			0.72	2.91	0	1.97	1.87
Liver			7.26	5.30	9.24	17.7	3.34
Yolk sac	3.05	34.9	48.5	30.4	0		

[a]Blank spaces in the table indicate that no determinations were made on these tissues

Patterns Observed in the Levels of Carnitine Acetyltransferase Activity During Development

The data obtained in these studies is presented graphically in Figures 8 and 9. Figure 8 shows the enzyme activities for each tissue, expressed on the basis of extract protein, plotted against

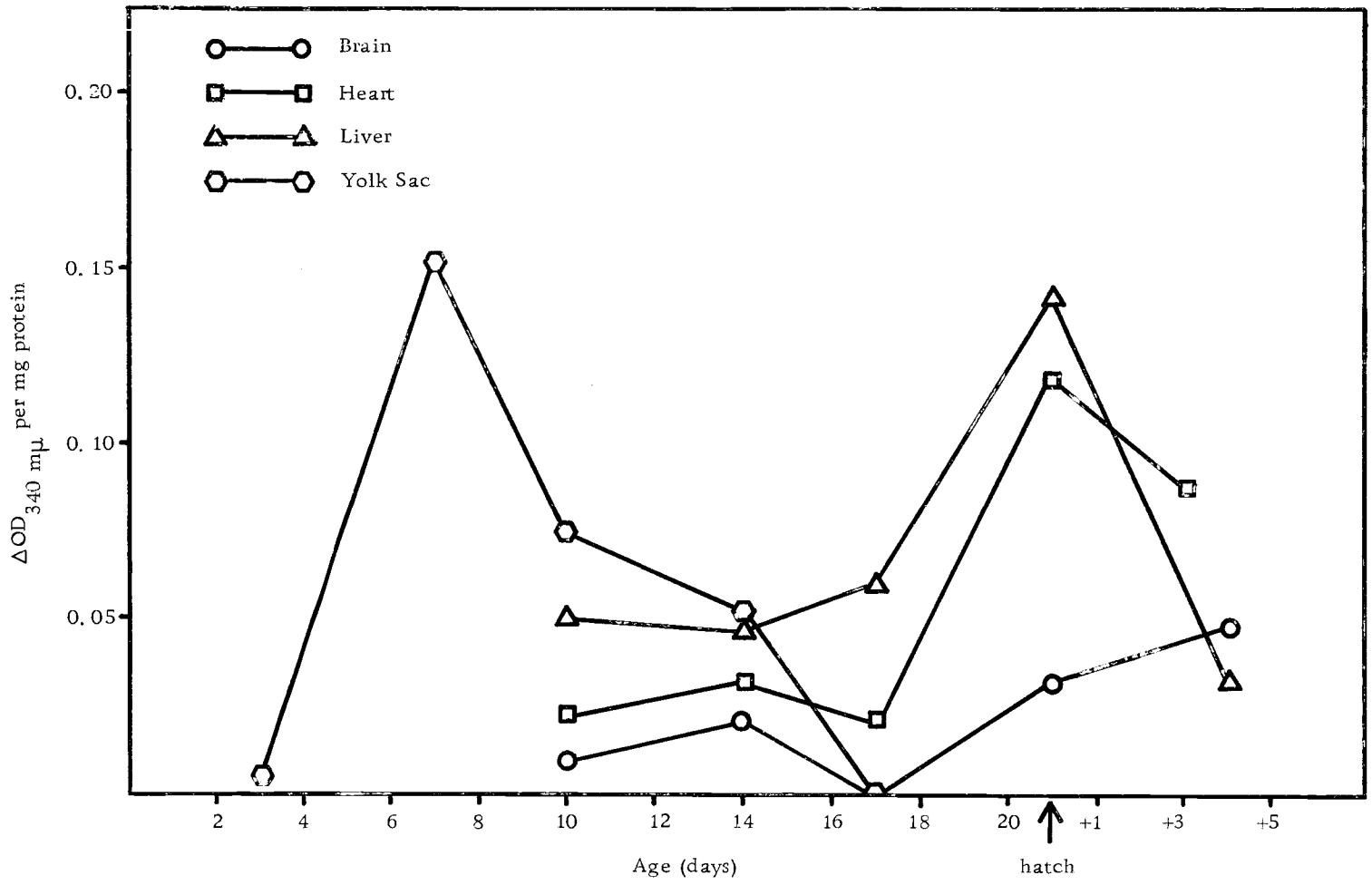


Figure 8. Levels of carnitine acetyltransferase activity in chick tissues during development. Activity is expressed relative to protein present in enzyme extract.

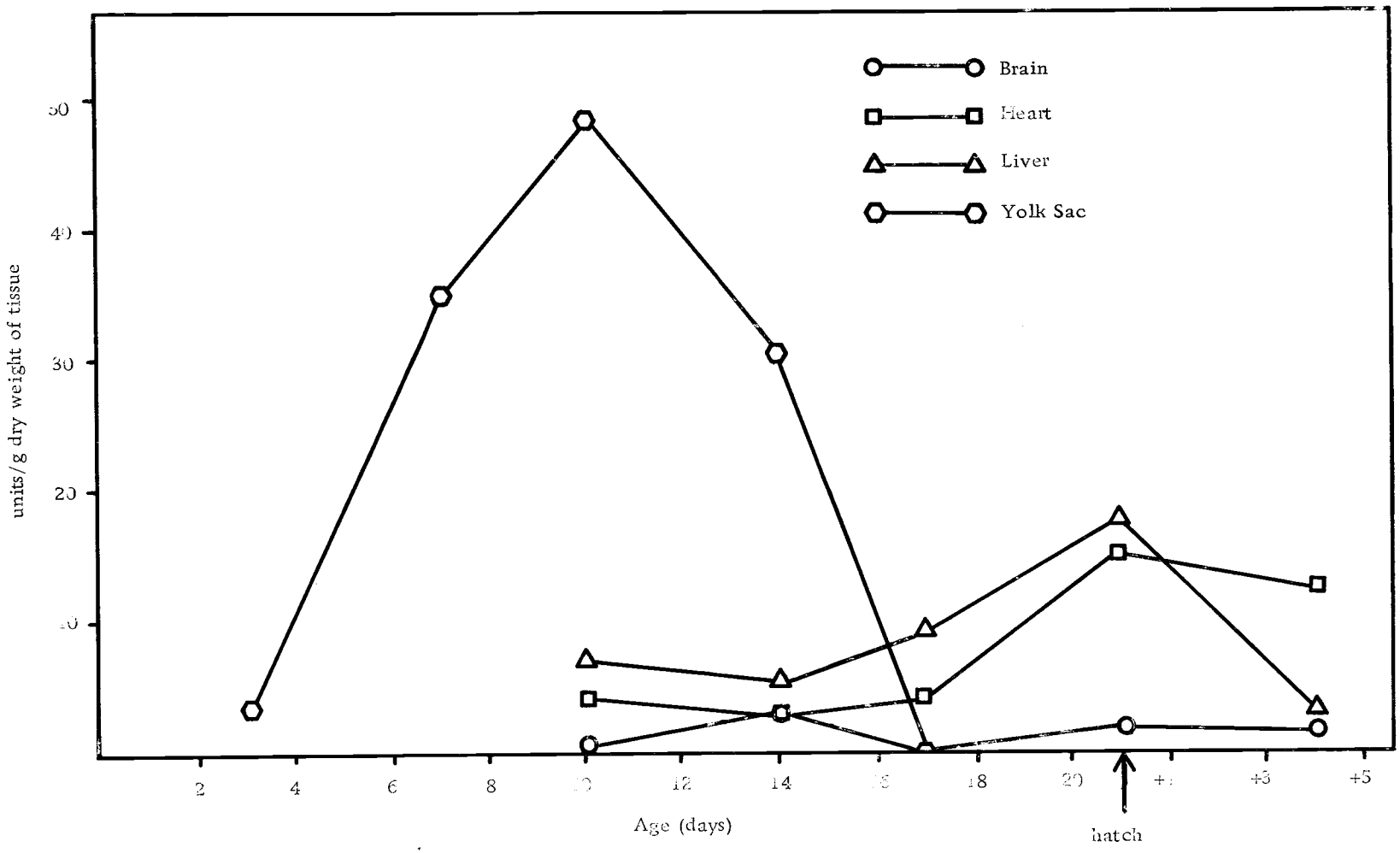


Figure 9. Levels of carnitine acetyltransferase activity in chick tissues during development. Activity is expressed relative to dry weight of tissue remaining after extraction of the enzyme.

developmental age. Figure 9 is analogous to Figure 8 except that total extractable enzyme activities are expressed on the basis of residual dry weights of tissues. The patterns shown on both graphs are similar except for the activities of the enzyme derived from the yolk sac. The explanation for this apparent discrepancy observed in the data can be found in the procedure used to prepare the enzyme extracts. As was mentioned in the methods section, early experiments were performed on yolk sacs which still contained the yolk. The results reported on the basis of protein present in these extracts reflect the presence of large amounts of protein extracted from the yolk. The yolk was later shown to contain no enzyme activity. In addition, the results reported on the basis of dry weights of residue account for the total amount of extracted enzyme. The total amount of enzyme extracted from the yolk sac was substantial when one considers the large amount of extraction solution required to homogenize the total yolk sac (about 40 ml, which compares to 10 ml used to extract the other tissues).

The patterns of enzyme activities as a function of development, show that in the case of the heart and the liver, carnitine acetyltransferase levels show a peak that begins at the fourteenth day of incubation and reaches a maximum on the day of hatch. In these organs there is a slight drop in the level of the enzyme at four days after hatch. These results are consistent with the pattern observed

in the levels of acetylcarnitine present in the embryonic tissues. As was shown previously, the appearance of substantial quantities of acetylcarnitine (more than 20% of the total carnitine) was observed after 17 days of incubation.

The levels of carnitine acetyltransferase observed in the brain during development do not conform to the pattern observed in the other tissues. As is shown in Figure 9, the transferase activity is present only at a constant, low level during development. These results may be due to the difficulties encountered in solubilizing the enzyme from brain tissue. Extraction of brain tissue with phosphate-deoxycholic acid buffer, as performed with other tissues, usually failed to show transferase activity in the extract. Extraction with distilled water was performed instead, and the levels of transferase activity reported in Table 19 were obtained using this procedure. A 5 mM solution of EDTA (pH 7.5) was tried and did not extract more enzyme activity than did the distilled water.

Carnitine Acetyltransferase in the Yolk Sac Membrane

The enzyme activity present in the yolk-containing, yolk sacs was shown to be localized in the yolk sac-membranes. The yolk was separated from the sac-membrane by the technique described in the methods. Both the yolk and the yolk sac-membrane were extracted with phosphate-deoxycholic acid extraction buffer and aliquots of these

extracts were assayed for carnitine acetyltransferase activity. The results of these experiments showed that all the detectable activity remained in the yolk sac-membrane. In one experiment that employed a yolk-containing, yolk sac-extract as a control, the total amount of activity present in the yolk sac-membrane accounted for only 50% of the total activity present in the control. However, there was no detectable activity present in the yolk removed from the yolk-sac membrane. Whether this result is due to variations in the amount of enzyme present in the two yolk sacs or is due to activation of the enzyme by yolk is not known at this time.

One attempt was made to measure carnitine compounds in 13 day yolk sacs using the perchloric acid extraction technique. The results of this experiment showed 110 and 115 μg free carnitine and 130 and 115 μg long-chain acylcarnitine for two different yolk sacs. Even though these results are in close agreement with the results reported by Mehlman and Wolf (50), the perchloric acid extracts of the yolk sacs showed even higher levels of inhibition than was present in the extracts of other embryonic tissues. As usual, the attempt to show acetylcarnitine present in the perchloric acid-soluble fraction of the extract by hydrolysis showed less free carnitine in the hydrolyzed samples than was present in the same samples before hydrolysis. Acetylcarnitine determinations by the coupled enzyme assay procedure showed barely detectable quantities of acetylcarnitine.

DISCUSSION

Inhibition of Carnitine Acetyltransferase by Perchloric Acid Extracts of Embryonic Chick Tissues

As mentioned previously, Marquis and Fritz (46) report that linearity exists between aliquot size and absorbancy change when using this same procedure for the determination of carnitine on adult rat tissue extracts. The results reported here (Table 7) substantiate their report since no inhibition of the enzymatic reaction was observed in the presence of rat tissue extracts. On the other hand, Childress, Sacktor, and Traynor (19) report that perchloric acid extracts of insect flight muscle inhibited carnitine acetyltransferase in their assays for carnitine and acetylcarnitine. They suggest that the inhibition could be overcome by including 5 mM EDTA in the assay media. The reaction media routinely used in our studies contained EDTA at this concentration but this had no effect on the inhibition observed. The possibility that the assay procedure for acetylcarnitine used by Childress *et al.* was not entirely satisfactory is suggested by their report of an approximate 30% loss in the recovery of acetylcarnitine added as an internal standard to frozen thoraces.

The identity of the inhibitor remains unknown and its identification may be worth pursuing in another study. Fritz and Schultz (33) have studied competitive inhibition of the transferase by carnitine

derivatives. The most potent competitive inhibitor found was (+) acetylcarnitine. Other competitive inhibitors included deoxycarnitine, γ-aminobutyrate, choline, acetyl β-methylcholine and carnitine nitrile. Various sulfhydryl reagents, including DTNB, inhibited the enzyme. That DTNB is not responsible for the inhibition observed in this study is proven by: (1) standard assays for carnitine performed in the presence of DTNB, but in the absence of tissue extract, showed no inhibition; and (2) assay for acetylcarnitine in tissue extracts, which does not require DTNB, showed the presence of the inhibitor. In addition to the inhibitors studied by Fritz and Schultz (33), Chase and Tubbs (18) have reported that the products of both the forward and back reactions are inhibitors of carnitine acetyltransferase.

The general properties of the inhibitor, summarized in the experimental and results section, suggest the possibility that this substance may be a "normal" metabolite present in the tissues. Final identification of this substance could be interesting in terms of recent knowledge obtained about the mechanisms of the regulation of enzymatic activity (71). Since carnitine acetyltransferase used in these enzymatic assays for carnitine and acetylcarnitine is presumably also an important enzyme involved in the metabolism of the chick embryo, the inhibitor may have a specific regulatory function in the metabolism of the embryo.

The results obtained from assays in which the extract concentration in the assay mixture is rigidly controlled (Tables 12 and 13) show that carnitine and acetylcarnitine can be accurately determined in the presence of limited amounts of the inhibitor. Data reported in the rate study experiments (Table 7) also support this conclusion.

Embryonic Tissues Levels of Free Carnitine, Acetylcarnitine, and Long-Chain Acylcarnitine During the Development of the Chick

It is very difficult to make meaningful comparisons between tissue levels of carnitine and its derivatives reported in this study and levels reported in similar studies in the literature. In the first place, this is the only detailed study of its kind involving embryonic tissues. Secondly, adult rats were used in the other investigations (7, 9, 46, 47, 61, 62). Finally, the results reported in the literature do not completely agree with each other and are at times contradictory. That the results reported for adult rat tissues is shown in Table 20. Although the results reported by Pearson and Tubbs and those reported by Marquis and Fritz for adult rat hearts and brains are not in close agreement, their results are more precise than those reported using chemical and bioassays (30). The discrepancies between the two rat studies shown in Table 20 may be only apparent since the original values reported by Pearson and Tubbs were expressed on the basis of mμg/g of frozen tissue. Their results

Table 20. Concentrations of Carnitine and its Derivatives in Various Animal Tissues[a]

Source	Tissue	Form of carnitine			Reference for analysis
		Free	Acetyl	Long-chain Acyl	
Rat	Heart	1.57	1.88	0.26	Pearson and Tubbs (61)[b]
Rat	Heart	4.0	1.1		Marquis and Fritz (47)
Rat	Liver	0.87	0.20	0.05	Pearson and Tubbs
Rat	Brain	0.22	<0.05		Pearson and Tubbs
Rat	Brain	0.5	0.2		Marquis and Fritz
2 d Chick Embryo	Heart	2.06	1.47	0.17	This study[c]
	Brain	1.49	0.83	0.15	This study[c]
	Liver	1.93	0.86	0.10	This study[c]

[a] μmoles carnitine/g dry weight, by various enzymatic methods

[b] Original data was presented in mμmoles/gram of frozen tissue. Results recorded were estimated by multiplying original concentration by five on the assumption that tissues were 80% water.

[c] Results recorded are one half the values recorded in Tables 16, 17 and 18 assuming that (-) carnitine is the only active isomer present in tissue extracts.

recorded in Table 20 were estimated by multiplying the original concentrations by five on the assumption that tissues were 80% water. One additional calculation was made to present the results obtained in this thesis on the same basis as other results shown in Table 20. Fritz, Schultz and Srere (34) suggest that (-) carnitine is the only isomer present in animal tissues. The results reported elsewhere (Tables 15, 16, and 17) in this thesis were calculated on the basis of standard curves (Figures 2 and 4) which were prepared from assays of DL mixtures. Therefore, the data from this study shown in Table 20 are one half the concentrations reported elsewhere in the thesis.

The only report in the literature concerning organ levels of carnitine in the developing chick embryo is that of Mehlman and Wolf (50). However, they report only one period of development. In order to make comparisons between their report and results reported in this thesis, two assumptions were made concerning their data. First, though not specifically stated by the authors, other data reported in the reference permits one to assume that their results were obtained using embryos incubated for 15 days. Secondly, since their original data is expressed on a wet weight basis, a dry weight expression was estimated by assuming that the tissues were 80% water. These comparisons are shown in Table 21.

Table 21. Comparison of Free Carnitine Determinations in Embryonic Chick Tissues

Reference	Carnitine in various organs (μmoles/g dry weight)		
	Hearts	Brains	Livers
Mehlman and Wolf	1.67	1.05	1.92
This study[b]	2.02[a]	2.58	2.27

[a]Value reported in reference is for muscle. No mention is made as to source

[b]Corrected to conform to assumption that tissues contain only (-) carnitine

In spite of the assumptions that must be made to make the data reported by Mehlman and Wolf comparable to that reported in this thesis, there is close agreement between values obtained with their chemical assay procedure and results obtained in this study employing an enzymatic assay. Mehlman and Wolf also report that all of the carnitine present in water homogenates of the tissues is removed by dialysis and is therefore "free carnitine." The data reported in their reference does not substantiate this claim since, in some cases, from five to ten percent of the carnitine remains in the dialysis bag after 45 hours of dialysis. They use this criteria, in the case of yolk sac homogenates, to show the presence of 20-45% of the total carnitine as being present as "bound carnitine." As discussed earlier, this "bound carnitine" was later identified as long-chain acylcarnitine (31, 67). This amount of "bound carnitine" present in the homogenates of

liver, brain and muscle is in agreement with the amount of long-chain acylcarnitine present in these tissues as reported in this thesis.

The results reported in this study are unique in one respect when compared to the results of similar studies performed with other animal tissues. In general, the studies (47, 61) performed with adult rats, have shown that the amount of carnitine present varies for different tissues (Table 20 as an example). Usually, the amount of total carnitine and the amounts of various carnitine pools are greatest in the heart. Liver contains an intermediate level of carnitine compounds (roughly, one third to one half of that found in the heart), and brain contains the least amount of carnitine found in these three tissues. Results presented in this thesis indicate that approximately the same amount of total carnitine is present in all the tissues studied. We are not able to say, at the present time, whether this is characteristic of embryonic tissues. The relative amounts of carnitine, acetylcarnitine, and long-chain acylcarnitine in each tissue, in embryos older than 17 days, compares favorably with the relative amounts of these compounds reported by others in studies on adult tissues (7, 9, 61).

The alterations in the acylation state of carnitine observed in these studies is not unique. The studies of Pearson and Tubbs (61, 60), and Bohmer (7, 9) have shown that alterations in the acylation state of carnitine accompany metabolic changes. The changes

observed in the acylation state of carnitine in these studies, presumably, reflect metabolic changes that occur in the embryo during development.

In general, Bohmer (7, 9) and Pearson and Tubbs (60) have reported an increase in the acetylcarnitine:free carnitine ratio during fasting, a nutritional state known to increase the rate of fatty acid oxidation. Similar results were observed when determinations were made on tissues from animals fed high-fat diets and on individual organs previously perfused with high levels of palmitate (61). On the other hand, these same authors do not completely agree with this generalization within their own publications. Pearson and Tubbs report an increase in acetylcarnitine in fasted rat hearts in one publication (60) but do not observe this in another (61). A similar situation is noted in the publications of Bohmer (7, 9). One possible reason for this apparent discrepancy is the fact that the measurement of carnitine and acetylcarnitine is complicated by changes in the concentrations of these compounds as a function of delay between excision of tissue samples and freezing (61). For this reason, tissue samples in the present study were excised and frozen in liquid nitrogen as fast as possible.

A comparison of the relative amounts of carnitine and its derivatives in rat tissues in various nutritional states and in embryonic livers during development is shown in Table 22.

Table 22. The Relative Amounts[a] of Free Carnitine, Acetylcarnitine, and Long-Chain Acylcarnitine in Organs of Animals in Different Nutritional States

Organ	Group	Form of carnitine			Reference
		Free	Acetyl	Long-chain acyl	
14 d liver		89	0	11	This study
17 d liver		67	28	5	
21 d liver		67	27	4	
Rat liver	Normal	73	25	2.2	Bohmer (7)
	Fasted	45	42	13	
Rat adipose tissue	Normal	81	17	2.1	
	Fasted	45	46	8.6	

[a]All values are given as percent of the total carnitine found in the organ

As shown in Table 22, correlations between the two studies do exist, but one must remember that since the measurements are made on two systems that differ markedly in terms of maturity and phylogeny such correlations must be made with some reservation. The data presented in Table 22 show increases in acetylcarnitine levels in rat tissues during periods of increased rates of fatty acid oxidation. This same pattern is apparent in the case of developing chick tissues if one considers the great increase in fat metabolism that occurs during the last week of embryonic development (52, p. 1163-1173). Another similarity noted in the carnitine-acetylcarnitine systems from the two different sources is that which exists in the levels of carnitine

acetyltransferase activity. Results obtained in this study show that the increase in acetylcarnitine concentration in embryonic livers can be correlated with a parallel increase in the activity of carnitine acetyltransferase. Norum (54) has noted a similar increase in the hepatic activity of this enzyme in fasted rats.

One observation that is consistent among reports in the literature is that the concentration of long-chain acylcarnitine increases in tissues of animals exposed to conditions which increase rates of fatty acid oxidation. The data presented in Table 22, in the case of embryonic chick liver, is not clear in this regard. The relative amounts of long-chain acylcarnitine shown in this table, and generally present in other embryonic tissues, are higher than those reported by Bohmer in the case of "normal" rat tissues. On the other hand, they are not as high as concentrations reported by him for tissues from animals exhibiting increased rates of fatty acid oxidation. However, one must remember that the long-chain acylcarnitine levels reported in this thesis are probably lower than the "true concentrations." For reasons previously discussed, determination of this carnitine derivative was sometimes performed under conditions known to inhibit the assay procedure.

Levels of Carnitine Acetyltransferase Activity in the Tissues of Developing Chick Embryos

The maximum levels of carnitine acetyltransferase activity reported in this study are in approximate agreement with those reported by Beenakkers and Klingenberg (2) for various animal tissues and, in general, are about ten times lower than those reported by Marquis and Fritz (47) for rat tissues. The generalization observed by other investigators (47), that activity levels of this enzyme tend to be highest in organs that are most responsive to stimulation of fatty acid oxidation by carnitine, may also be true in the case of the chick embryo. An almost exclusive dependence on lipids for oxidative purposes exhibited by the chick embryo (52, p. 997), coupled with the relatively high tissue levels of carnitine found in this study, support this generalization. Furthermore, the extremely low level of transferase activity in embryonic chick brain also seems to be in agreement with this generalization, and is also in agreement with the results reported by Marquis and Fritz for rat brain (47). The need for a detailed study of the fatty acid oxidase system in the developing chick embryo is apparent. It would be very interesting to know to what degree these tissues are dependent on carnitine for fatty acid oxidation.

A parallel relation between the developmental pattern of carnitine acetyltransferase activity and the developmental pattern of

acetylcarnitine in embryonic hearts and livers is shown in Figures 5, 7, and 9. The studies on embryonic brain, however, did not show this relationship. In this organ, levels of transferase activity remained at a constant low level. Whether this is a real difference in enzyme activity *in vivo* or is due to problems occurring in the enzyme extraction procedure is unknown. It is interesting to note that the appearance of acetylcarnitine in the organs studied and the increase in transferase activity in heart and liver parallel the "awakening of fat metabolism" described by Needham (52, p. 1166). In comparing the growth of fatty substances in the embryo, Needham noted that all the curves showing fatty acid content as a function of development possessed an inflection point about the fourteenth day.

In summary, the data reported in this thesis for intra-embryonic organs generally indicates that the carnitine-acetylcarnitine system is involved in fatty acid oxidation. This observation is in agreement with the majority of opinions reviewed by Fritz (29). The possibility reported by others (10, 12, 13) that the carnitine-acetylcarnitine system functions to translocate acetyl groups from mitochondria to sites of fatty acid synthesis appears remote in the case of the developing chick embryo. Goodridge (36) has reported that the rate of fatty acid synthesis in chick livers is very low during the embryonic period.

The hypothesis presented by Fritz (29) that the short-chain

transferase system functions to transfer acetyl groups within mitochondrial compartments is helpful in explaining the results reported in this study. Fritz postulates that the site of fatty acid oxidation lies in a different compartment from the site of the tricarboxylic acid cycle. The carnitine acetyltransferase system would then serve to translocate "active acetate" units derived from fatty acid oxidation to the site of NADH production. One would expect, on the basis of this hypothesis, to observe a general activation of the carnitine acetylcarnitine system during periods of increased rates of fatty acid oxidation.

The discovery of relatively high levels of carnitine acetyltransferase activity in the yolk sac membrane (Figure 9) may reflect an entirely different function from the mitochondrial function described for this enzyme. The primary purpose of the yolk sac membrane is the absorption of nutrients from the yolk which then pass into the embryo via the vitelline circulation. It is interesting to note that the very large increase in yolk sac transferase activity with development has a peak on the tenth day. This coincides very well with the large peak in the absorption curve for fat which also occurs on the tenth day (52, p. 925). The localization, substrate specificity, and function of this yolk sac membrane transferase is a subject for future studies. Although Fritz et al. (34) suggest that carnitine acetyltransferase is specific for short-chain acyl groups, Beenakkers

and Klingenberg (2) are of the opinion that this enzyme from rat hearts, and also from locust flight muscle, can also transfer acyl groups of medium and long carbon chains. If this is true in the case of the yolk sac enzyme then one may speculate that this enzyme functions in the transfer of acyl groups from the yolk sac into the circulatory system of the embryo.

There are reports which suggest the possibility that carnitine-mediated transport of fatty acids across other cell membrane barriers may occur in a fashion analogous to that proposed in the case of the mitochondria. Wittels and Hochstein (76) have provided evidence that carnitine palmityltransferase is an integral part of the membrane of the mature human erythrocyte. They suggest the possibility that long-chain fatty acylcarnitine might play a role in translocating long-chain fatty acids across cell surface membranes. In another study, Wittels and Blum (75) have demonstrated that Euglena grown with acetate as a sole carbon source can adapt, after an extended lag period, to grow in a medium containing palmitate as a sole carbon source. In addition, carnitine palmityltransferase activity was about three times higher in the cultures grown with palmitate than in cultures grown with acetate. The authors suggest that carnitine palmityltransferase would be a logical site for enzymatic adaptation in the metabolic readjustment of Euglena for growth on palmitate.

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