AN ABSTRACT OF THE THESIS OF

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Title: KINETICS OF SYNTHESIS AND DEGRADATION OF CHICKEN LIVER XANTHINE DEHYDROGENASE

Abstract approved: Robert W. Newburgh

The role of synthesis and degradation in controlling enzyme concentrations was studied.

Concentrations of chloride and arginine were determined for blood and liver at various stages of development from the embryo to the 8 day old chicken. The chloride concentrations were used to obtain the volume of extracellular blood in the liver. Using these values together with the data for total arginine, it was possible to calculate the intracellular and extracellular arginine concentrations in the liver. Extracellular arginine represented 19%, 15% and 26% of the total arginine in the 14 day embryo, 1 day old chick and 8 day old chick respectively.

The kinetics of incorporation of L-(guanido-^{14}C)-arginine and L-valine-^{14}C(u) into the blood amino acid pool and intracellular liver amino acid pool of the chick during various stages of development

were measured. Intravenous injection of isotopic amino acid leads to results similar to a pulse labeling pattern, since more than 80% of the tracer is consumed after 60 minutes. The time-integrated radioactive amino acid pools were determined for each age of chick.

Thirty minutes after injection of radioactive arginine or valine, 40% of the radioactivity remaining in the amino acid pools is found in compounds other than arginine or valine. These results are indicative of probable matabolism of these amino acids into other compounds.

Xanthine dehydrogenase concentrations and the wet weight of liver also were measured during development. The liver wet weight increases most rapidly in the embryo. The enzyme activity is low in the embryo, rises rapidly upon hatching and reaches a high level by the eighth day after hatch.

The rate constants of synthesis and degradation were determined for xanthine dehydrogenase during chick liver development, by purifying the labeled enzyme and measuring the rate of incorporation of label from arginine or valine into the protein. The rate constants for synthesis using L-(guanido-^{14}C)-arginine are 3.7×10^{-12}, 2.8×10^{-12} and 0.8×10^{-12} moles XDH per minute per gram of liver for the 8 day chick, 1 day chick and 14 day embryo respectively. The $t_{\frac{1}{2}}$ values for the 8 day chick, 1 day chick and 14 day embryo are 17, ∞, and ∞ hours respectively. When L-valine-^{14}C(u) is used as substrate, the rate constant for synthesis is 7.0×10^{-12} moles XDH per

minute per gram of liver for the 1 day chick. The rate of synthesis of XDH relative to the rate of synthesis of total soluble protein for the 1 day chick is 1.7 for valine as substrate and 0.9 for arginine. The role of synthesis and degradation in determining enzyme concentrations is discussed. Also included is a critique of the methodology.

Kinetics of Synthesis and Degradation of Chicken Liver Xanthine Dehydrogenase

by

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Chairman of Department of Biochemistry and Biophysics

Dean of Graduate School

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TO CHANA

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Abbreviations

ATP	adenosine triphosphate
AMP	adenosine monophosphate
CoA	coenzyme A
EDTA	ethylene diamine tetraacetic acid
LDH	lactic dehydrogenase
NAD, NADH	the oxidized and reduced forms of nicotinamideadenine dinucleotide
PMS	phenazine methosulphate
POPOP	1, 4 bis(2-(5-phenyloxazolyl))benzene
PPO	2, 5 diphenyloxazole
TCA	trichloroacetic acid
Tris	tris(hydroxymethylamino)methane
XDH	xanthine dehydrogenase
λ	time constant for carbon-14, 2.37×10^{-10}/minute

Definitions

Enzyme unit	amount of enzyme catalyzing the reduction of one μmole of substrate per minute at 25°C

KINETICS OF SYNTHESIS AND DEGRADATION OF CHICKEN LIVER XANTHINE DEHYDROGENASE

INTRODUCTION

Studies on the Controls of Enzyme Levels

One early interest in the area of developmental biochemistry consisted of observing the changes in enzyme levels caused by hormonal and dietary variations. In such studies, it was thought that compounds which act during particular stages of development may be influencing cellular control mechanisms. In particular, investigators were interested in those factors which lead to the appearance and disappearance of cellular components in a manner similar to that which occurs during differentiation.

A variety of effects were observed such as a dietary influence on the concentration of rat liver arginase and ornithine transcarbamylase (87), an accumulation of rat liver ferritin (64) by iron, a regulation of hexokinase activity by glucose, glucose-6-P, and P_i (20), hormonal affects on enzyme levels occurring at birth in rat liver (32), an elevation of the level of tyrosine transaminase by glucocorticoids (61), and a 100 fold increase of threonine dehydratase, when rats are maintained on a protein-free diet for 5 days and then fed a casein hydrolysate (79).

Following the observations that changes occur, attempts were

made to find the mechanisms involved. Kenney (52) showed that glucocorticoids increased the concentration of enzyme by increasing its rate of synthesis. Glucagon and cyclic AMP increase the rate of synthesis of serine dehydratase (50). Estrogen stimulates RNA synthesis in rat uterine cells (16). These studies showed the importance of synthesis in changing levels of enzymes.

From the results of another study (92), another mechanism for the control of enzyme levels was found. Tryptophan pyrrolase activity in vivo can be increased by hydrocortisone, as well as by its own substrate tryptophan. Hydrocortisone was found to increase the rate of synthesis of the enzyme, while tryptophan has no effect on the rate of synthesis. It was found, however, that tryptophan decreased the rate of degradation of the enzyme.

It is apparent that changes in enzyme levels in higher organisms may result from an alteration in the rate of synthesis, or in the rate of degradation, or both. Further studies have subsequently confirmed this concept (23, 89, 90).

Dietary changes affect the rate of degradation of arginase. For example, starvation causes the rate of degradation to become zero (89). On the other hand, starvation increases the rate of acetyl CoA carboxylase degradation and decreases its synthesis. A change from a fat free diet to a 12% fat diet leads to a decreased rate of synthesis of acetyl CoA carboxylase, while the rate of degradation remains

unchanged, indicating that independent factors affect the rates of synthesis and degradation (67). Fritz (23) found that the difference in enzyme levels of the isozyme LDH-5 between rat liver, heart and muscle was due to differences in its rate of degradation in the various tissues. Ganschow (26) found a mutation in mice which leads to a regulation of the concentration of liver catalase by altering its rate of degradation. Herrmann (40) showed that, under conditions of rapid growth in chick embryo explants, there was no degradation of proteins, but, under conditions of minimal growth, there was a substantial degradation of total proteins.

An excellent review on the control of enzyme levels is that of Schimke (90). The review covers the roles of hormones in increasing the rates of synthesis of specific enzymes, the regulation of the rates of synthesis and degradation of proteins by genetic and dietary alterations, and the influence of drugs in stimulating enzyme synthesis.

Xanthine Dehydrogenase as a Model System for Studying Controls at the Level of Synthesis and Degradation

Of interest in the area of developmental biochemistry are the changes in individual protein levels during development. These changing patterns may provide systems for studying the control mechanisms involved during the process of differentiation. A study of the rates of synthesis and degradation of proteins during development

could be used to test the theory that enzyme levels can be controlled by a change in the rate of synthesis or degradation or both. Of particular interest are the controls that determine when the activity of a certain enzyme is to be at a low but constant level, when the level should increase rapidly and when the level should be maintained at a high and constant level. In all instances, we are referring to activity per cellular unit.

Chicken liver xanthine dehydrogenase (XDH), which catalyzes the reduction of NAD^+ by oxidizing the purines xanthine and hypoxanthine to uric acid, exhibits large changes in activity during development. It is at a very low but constant level during the embryonic stages, increases dramatically upon hatching and reaches a high and constant level by the eighth day after hatch (101).

These changes in enzyme levels could be brought about by alterations in the rate of synthesis or degradation or both. Recently, Murison (72) published a paper on the relative rates of synthesis and degradation of chicken liver XDH after hatching. This is a period of time when the level of the enzyme is increasing rapidly. He used 6 hours, 24 hours and 96 hours after hatch to represent times at which XDH is at a low, intermediate and high level respectively. Actually, the use of 6 hours after hatch for the low level was not a proper choice for studying XDH as a time representing a low rate of synthesis. Strittmatter (101) showed previously, that immediately upon hatching,

the level of XDH increases dramatically. Because Murison did not study the embryo, which has a reduced rate of synthesis, he could not prove that the level of XDH increases upon hatching because of an enhanced rate of synthesis.

In this thesis, 14 day old embryos, 1 day old chicks and 8 day old chicks were chosen for determining the absolute rate of synthesis and degradation of XDH during low, intermediate and high levels of enzyme content.

Kinetics of the Rate of Synthesis and Degradation

Synthesis may be represented by the following formula:

$$A \xrightarrow{k_1} P$$

where

A = substrate (amino acid)

P = product (protein)

k_1 = a rate constant for synthesis.

In this equation certain assumptions are necessary and include the following:

(1) Degradation of labeled protein does not occur during the pulse labeling period. In order to obtain the true rate of synthesis, it is necessary that the time of the pulse labeling be short relative to the half life of the enzyme.

(2) The rate constant of synthesis is zero order (90) with respect to the total amino acid pool and is independent of the protein concentration already present.

(3) Only free amino acids from the amino acid pool are incorporated into protein (23).

(4) The concentration of enzyme, protein and amino acids measured in the tissue extracts are a true measure of the intracellular concentration of these substances (23).

(5) The rate of incorporation of tracer into protein is proportional to the specific radioactivity of the intracellular amino acid pool at time t. Since the specific radioactivity of the amino acid pool varies during the pulse labeling period, the total radioactivity incorporated into protein at any time t must be proportional to the time integrated amino acid specific radioactivity pool to time t (43).

(6) A steady state exists during the brief labeling period.

Taking these assumptions into consideration permits a simplified treatment of the kinetic problem. The rate of synthesis may then be expressed as:

$$\frac{dP^*}{dt} = k_1 a_t^*$$

$$P_t^* - P_0^* = k_1 \int_0^t a_t^* dt$$

where

P_t^* = moles of radioactive protein per gram of liver at time t

P_0^* = moles of radioactive protein per gram of liver at time 0

k_1 = rate constant for synthesis

a_t^* = specific radioactivity of the intracellular amino acid pool at time t

t = time.

Degradation may be represented as follows:

$$P \xrightarrow{k_2} A$$

where

A = amino acid

P = protein

k_2 = rate constant for degradation.

The following assumptions are necessary:

(1) The labeled amino acid pool becomes diluted with time and eventually the specific radioactivity of the added radioactive amino acid becomes negligible compared to the initial specific radioactivity (92). For meaningful results, the rate of degradation should be determined when the radioactive amino acid pool becomes negligible.

(2) The rate of degradation of protein is first order and

degradation occurs in a random fashion without regard to the age of the protein (90).

(3) The rate of reutilization of the isotope for synthesis is negligible compared to the rate of degradation.

(4) The exponential decrease in the total radioactivity of a specific protein in a growing system is a measure of its degradative rate (56).

These assumptions permit a simplified treatment of the kinetic problem. Using these assumptions, the rate of degradation is expressed as follows:

$$\frac{dP_t^*}{dt} = -k_2 P_t^*$$

$$\ln P_t^* - \ln P_0^* = -k_2 t$$

where

P_t^* = total radioactivity in specific protein at time t

P_0^* = total radioactivity in specific protein at time 0

k_2 = first order rate constant of degradation

t = time.

Intracellular Amino Acid Pools

One of the assumptions used in the calculation of the rates of synthesis of proteins is that the rate of incorporation of tracer is

proportional to the specific radioactivity of the intracellular amino acid pool. Since all tissues (25) contain extracellular blood and fluids, data for the intracellular pool must be corrected for the extracellular amino acids. This can be done by measuring the chloride space and assuming that the extracellular amino acids have the same specific activity as that found in the blood. The determination of chloride for measuring the extracellular space is based on the assumption that chloride does not permeate the cell wall and that it remains in the extracellular fluids in a concentration equal to that found in the blood plasma (42). The concentration and specific activity of the intracellular amino acid pool is obtained by subtracting the determined extracellular amino acid from the values measured for total tissue amino acids.

The Use of Arginine and Valine for Estimating Turnover of Proteins

The re-utilization of a C-14 amino acid for protein synthesis is one of the major limitations in turnover studies and results in an apparent prolonged half life. As a result, the degree of overestimation for protein turnover is greater for more rapidly degraded proteins (25, 64, 90, 102, 103).

Re-utilization of the guanidine group of arginine was shown by Swick (102) to be at a minimum in rat liver. In fact, 98% of the free

guanidine groups represent a newly synthesized compound in rat liver. The guanidine portion of arginine is converted to urea and excreted. For this reason, arginine is presumably the best amino acid for studying turnover of proteins in mammals. In addition, the guanido group of arginine is not converted to other amino acids. Thus, if L-(guanido-^{14}C)-arginine is used, the isotope will appear only in arginine in mammals (90).

However, uniformly labeled valine, which was used by Murison (72), is presumably not turned over as rapidly as the guanido portion of arginine and would lead to a greater re-utilization of isotope. In addition, valine, being a glycogenic amino acid, is broken down and converted into other amino acids, thus leading to labeling of other amino acids in the pool.

MATERIALS AND METHODS

Fertile Eggs and Chicks

Hy Line 950A fertile eggs were obtained from Jenk's hatchery, Tangent, Oregon. The eggs were incubated at 99°F dry bulb and 86°F wet bulb in a Jamesway incubator, Model 252-B. The eggs were turned automatically every two hours. Chicks were hatched in either the incubator or in a Leahy brooder. The chicks were maintained on a diet of Purina Commercial chick starter (medicated) ZO 6084.

Chemicals

Creatinine, thymine, l-arginine hydrochloride, xanthine and nicotine-adenine dinucleotide were purchased from Calbiochem. Bio-Rex 70(200-400 mesh, sodium form), was obtained from Bio Rad. α-naphthol and 2-methoxyethanol were purchased from Matheson, Coleman, Bell. Trichloroacetic acid and anhydrous ethyl ether were purchased from Mallinckrodt. L-(guanido-^{14}C)-arginine (specific activity 49 mC/mmole) was a product of Schwarz Bioresearch. L-valine-^{14}C(u) (specific activity 248 mC/mmole) was obtained from New England Nuclear. NCS solubilizer was obtained from Amersham Searle. 2, 5 diphenyloxazole and 1, 4 bis(2-(5-phenyloxazolyl)) benzene was purchased from Packard Instrument Company. Toluene,

acetone and disodium ethylenediaminetetraacetate were acquired from J. T. Baker Chemical Company. β-alanine, phenazine methosulphate, nitroblue tetrazolium, sodium heparin and bovine serum albumin were procured from Sigma Chemical Company. Dialysis tubing was a product of Union Carbide and was pre-cleaned by boiling in an EDTA solution before use. All materials for disc gel electrophoresis were purchased from Eastman Kodak Company. Acrylamide and bisacrylamide were re-crystallized according to Loening (63). Ammonium sulphate-ultra pure was purchased from Mann Research Laboratories. All other chemicals were of reagent grade and were obtained from commercial sources.

Operational Assay

Enzymatic assays were performed in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 0.15 mM xanthine, 0.5 mM DPN^+; in a total volume of 3 ml; at 25°C and at 340 mμ. A Beckman spectrophotometer with a Gilford attachment was used for obtaining initial rates.

$$\text{UNITS} = \frac{\frac{\Delta \text{O. D.}_{340\ m\mu}}{\text{minute}} \times 3}{6.2}$$

1 unit = 1 μmole NAD^+ reduced per minute

Chloride Determination

The Van Slyke method (39, p. 627) was used to determine chloride in the liver and blood. Livers from chickens were removed from the animal, blotted to remove adhering liquids, frozen in liquid nitrogen and stored at -20°C for later analysis. One tenth to 2.0 grams of frozen liver were placed in 50 ml erlenmeyer flasks. Five ml of standard $AgNO_3$, 0.0177 N, was added followed by 10 ml of concentrated HNO_3. To this mixture was added a few drops of n-octyl alcohol to prevent foaming. The mixture was then incubated in an 85°C water bath for 4.5 hours. After cooling, approximately 1 gram of ferric ammonium sulphate in 20 ml of water was added. The mixture was cooled to 5°C in an ice-water bath and titrated with standard potassium thiocyanate, 0.019 N. Water was used as a blank. The results were converted to mg Cl^-/gram of liver.

Blood, from hatched chicks, was obtained by decapitation followed by collection in a graduated centrifuge tube to which was added either sodium heparin or sodium citrate as anticoagulants (39). Blood from embryos was obtained by withdrawal from veins using a 30 gauge needle fitted to a disposable syringe. For the determination of chloride, 0.1 to 0.2 ml of blood was placed in a 50 ml erlenmeyer flask and the amount of chloride present determined as above.

Determination of Arginine and Creatinine Pools

Arginine was estimated quantitatively by the Sakaguchi reaction (109). Monosubstituted guanidines react positively in this reaction and certain substances such as glutathione, cysteine, creatine, and creatinine serve as inhibitors of the reaction.

Removal of guanidoacetic acid, a monosubstituted guanidine, was achieved by passing the free amino acids through a Bio-Rex 70 column, which retains arginine. Glutathione, cysteine and creatine are not retained. Creatinine, however, is also absorbed onto the column and is eluted with the arginine. According to Wu (109) 13 μmoles of creatinine causes a 50% reduction in the absorbance obtained from 0.05 μmoles of arginine. An estimate of the creatinine concentration was therefore necessary.

Quantitative determination of creatinine is based on the Jaffe' reaction, which leads to the formation of a red tautomer of creatinine picrate in the presence of picric acid in alkaline solution (3, 39, p. 899-902). The concentration is determined by placing protein free extracts of either blood or liver into a 50 ml erlenmeyer flask, adding 5 ml of freshly prepared 1% alkaline picrate reagent with mixing and allowing to stand at 30°C for 15 minutes. The absorbance is read at 520 mμ.

For the estimation of arginine, liver and blood were

deproteinized with TCA according to the procedures of Wu (109). To the Bio-Rex 70 columns, which were pre-treated with 1 N NaOH and subsequently washed with distilled water until the pH of the washings were about 10, was added the protein free extracts. Fifteen ml of water was used to wash the columns and was followed by 10 ml of 0.5 M sodium acetate, which was sufficient to elute all of the arginine from the column. The flow rate through the column was 1/2 ml per minute. Faster flow rates usually resulted in incomplete retention of arginine on the column. The clear eluant, containing the arginine, was used for the color determination. As much as 0.2 μmole of standard arginine can be quantitatively recovered from a Bio-Rex 70 column containing 2 cc of resin. The acidity and volume of the sample applied are important in determining the retention of arginine on the column. For example, 16 ml of 4% TCA will elute arginine completely, while 1 ml of 4% TCA has no effect.

The spectrophotometric procedure of Wu (109) for determining arginine was used. It is essential that all reagents be kept at 4° C and that, immediately following the addition of each reagent, the mixture is thoroughly mixed. Addition of reagents without immediate and thorough mixing results in a non-linear standard curve. A standard curve for arginine which is linear can be obtained in the region of 0-0.2 μmoles arginine. The absorbance of the reaction mixture decreases with time and should be determined after a 15 minute

incubation period.

Carbon-14 Incorporation into Total Protein

The procedure of Mans (68) was used to follow the incorporation of C-14 amino acids into soluble protein. One tenth ml aliquots from a 100,000 x g supernatant were absorbed onto filter paper discs and the discs washed in succession with 10% TCA and 0.1 M arginine at 4°C for 60 minutes, 5% TCA at 90°C for 30 minutes, ether-ethanol (1:1) at 37°C for 30 minutes and ether at room temperature for 15 minutes. After the final ether step, the discs were air dried and then placed in a 20 ml low potassium glass counting vial and 1 ml of NCS solubilizer was added to each vial and incubated at 37°C for 12 hours to solubilize the protein from the discs. Ten ml of scintillator mixture were then added. The scintillation fluid contained 4 grams of PPO, 0.1 gram of POPOP in 1 liter of toluene. The samples were counted in a Model 3003 Packard Tri-Carb liquid scintillation spectrometer at 12% gain and 50-1000 window. The channels ratio method was used for quench determination. Settings of 12% gain and 200-1000 window were used. The radioactivity observed was a linear function of the amount of radioactive protein added.

Carbon-14 in the Free Amino Acid Pools of the Liver and Blood

The procedure followed was that of Gan (25). To either a liver

homogenate or blood was added an equal volume of cold 20% TCA and the precipitate centrifuged at 600 x g for 10 minutes. The precipitate was washed twice with 10% TCA. The supernatants were combined and passed through a 0.22 μ millipore filter. To the supernatant was added an equal volume of ether and this was shaken thoroughly with a vortex mixer, and then the ether was removed. This was repeated an additional four times to remove all the TCA. The residual ether then was distilled off in a constant temperature water bath at 40°C. The TCA-free amino acid supernatant was then transferred to counting vials and counted using a flour containing 8 ml of 2-methoxyethanol and 10 ml of the scintillation flour described previously. A linear standard curve was obtained when plotting dpm versus μliters of C-14 amino acid.

Purification of Xanthine Dehydrogenase

The purification procedure followed was that of Rajagopolan (82), with the last two steps of ion exchange and molecular sieving chromatography replaced by disc gel electrophoresis.

The purification scheme was easily scaled down so that livers from 3 chickens were adequate for the enzyme purification. Embryonic chicken liver XDH, labeled with a C-14 amino acid, was purified by mixing the embryonic liver homogenate with a homogenate from uninjected adult livers. The total XDH activity in the embryonic

livers and the adult livers was measured prior to mixing.

All of the purification steps were performed at 4°C, except the acetone step, which was done at -5° C. The livers were homogenized with 3 volumes of glass distilled water in a ground glass homogenizer and then centrifuged at 100,000 x g in a Spinco model L ultracentrifuge for 90 minutes. Aliquots were removed from the supernatant after centrifugation for determining the C-14 amino acids or the C-14 soluble total protein. The supernatant was then heated with stirring to 70° C in a boiling water bath, immediately removed and transferred to a constant temperature bath at 70° C. The supernatant was held at this temperature for 2 minutes without stirring. It was then brought to 55° C by the addition of ice. Further cooling to 15° C was achieved by placing the homogenate in an ice bath. The heated homogenate was centrifuged at 10,000 x g in a refrigerated Sorvall centrifuge. To the clear red supernatant was added sufficient ammonium sulphate to give a concentration of 36 gram per 100 ml of supernatant. After sitting for 90 minutes, the solution was centrifuged at 10,000 x g for 15 minutes. The precipitate then was suspended in 0.05 M phosphate buffer, pH 7.9, containing 0.1 mM EDTA.

Sufficient acetone, at -5° C, then was added to make a 32% solution (v/v). The mixture was immediately centrifuged at -5° C at 10,000 x g for 15 minutes. To the supernatant was added enough acetone to make a 40% solution (v/v) and the mixture centrifuged as

before. The precipitate was resuspended in a 0.05 M phosphate buffer, pH 4.7, containing 0.1 mM EDTA.

Solid ammonium sulphate (23 gm/100 ml) was added to the solution with stirring. This was allowed to stand for 90 minutes before centrifugation at 10,000 x g for 15 minutes. Additional ammonium sulphate (13 gm/100 ml of original extract) was added with stirring, and after standing for 90 minutes the solution was centrifuged. The precipitate was resuspended in 0.05 M phosphate buffer, pH 4.7, containing 0.1 mM EDTA.

The suspension was then dialyzed against 1 liter of a 1/10 dilution of buffer containing 6.0 grams of Tris, 28.8 grams of glycine and 0.37 grams of NaEDTA in 1 liter of glass distilled water for 12 hours at 4°C. To the dialyzed material was added sucrose to make a 10% w/v solution. Aliquots of the sucrose solution were then layered on top of polyacrylamide gels for electrophoresis.

Polyacrylamide gels were prepared according to the procedures of Davis (15), with several modifications. Solution "C" was made with 36 grams of recrystalized acrylamide to make a 9% separating gel. In place of a sample gel, protein was layered on top of the stacking gel and the gel tubes were 0.5 cm internal diameter and 6 cm long.

Approximately 100-200 μg of protein was used in each electrophoresis tube. A gel holder and chamber was used that held 20 tubes. The electrophoresis was run for 8 hours at 2.5 mamps/tube. Gels

were removed, scanned at 280 mμ in a Gilford 2410 Linear transport, which was mounted to a Beckman DU monochromator. An aperature plate of 0.05 x 2.36 mm and a scan rate of 2.5 cm/minute was used. Areas under the curves were estimated by triangulation.

Unstained gels, that were previously scanned at 280 mμ, were incubated with an activity stain to localize active XDH. The activity stain contained 0.05 M phosphate buffer, pH 7.8, 0.1 mM EDTA, 0.15 mM xanthine, 0.5 mM DPN^+, 1 mg PMS, 2 mg nitroblue tetrazolium in 10 ml of water at room temperature. The time of incubation was from 3 to 5 minutes in the dark.

In the C-14 experiments, XDH was localized on the gel and removed by slicing the gel with a razor blade. The slices were then placed in counting vials, mascerated with a glass stirring rod and incubated at 37°-40°C in the presence of from 5-7 ml of NCS solubilizer for at least 24 hours (6, 104). Ten ml of scintillation flour containing 4 gm of PPO, 0.1 gm of POPOP in 1 liter of toluene were then added to the vials. The vials were counted for 100 minutes at 12% gain and a 50-1000 window. Vials were then spiked with standard C-14 toluene to determine the counting efficiency.

Gels were also stained with amido swarz and were destained by diffusion in 7% acetic acid. These gels were scanned at 610 mμ in the same gel scanner, as mentioned above.

Polyacrylamide Gel Electrophoresis--Different Gel Concentrations and pH

The procedure for electrophoresis at pH 9.3 was that of Davis (15), except for solution "C" which was modified to give different separating gel concentrations according to the formula

$$\frac{30 \text{ gm}}{x} = \frac{7.5\%}{\text{desired concentration}}$$

In addition, the samples were layered on top with sucrose, eliminating the need for a sample gel.

The procedure for electrophoresis at pH 4.3 was obtained from Canalco Corporation, and is listed in the Appendix.

Re-electrophoresis of Active Xanthine Dehydrogenase

Active XDH, localized on polyacrylamide gels using the activity stain, was removed by slicing as above and placed on top of a stacking gel of a wider internal diameter than the removed gel slice. Electrophoresis was then performed at pH 9.3 and pH 4.3 for 2 and 3 hours respectively, at 3 mamps/tube into a $7\frac{1}{2}\%$ separating gel. These gels were stained with amido swarz and destained by diffusion in $7\frac{1}{2}\%$ acetic acid.

Determination of Xanthine Dehydrogenase Pool Sizes

Fresh livers were blotted and weighed on a Mettler balance. They were then homogenized in a glass homogenizer with 9 volumes of 0.05 M phosphate buffer, pH 7.0, containing 0.1 mM EDTA. Homogenates were centrifuged at 100,000 x g for 60 minutes in a Model L ultracentrifuge. Supernatant volumes were measured, then assayed in 0.05 M phosphate buffer, pH 7.8, 0.1 mM EDTA, 0.15 mM xanthine, 0.5 mM DPN^+, 0.2 mM KCN, in a volume of 3 ml, at 25°C and at 340 mμ. Potassium cyanide was added to inhibit NADH reoxidation in the crude homogenate (101).

Wet Weight of Chicken Liver

Livers were dissected from the animal, blotted to remove excess fluids and the wet weight determined on a Mettler balance. Embryonic chicken livers were blotted by moving the liver over a dry glass surface.

Chromatography of C-14 Amino Acids

Protein from liver homogenates and blood were precipitated with 20% TCA. The supernatants were passed through 0.22 μ millipore filters, and then mixed 5 times with equal volumes of ether to remove the TCA. The supernatants were then spotted onto 5 x 45 cm

Whatman #1 paper strips. The solvent systems for descending chromatography were: for C-14 arginine, n-butanol:acetic acid:water (5:1:4) and 88% formic acid:t-butanol:water (15:70:15); and for C-14 valine, phenol:water (100:39;w:v) plus 0.04% 8-hydroxyquinoline and n-butanol:acetic acid:water (5:1:4). Strips with 2000 cpm or more were scanned and the counts integrated on a Packard Radiochromatogram scanner Model 385, with a 2 mm slit, 1 cm/min scan rate, linear scale of 300 cpm, and a time constant of 10 seconds. On strips with less than 2000 cpm applied, regions were cut into small pieces, placed in vials and counted in a liquid scintillation counter at a 12% gain and 50-1000 window. The scintillation flour used contained 4 grams of PPO, 0.1 gram of POPOP in 1 liter of toluene.

Arginine could be detected on the paper strips by spraying them with the Sakaguchi reagents (1). These reagents are prepared as follows:

(a) 0.01% of α-naphthol in 95% ethanol containing 5% urea.

(b) 5% sodium hypobromite solution (2 grams of Bromine in 100 ml of 5% sodium hydroxide).

Five pellets of sodium hydroxide are added to 10 ml of solution (a) and the solution is used to spray the chromatogram. After drying at room temperature the paper is sprayed lightly with solution (b).

Protein Determination

The microbiuret method described by Itzhaki (46) was used to estimate protein in solution. Crystalline bovine serum albumin was used as the standard.

Valine Pool Size in the Blood

One ml of blood, containing sodium heparin, was deproteinized with an equal volume of 20% TCA and centrifuged at 600 x g for 15 minutes. The precipitate was washed twice with 10% TCA and all the supernatants combined and passed through a millipore filter. TCA was removed by extracting 5 times with an equal volume of ether. The solution was lyophilized and the residue analyzed for valine on an amino acid analyzer.

Injection of C-14 Amino Acids into the Chick Embryo and Hatched Chick

The hatched chick, in all cases, was injected intravenously into the wing vein with 0.1 ml of sterile 0.7% sodium chloride containing the radioactive amino acid. A 0.25 cc glass syringe with a 30G needle was used for all injections.

Air space injections into the eggs involved injecting labeled amino acid through a hole punched into the large end of a candled egg. The label was layered on top of the allantoic membrane.

Intravenous injection into embryos involved candling the egg to find large straight veins, swabbing the egg with 70% ethanol, cutting a window into the egg shell leaving the membrane intact, placing mineral oil on the membrane to expose the vein and injecting into the vein with a 30G needle. Vessels on the surface of the egg are usually veins. Arteries are usually on the inside and float around freely.

Collection of Blood from the Hatched Chick and Embryo

Hatched chicks were decapitated and their blood collected directly in graduated centrifuge tubes containing an anticoagulant (sodium heparin 0.2 mg/ml of blood or sodium citrate 5 mg/ml of blood).

Embryo blood was obtained by inserting a 30G needle fitted with a syringe into a vein which had been previously exposed and withdrawing the blood by suction. In order to keep the needle stationary, the syringe, fitted with the needle, was attached to a microscope stage that had micrometer dials for up, down, backward, forward, left and right movements.

EXPERIMENTAL AND RESULTS

Chloride Levels in Chick Liver and Blood During Development

Since chloride is assumed not to penetrate the cell wall (42) and is found only in the blood, a knowledge of the chloride content in the liver and blood can be used to calculate the ml of blood per gram of liver, by simply dividing the chloride content in the liver by the chloride content in the blood.

The results obtained using the Van Slyke method (39, p. 625-629) for determining free chloride in blood and liver are summarized in Table I.

These results show that the extracellular space decreases 15% from the 14 day embryo to the newly hatched chick and then increases 35% as the chick develops, presumably by an increase in the blood supply throughout the liver.

The standard error of the mean was rather constant for the blood, but varied more with the liver determinations with the largest standard error occurring with the embryo. This is most likely due to the difficulty in blotting chick embryo livers to remove excess fluids, thereby giving rise to errors in wet weight determinations. Chick embryo livers were blotted by moving the livers over a dry glass surface until no more fluid remained, whereas hatched chick livers

Table I. Levels of extracellular blood found in the liver during development.[a]

Age of Chick	Number of Chicks	Chloride Content in Liver (mg Cl^-/g liver)	Number of Chicks	Chloride in Blood (mg Cl^-/ml blood)	Milliters of Blood in Liver (ml/g liver)
14 day embryo	19	1.71 ± 0.094	10	5.04 ± 0.063	0.339 ± 0.019
1 day chick	20	1.62 ± 0.019	10	5.70 ± 0.062	0.284 ± 0.004
8 day chick	14	2.22 ± 0.064	10	5.77 ± 0.043	0.385 ± 0.011

[a]The measurements include the mean and standard error of the mean.

could be easily blotted by absorbtion on paper towels.

Creatinine Levels in the Blood and Liver of the Hatched Chick and its Influence on the Arginine Color Reaction

Arginine as measured colorimetrically by the Sakaguchi reaction is inhibited by creatinine (109). Wu estimated that 13 μmoles of creatinine causes a 50% reduction in absorbance obtained with 0.05 μmole of arginine. The concentration of creatinine in the blood and liver of the chick was determined in order to determine if the levels of creatinine would be high enough to inhibit the blood and liver arginine color reaction.

Table II shows the results obtained in the determination of creatinine levels in the blood and liver of hatched chicks.

Table II. Creatinine concentrations in the blood and liver of the hatched chick.

Source of Extract	Number of Chicks	Concentration of Creatinine μmole/ml blood or μmole/g liver
Blood	3	0.035
Liver	3	0.17

These and higher levels of creatinine were then added to a standard arginine solution containing 0.01 μmoles and the degree of color inhibition determined. The results are shown in Table III.

Table III. Inhibition of color formation in the Sakaguchi reaction by creatinine.

Quantity of Arginine in Assay (μmoles)	Quantity of Creatinine Added (mg)	Total Volume in Assay (ml)	Optical Density at 500 mμ	Percent Inhibition
0.01	0.000	4	0.025	0
0.01	0.003	4	0.025	0
0.01	0.03	4	0.024	4

Creatinine, 0.03 mg, inhibits the color reaction by only 4%, while 0.003 mg does not appear to inhibit the color reaction.

One hundred mg of liver and 0.2 ml blood were used in the arginine determination, representing 0.0017 mg and 0.0003 mg creatinine in the liver and blood respectively. Since the levels of arginine in Table IV are about 0.1 μmole per 100 mg of liver and 0.05 μmole per 0.1 ml blood, the levels of creatinine are not significant enough to hinder the quantitative estimation of arginine in the liver and blood.

Arginine and Valine Pools in the Blood and Liver of the Embryo and Hatched Chick. Determination of the Intracellular and Extracellular Pools

Blood and liver pool sizes of valine or arginine are given in Table IV, together with the results obtained by Murison (72). Blood and liver arginine pools in both sets of data show an increase in the pool size upon hatching. Presumably, this change in the amino acid pool is due to the digestion of proteins present prior to hatching and/or

Table IV. Arginine and valine pool sizes in liver and blood in the chick during development.[a]

Age of Chick	Amino Acid Studied	Total Pool of Amino Acid in Liver (μmole/g liver)	Total Pool of Amino Acid in Blood (μmole/ml blood)	Extracellular Pool of Amino Acid (μmole/g liver)	Intracellular Pool of Amino Acid (μmole/g liver)
14 day embryo	Arginine	0.84 ± 0.17	0.48 ± 0.001	0.16 ± 0.009	0.68 ± 0.17
19 day embryo	Arginine	0.60[b]			
1 day chick	Arginine	1.08 ± 0.04	0.57 ± 0.001	0.16 ± 0.002	0.92 ± 0.04
	Arginine	1.38[b]			
	Valine	0.82[b]	0.45	0.13	0.69
4 day chick	Arginine	1.38[b]			
8 day chick	Arginine	0.806 ± 0.063	0.500 ± 0.001	0.21 ± 0.006	0.596 ± 0.06

[a]Arginine values obtained in this thesis are given in terms of mean and standard error of the mean.

[b]Values obtained from Murison (72).

amino acids in the yolk sac which are incorporated into the pools on hatching. The maintenance of the high level of liver arginine 3 days after hatching and a decrease 8 days after hatching could be due to a rapid absorption of yolk during the first 4 days, its complete disappearance by the eighth day (84), and an accompanying changeover to a food diet.

In comparison to Murison's values for arginine, the slightly higher values for arginine in the embryo liver and lower value in the 1 day chick liver may be due to differences in the strains of chickens used. In addition, since arginine as well as valine, are both essential amino acids for the chick (73), any differences in the diet could affect the levels of the amino acid in the blood and liver amino acid pools.

The intracellular arginine and valine pools are determined by subtracting the amino acid pool found extracellularly from the total pool which is composed both of the intracellular and extracellular pools.

The extracellular pools is obtained as follows:

$$\frac{\text{extracellular amino acid pool}}{\text{gram of liver}} = \frac{\text{amino acid pool}}{\text{ml of blood}} \times \frac{\text{ml of blood}}{\text{gram of liver}}$$

The intracellular pool is obtained as follows:

$$\frac{\text{total amino acid pool}}{\text{gram of liver}} - \frac{\text{extracellular amino acid pool}}{\text{gram of liver}} = \frac{\text{intracellular amino acid pool}}{\text{gram of liver}}$$

The intracellular and extracellular pool results are summarized in Table IV. The intracellular arginine pool, like the total arginine pool, increases upon hatching and then decreases by the eighth day to approximately the same value as that found in the embryo. The greatest source of error is again found in the embryo.

Table V shows the percentage of the total arginine found in the liver that is either extracellular or intracellular.

Table V. The percent of total arginine in the liver that is due to intracellular and extracellular arginine.

	Percent of Total Arginine Pool	
Age of Chick	Intracellular Arginine	Extracellular Arginine
14 day embryo	81	19
1 day chick	85	15
8 day chick	74	26

The contribution of the extracellular pool to the total pool is significant, being 19% in the embryo, 15% in the 1 day chick and 26% in the 8 day chick. Therefore, a correction is required if meaningful results for intracellular pools are to be obtained.

Chromatographic Separation of C-14 Amino Acids

In order to determine if the injected amino acid was metabolized, an aliquot of the TCA supernatant was subjected to paper chromatography and the strips with 2000 cpm or more scanned with a Packard

Radiochromatogram scanner, which counts and integrates the counts under each peak. Strips with less than 2000 cpm were cut into small pieces and counted in a liquid scintillation counter as described in the methods. The results are given in Table VI. The results show that after approximately 30 minutes the ratio of the radioactivity due to arginine or valine to that of total radioactivity is relatively constant.

At least two possibilities exist to explain why an injected amino acid represents a smaller percent of the total activity with time. One is that the original solution injected into the chick is not radiochemically pure. If the contaminant remains at a constant level in the pool or is not incorporated into the enzyme studied then it will have little effect on the results. However, it is necessary to correct for this amount of radioactivity in the calculations, since the calculation of the rate of synthesis assumes incorporation of the particular amino acid injected. Another explanation is that the injected amino acid is metabolized and the radioactivity of the metabolites is included in the determination of total radioactivity. Again, if the metabolite is not incorporated into the enzyme being studied, it will not alter the results. That is, providing that the actual pool size of the injected amino acid is known at all times studied. The formula used to calculate the radioactivity of either arginine or valine at time t was as follows:

Table VI. Paper chromatographic separation of radioactive liver amino acids in different solvent systems.[a]

Age of Chick	Radioactive Amino Acid Injected	Length of time label was in the liver prior to analysis (min)	Percentage of the total radioactivity present at time t remaining as the injected amino acid			Average Percent
			Solvent I[b]	Solvent II[c]	Solvent III[d]	
8 day chick	L-(guanido-^{14}C)-arginine	5	85		83	84
		30	60		59	59
		60	56		55	55
		120	52		54	53
1 day chick	L-(guanido-^{14}C)-arginine	5	84		85	85
		30	56		57	56
		60	55		60	57
		120	53		47	50
1 day chick	L-valine-^{14}C(u)	5	88	90		89
		30	70	65		67
		60	64	58		61
		120	53	62		58
		240	47	47		47
14 day embryo	L-(guanido-^{14}C)-arginine	20	85		78	81
		60	62		52	56
		120	56		47	51
		240	54		57	55

[a] Blank spaces in the table indicate that no determinations were made.

[b] Solvent I n-Butanol:acetic acid:H_2O (5:1:4)

[c] Solvent II Phenol:H_2O (100:39;w:v) plus 0.04% of 8-hydroxyquinoline

[d] Solvent III 88% Formic acid:t-butanol:H_2O (15:70:15)

Radioactivity of arginine or valine at time t in the amino acid pool = total counts in TCA supernatant x percentage of the total counts in the TCA supernatant due to arginine or valine

The pathway for valine degradation is described by Mahler (66, p. 690). It is a glycogenic amino acid, having as an end product succinyl-CoA, which via the tricarboxylic acid cycle can be catabolized to pyruvate and then to glucose. The catabolic products of glucose can then become the substrates for other amino acids and other substances such as fats, nucleic acids, etc.

The guanidine portion of arginine is rapidly turned over into urea in the rat liver (102), and is therefore an excellent tracer for use in that system. The renewal rate of the guanidine group of arginine in the chicken, however, is not well established. The chicken being a uricotolic animal excretes mainly uric acid with only a small portion being urea (84). Very low arginase levels have been measured in the whole chick embryo (84) and in the liver of hatched chicks (31).

Changes in L-(guanido-^{14}C)-arginine and L-valine-^{14}C(u) in the Intracellular Liver Amino Acid Pools and Blood Amino Acid Pools and their Incorporation into Total Protein

Carbon-14 labeled arginine or valine was injected intravenously into hatched chicks or into the air space of chick embryos. The

incorporation into blood amino acid pools or soluble proteins was determined and the results are shown in Table VII. Figures 1, 3, 4, and 5 show the incorporation into the intracellular liver amino acid pools.

In the experiment, where 0.1 μc of C-14 arginine is injected into the air space of the embryo, the isotope enters the blood amino acid pool very rapidly during the first 20 minutes and then decreases rather rapidly up to 75 minutes followed by a slow decrease. The incorporation into soluble proteins is slower, in that a rapid increase occurs up to 45 minutes followed by a slower increase and a leveling off after 75 minutes. The incorporation of C-14 arginine into the intracellular amino acid pool (Figure 1) follows closely the pattern of the incorporation into the blood amino acid pool. The nearly identical pattern of the changes in radioactivity in the blood and intracellular liver amino acids indicates that there is a rapid equilibrium set up between intracellular and extracellular amino acids in the embryonic liver.

The relatively constant level of radioactivity in the blood and liver amino acids after 2 hours is likely due to a slow transport of arginine from the albumin and yolk sac into the blood supply. Injection into the air space leads to a distribution of label into the blood system as well as into the yolk sac and albumin. This is desirable for studying rates of protein synthesis, where a knowledge of the

Table VII. Kinetics of incorporation of radioactive amino acid into total soluble protein and blood amino acids.[a]

Age of chick	Number of Chicks	Means of Injection	Radioactive Amino Acid Injected	Amount of Label (μc)	Time of Study (min)	Radioactivity in Blood dpm / ml blood	Radioactivity in Total Soluble Protein dpm / g liver
8 day chick	2	intravenous	L-(guanido-^{14}C)-arginine	1	5	21,200	13,200
	2				10	16,100	18,800
	2				15	8,170	16,100
	2				30	6,780	24,100
	2				45	4,025	24,200
	2				60	2,300	16,100
	2				120	1,430	22,300
	2				1500	260	15,300
1 day chick	2	intravenous	L-(guanido-^{14}C)-arginine	1	5	45,200	14,700
	2				10	28,500	16,000
	1				25	14,400	22,389
	1				30	11,000	27,099
	2				45	8,850	30,144
	1				60	11,700	35,427
	1				120	5,500	29,160
	2				1440	1,690	23,630
1 day chick	2	intravenous	L-valine-^{14}C(u)	1	5	35,500	
	2				10	31,400	
	2				20	17,000	
	1				60	4,430	
	1				120	2,640	
	1				240	1,170	
14 day embryo	1	air space	L-(guanido-^{14}C)-arginine	0.1	6	1,760	1,125
	1				14	4,540	1,890
	1				20	5,500	2,768
	2				30	3,770	3,100
	2				45	1,950	5,600
	2				75	1,050	7,800
	2				120	865	7,300
	2				165	750	6,080
	2				190	930	7,000
	2				240	650	

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

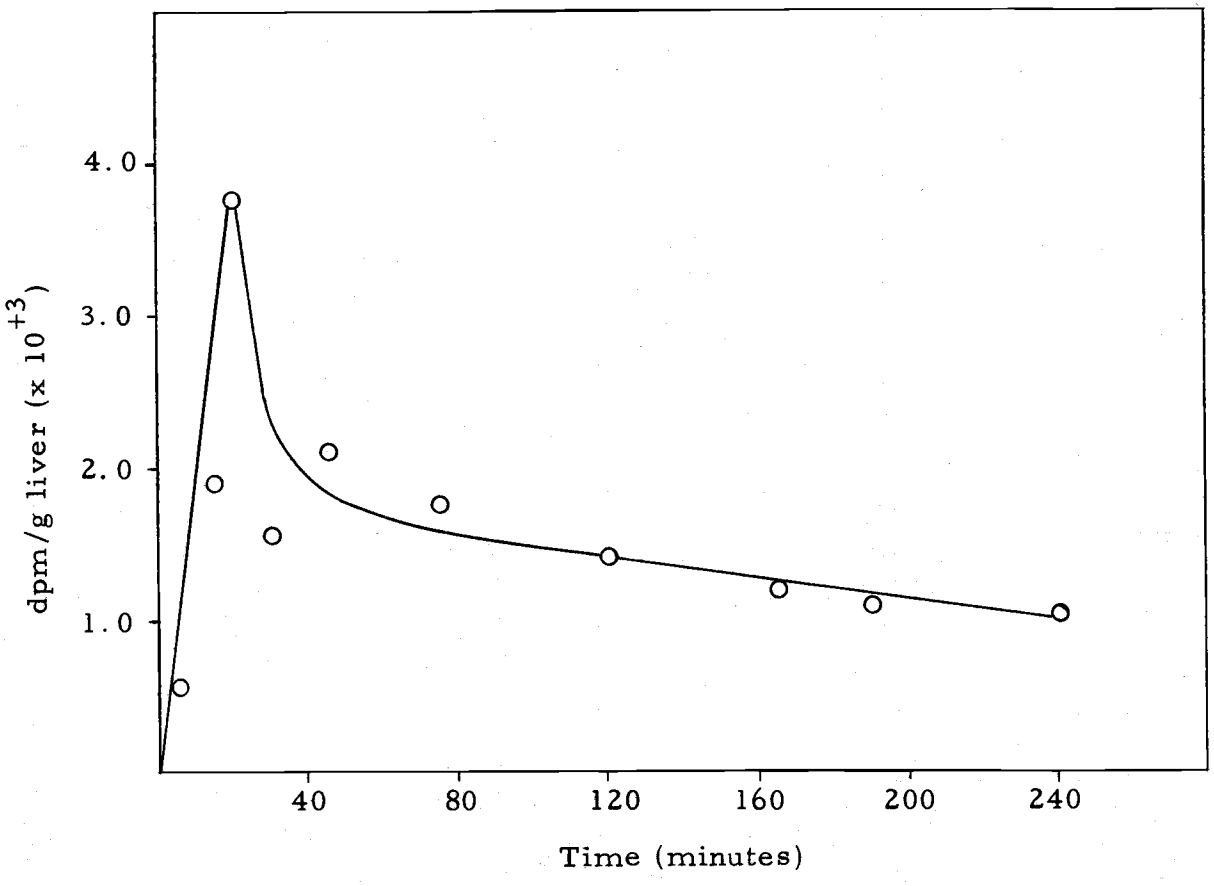

Figure 1. Kinetics of L-(guanido-^{14}C)-arginine in the intracellular liver amino acid pool of the 14 day embryo.

specific radioactivity of the amino acid pool is required, but is less desirable for studying rates of degradation of protein. For the latter, the best situation is to have a period of rapid labeling followed by a period in which non-labeled amino acid dilutes out the radioactive pool. One way to achieve the latter condition in the embryo is to inject the labeled compound intravenously and allow sufficient time for the label to be taken up into protein and be diluted out with cold amino acids transported to the blood from the yolk. Figure 2 shows a comparison between an intravenous and an air space injection with labeled arginine. Sixty minutes after an intravenous injection, the level of C-14 was 17,000 cpm per gram of liver and after 24 hours it was 2500 cpm per gram of liver, indicating that greater than 90% of the label had been "chased out" of the system after 24 hours. In the same figure is shown the results of an air space injection. In contrast to the intravenous injection, the C-14 remaining in the liver after 24 hours is about 66% of that found in the liver after 80 minutes of incubation, indicating that a slow but continuous supply of radioactive label was being transported to the blood from the yolk sac and albumin for more than a 24 hour period of time. Therefore, the best approximation to a chase condition is a pulse label via an intravenous injection and a subsequent period of 24 hours to allow sufficient time for the C-14 pool to be diluted out in the embryo.

The results in Table VII show that an intravenous injection into

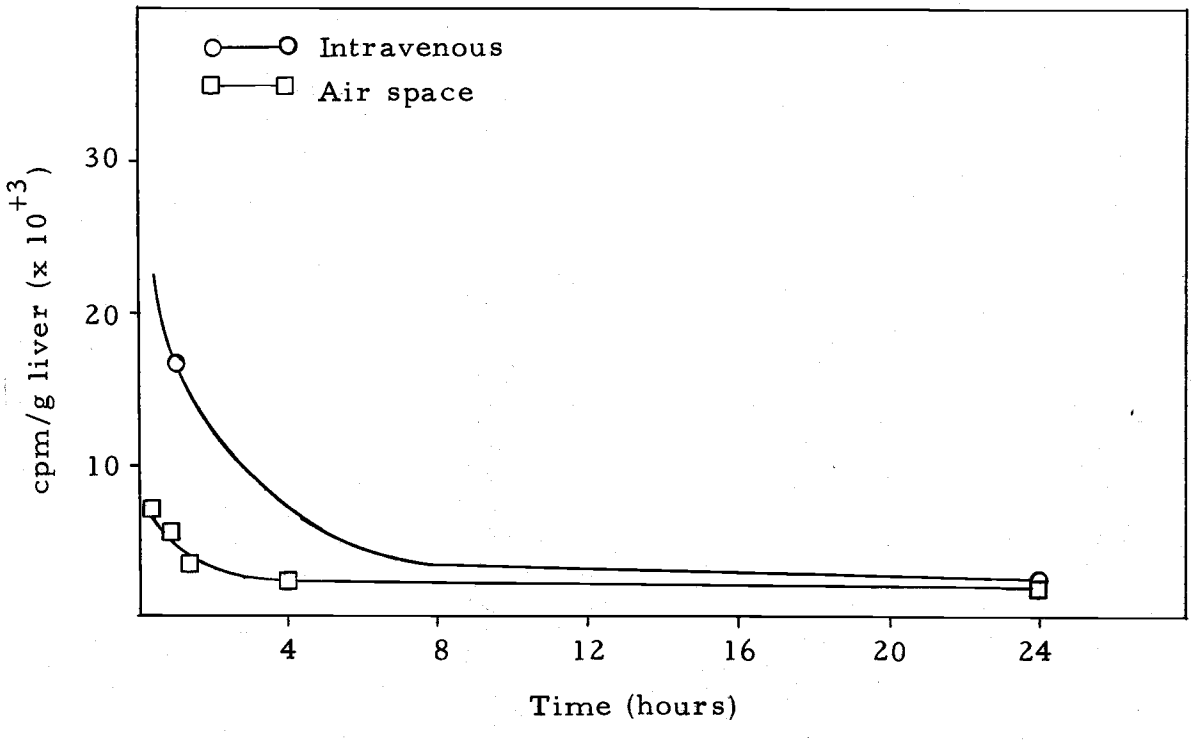

Figure 2. Kinetics of L-(guanido-^{14}C)-arginine in the total liver amino acid pool of the 14 day embryo.

the wing vein of 1 day and 8 day chicks of 1 μc of C-14 arginine results in a rapid labeling of the liver soluble protein pool. By 60 minutes, the labeling has reached a plateau. After 60 minutes, the rate of increase is very slow, indicating that C-14 arginine from the intracellular amino acid pool has been nearly depleted.

That the intravenous injection behaves as a pulse is seen from the labeling patterns of the intracellular liver amino acid pools (Figures 3, 4, 5). A dramatic increase in C-14 occurs during the first 5 minutes followed by a nearly exponential decrease of C-14 from the pool during the subsequent incubation period. The levels of radioactivity after 24 hours (Table VII) represents less than 5% of the amount of C-14 present at 5 minutes after injection. The loss of label from the pool occurs via amino acid metabolism, uptake of amino acid through protein synthesis and dilution due to uptake of cold amino acids during the metabolism of food or yolk protein.

XDH Pool Sizes in the Embryo and Hatched Chick

The rise in activity of chicken liver XDH upon hatching described by Strittmatter (101) was confirmed in this thesis (Table VIII).

The level of XDH in the embryonic liver is very low and at a rather constant value of 0.016 units per gram of liver. The large standard deviation and range of values in the embryo are due to errors associated with determining the wet weight of the embryonic liver and

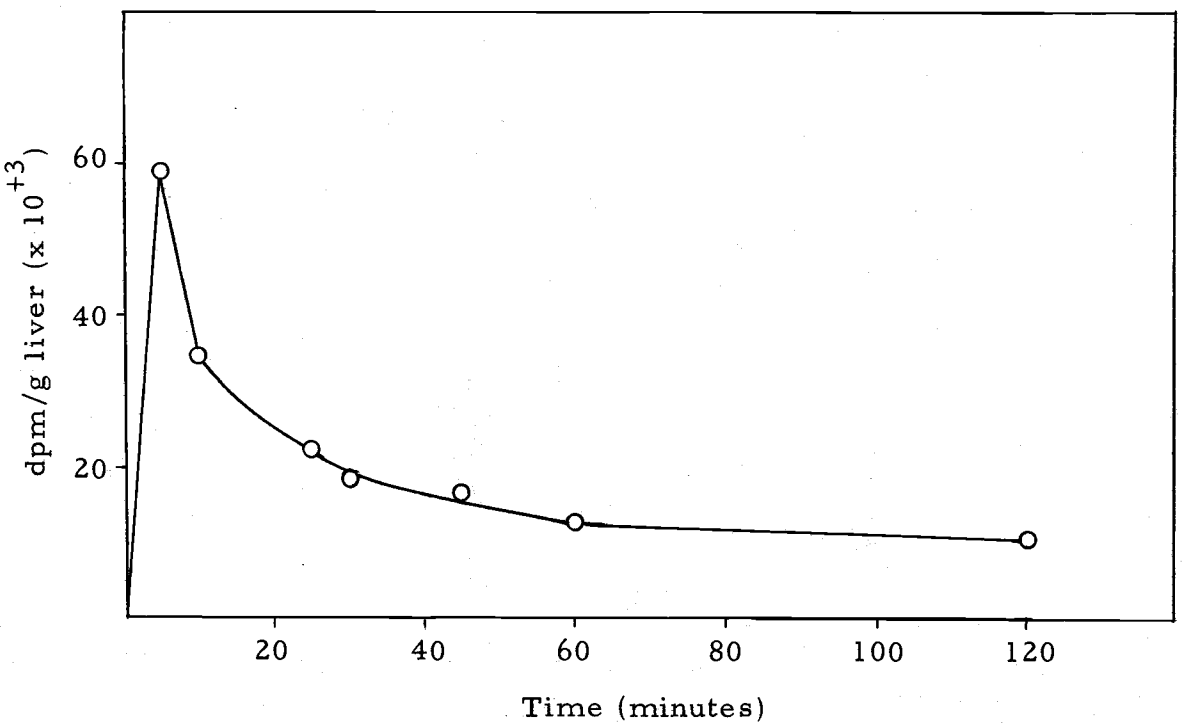

Figure 3. Kinetics of L-(guanido-^{14}C)-arginine in the intracellular liver amino acid pool of the 1 day chicken.

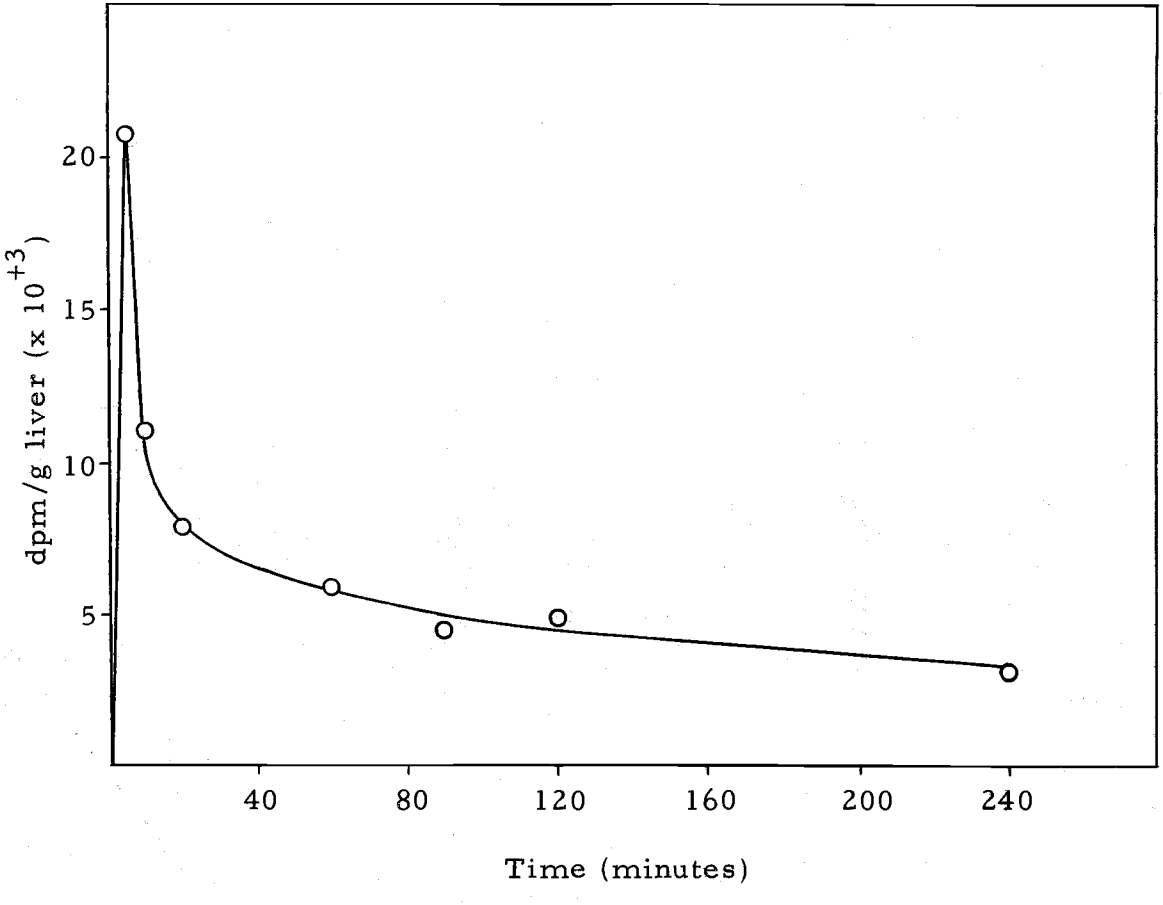

Figure 4. Kinetics of L-valine-^{14}C(u) in the intracellular liver amino acid pool of the 1 day chicken.

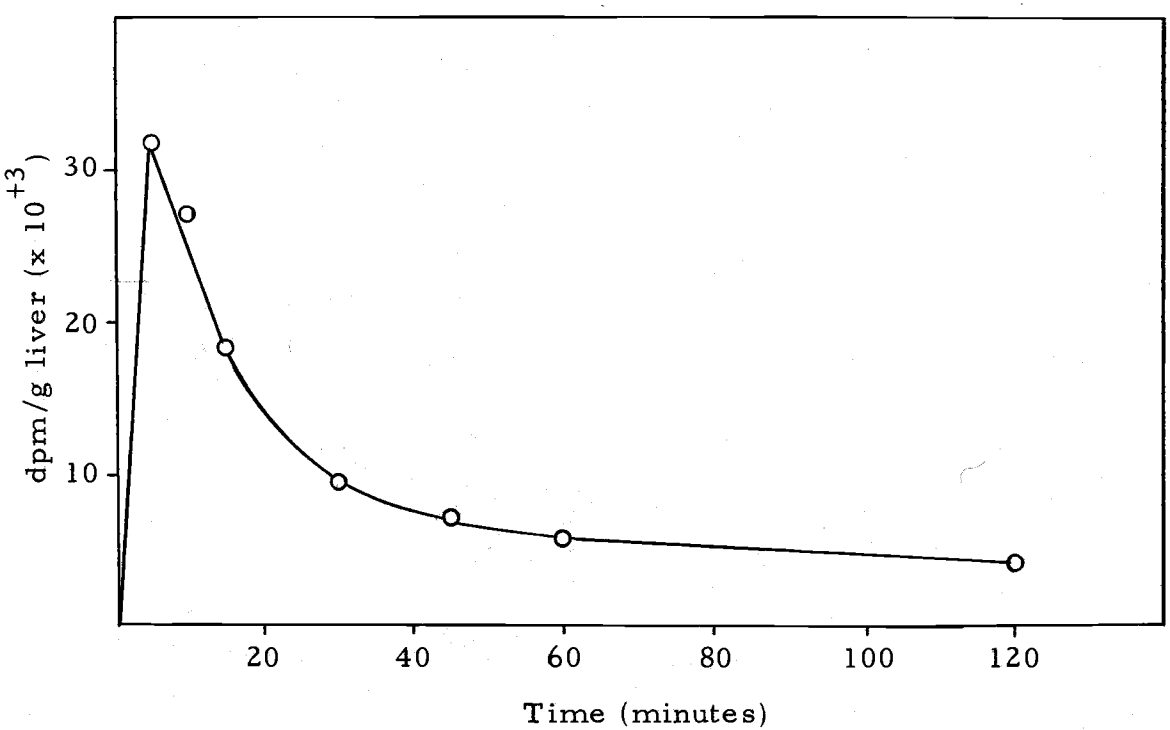

Figure 5. Kinetics of L-(guanido-^{14}C)-arginine in the intracellular liver amino acid pool of the 8 day chicken.

Table VIII. The level of xanthine dehydrogenase in chick liver during development.

Age of Chick	Number Used for Analysis	Xanthine Dehydrogenase Activity (units/g liver) Mean Value	Range	Standard Deviation	Reference for Analysis
14 day embryo	5	0.016	0.26-0.008	±0.007	This study
15 day embryo	8	0.011	0.017-0.006	±0.004	This study
19 day embryo		0.017[a]	---	---	Strittmatter
1 day chick	8	0.177	0.26-0.09	±0.052	This study
1 day chick		0.195[a]	---	---	Strittmatter
2 day chick	8	0.436	0.52-0.30	±0.076	This study
2 day chick		0.40[a]	0.45-0.35[a]	---	Strittmatter
8 day chick	4	0.84	0.91-0.80	±0.052	This study
8 day chick		0.70[a]	0.75-0.65[a]	---	Strittmatter
9 day chick	10	0.651	0.98-0.40	±0.171	This study
9 day chick		0.70[a]	0.75-0.65[a]	---	Strittmatter

[a]Strittmatter defines activity as mμmoles uric acid formed per minute per mg liver (wet weight).

with the errors in measuring the very low levels of enzyme activity.

Upon hatching the liver XDH level increases by 250% during a 24-48 hour period, going from 0.177 to 0.436 units per gram of liver. The large variation in measuring the XDH activity in the 1 day and 2 day chicks was due to the rapid increase in the level of XDH during this period of time and the difficulty in determining the exact time of hatching. Hatching was defined as the period when the shell was broken into 2 separate pieces with a complete circle about the egg. In order to see the time of hatching, a hatching incubator was used that had a window through which the hatching process could be viewed. Chicks that were 23-25 hours post hatching were defined as 1 day old and chicks 47-49 hours post hatching were defined as 2 days old.

A plateau in XDH activity in the liver is reached 8 days after hatching according to Strittmatter (101) and is confirmed by the data shown in Table VIII. The higher mean value for the 8 day chick than the 9 day chick could have been due to differences in the dietary state of the birds chosen, since the determinations were done on different lots of birds. Previous work (18, 93, 100) has shown that XDH pool sizes per gram of liver are affected by the dietary intake of the adult chick. All chicks used in this thesis were feed ad libitum and given water freely. Large fluctuations about the mean were also shown in Strittmatter's (101) quantitative study of chick liver XDH.

In summary, the results show that the concentration of chick

liver XDH in the embryo is very low and constant, it rises rapidly upon hatching and reaches a plateau region 8 days after hatch where the level is high and constant.

Determination of Wet Weight of Chick Liver During Development

The results of the wet weight determinations of the chick liver during development are shown in Table IX. The values are given in terms of the mean and standard error of the mean. Also, included are the wet weight values reported by Romanoff (84).

The differences between the wet weight values obtained by Romanoff (84, p. 68) and those obtained in this thesis might be ascribed to the possibility that the embryos used in this thesis were up to 12 hours younger than those described by Romanoff. That this might be the case is shown by a comparison of toe lengths of the embryos. According to Lillie (60, p. 90) 14 day old chick embryos at stage 40 should have a toe length of 12.7 ± 0.5 mm and 15 day old embryos at stage 41 should have a toe length of 14.9 ± 0.8 mm. In this thesis, 14 day old chick embryos had a toe length of 11.5-12.2 mm and 15 day embryos had a toe length of 13-14.5 mm. The percent increase in wet weight over a 24 hour period of time, however, was nearly the same in both cases. In this thesis, there was a 30% increase in wet weight while Romanoff (84, p. 68) showed a 39% increase.

Table IX. Wet weight of liver during chick development.

Age of Chick	Number in Sample	Wet Weight of Whole Liver[a] (gram)	Embryo Toe Length (mm)	Wet Weight of Whole Liver[b] (gram)
14 day embryo	9	0.168 ± 0.004	11.5-12.2	0.209
15 day embryo	9	0.22 ± 0.003	13.0-14.5	0.290
1 day chick	5	0.902 ± 0.04		
2 day chick	8	1.147 ± 0.060		
8 day chick	10	1.68 ± 0.08		
9 day chick	8	1.78 ± 0.04		

[a]Values are reported as mean and standard error of mean.

[b]Values were obtained from Romanoff (84, p. 68).

The results show that the liver increases most rapidly during embryonic stages where a 31% increase in wet weight occurs during a 24 hour period from day 14 to day 15. Upon hatching, the liver is still increasing rapidly with a 27% increase in a 24 hour period of time. By 8 days after hatch, the liver is increasing by only 1% in a 24 hour period of time.

Purification of Chicken Liver Xanthine Dehydrogenase

The results of the purification scheme are shown in Table X, and are compared with that obtained by Murison (72).

The final specific activity of XDH, in this thesis, is slightly higher than that obtained by Murison. The latter author claims to have

Table X. Purification of xanthine dehydrogenase from chicken liver.[a]

Purification Step	Volume (ml)	Protein (mg/ml)	Specific Activity[b] ($\times 10^{-3}$)	Total Units ($\times 10^{+1}$)	Yield in Percent
A. This thesis					
100,000 x g supernatant	250	100.0	2.4	6.00	100
Heat 70°C	250	10.8	22.2	6.00	100
Ammonium sulphate 36 gm/100 ml	40	12.0	116.0	5.55	92
Acetone 32-40% v/v	50	5.1	179.0	4.56	75
Ammonium sulphate 23-36 gm/100 ml	10	4.8	483.0	2.32	38
Analytical disc gel electrophoresis[c] pH 9.3			2400[d]		

continued on next page

Table X continued.

Purification Step	Volume (ml)	Protein (mg/ml)	Specific Activity[f] (units/mg protein)	Total Units[f] (x 10^{+3})	Yield in Percent
B. Murison's results[e]					
105,000 x g supernatant	500	160	1.7	136	100
Heat 70°C	500	17.0	17.2	145	107
60% Ammonium sulphate	250	16.0	28.4	112	83
50% Acetone	70	23.0	55.2	89	65
60% Ammonium sulphate	24	19.0	95.6	44	33
DEAE-Cellulose eluate	20	6.5	240.0	31	23
Hydroxylapatite eluate	3.5	2.4	1710	14	10

[a] Blank spaces in the table indicate that no determinations were made.

[b] Specific activity is defined as units/mg protein and a unit is defined as µmole NADH formed/minute.

[c] No attempt was made to perform analytical disc gel electrophoresis of all the protein.

[d] This value is an estimate, based on the degree of purification obtained during disc gel electrophoresis.

[e] This table was obtained from Murison (72).

[f] A unit is defined by Murison as mµmole Uric acid formed/minute.

a single protein species. The slight differences in the final specific activity may be explained by the dissimilarity in the methods used to calculate the final specific activity. Murison assayed his final purified enzyme in 0.05 M Tris-HCl buffer pH 8.0 containing 0.001 M EDTA, in a 1 ml cuvette (1 cm light path) with 0.2 mM xanthine and 0.7 mM NAD^+ final concentrations. The assays were performed at 30°C and at 290 mμ. The absorbance units were divided by 11, which is the millimolar absorbance for uric acid at 290 mμ. Protein was determined by the biuret method (30) and by the method of Lowry (65), with crystalline bovine serum albumin as the standard. In this thesis, the final specific activity was obtained by multiplying the specific activity of the last ammonium sulphate (23-36 gm/100 ml) step by a factor of 5. The factor 5 comes from the increase in purification of XDH obtained by disc gel electrophoresis (Figure 6). As can be seen in Figure 6, the absorption area under the active XDH peak is 1/5 that of the total protein absorption spectrum in the gel. The area under the curves was estimated by triangulation.

Criteria of Purity of Xanthine Dehydrogenase

Disc gel electrophoresis was used as the last step in the purification of XDH from chick liver.

Figure 7 shows two bands. Band 1 is closest to the - pole (top) and band 2 is closest to the + pole (bottom). From the densitometer

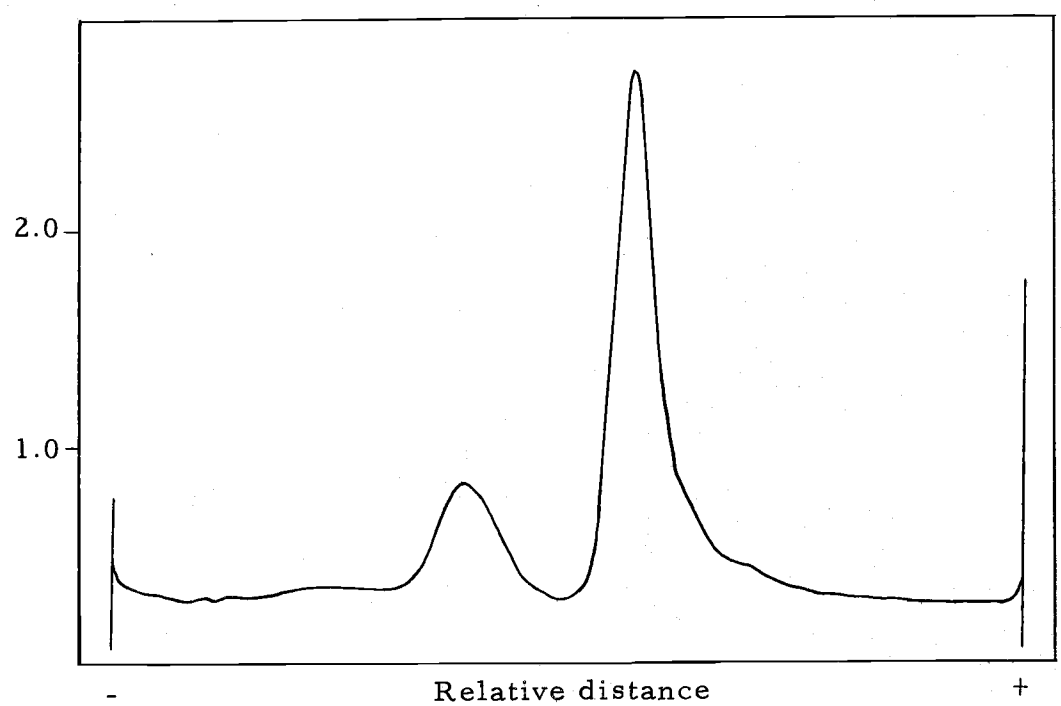

Figure 6. Densitometer tracing of protein on polyacrylamide gels at pH 9.3. 120 μg of protein was applied to an $8\frac{1}{2}$% separating gel with the standard pH 9.3 buffer system of Davis (15). Migration of the enzyme is toward the anode. Specific activity of the enzyme was 440 munits per milligram of protein before electrophoresis. The gel was scanned at 610 mμ in a Gilford 2410 Linear Transport, which was mounted to a Beckman DU monochromator. An aperture plate of 0.05 x 2.36 mm and a scan rate of 2.5 cm/minute was used. Full scale was 3.0 O.D. Areas under the curves were estimated by triangulation.

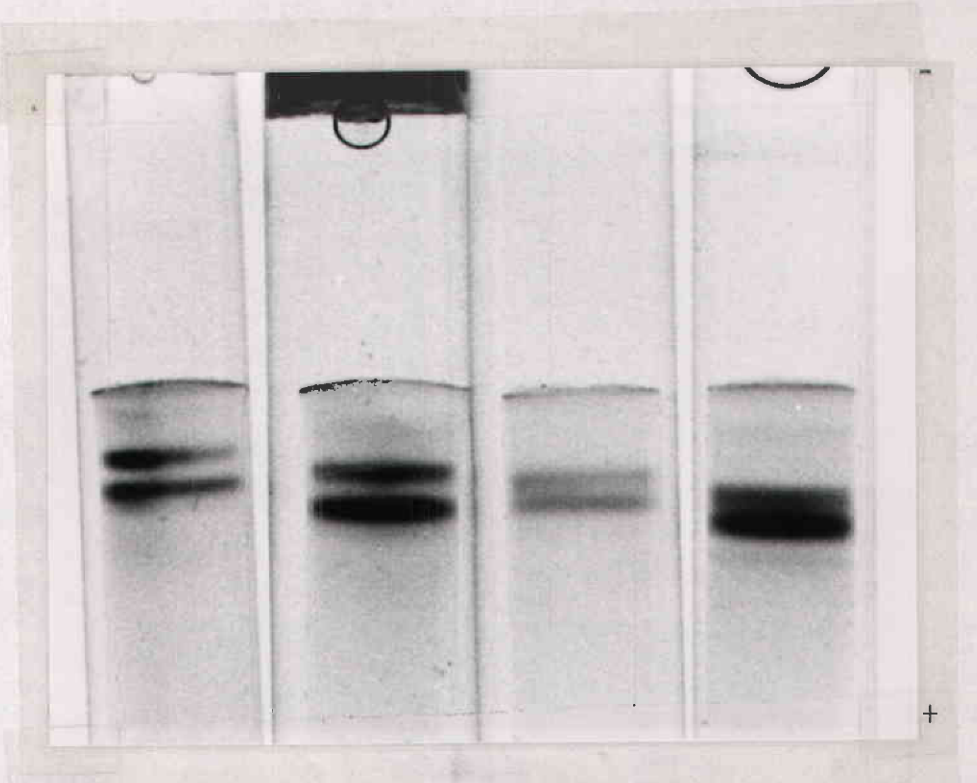

Figure 7. Disc-gel electrophoresis of partially purified xanthine dehydrogenase in different gel concentrations. Approximately 50 μg of protein, specific activity of 480 munits per minute per milligram of protein, was electrophoresced for 1 hour at 3 mamp/tube, in the standard buffer system (pH 9.3) of Davis (15). The gel concentrations for the separating gels are from left to right: 9%, 8½, 8%, and 7½%.

tracing in Figure 6, it is obvious that band 2 contains several species, since there is a shoulder in the peak. Band 2 after a longer electrophoresis time was resolved into two bands.

As can be seen from Figure 8, XDH is present in band 1 when the gel is stained specifically for XDH. Band 2, after a long incubation time yielded a slight positive activity stain without substrate, while band 1 only was positive in the presence of substrate. Infrequently, a minor second active XDH band would appear at the top of the gel. This is probably the enzymatically active dimer of XDH which was observed by Murison (72) in his disc gel electrophoresis of XDH.

Attempts to resolve band 1 (active XDH) into more than one band by varying the gel concentrations and length of time of migration are shown in Figures 7-9. As can be seen, only one symmetric protein band was obtained in all cases.

Another test of the purity of the band was obtained by electrophorescing the active XDH (band 1) at two different pH values. Band 1 was sliced from the gel and electrophoresced on other gels at pH 9.3 and at pH 4.3. As can be seen in Figure 10, only a single band was obtained. All the results indicate that the active XDH band is homogeneous with respect to changes in pH, gel concentration and length of time of electrophoresis.

Beside the fact that a homogeneous band of XDH was obtained by

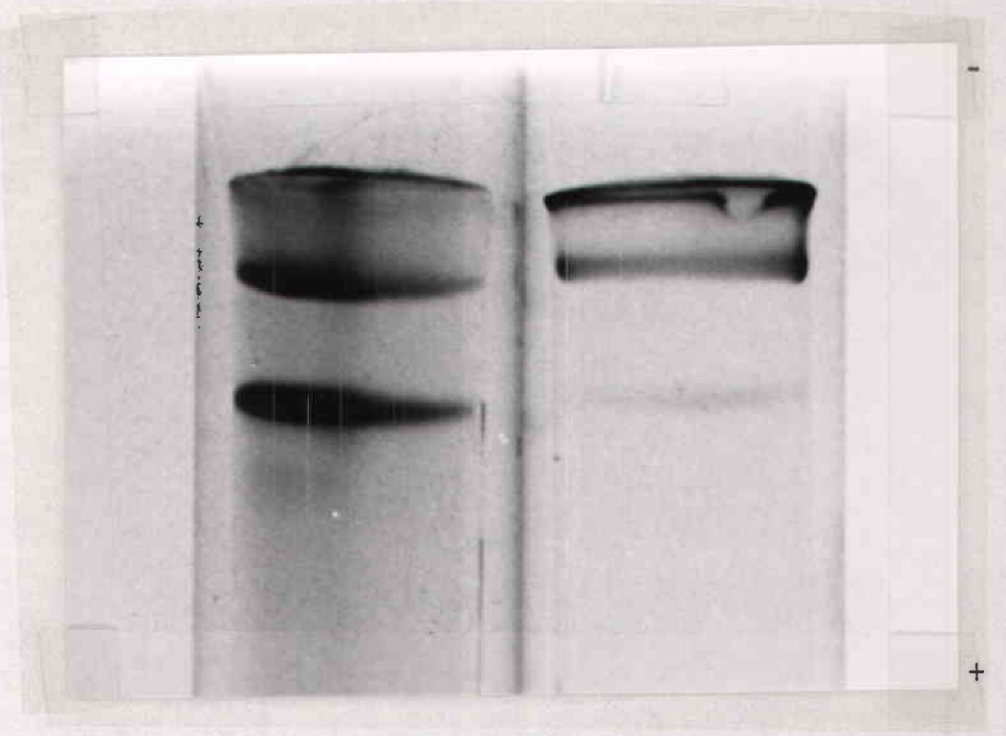

Figure 8. Disc-gel electrophoresis of partially purified xanthine dehydrogenase. Approximately 50 μg of protein, specific activity of 480 munits per minute per milligram of protein, was electrophoresced for 3 hours at 3 mamp/tube, in a 13% separating gel, in the standard buffer system (pH 9.3) of Davis (15). One gel (left) is stained for protein and the other (right) for enzyme activity.

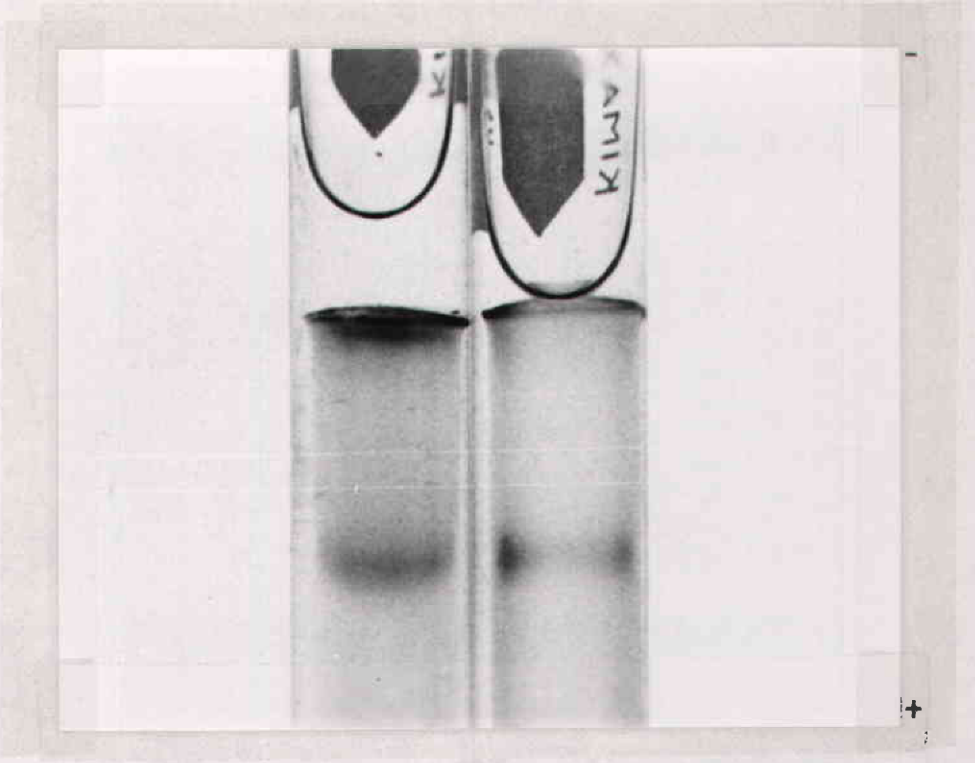

Figure 9. Disc-gel electrophoresis of partially purified xanthine dehydrogenase. Approximately 50 μg of protein, specific activity of 480 munits per minute per milligram of protein, was electrophoresced for 20 hours at 3 mamp/tube, in a 13% separating gel, in the standard buffer system (pH 9.3) of Davis (15). One gel (left) is stained for protein and the other (right) for enzyme activity.

Figure 10. Disc-gel electrophoresis of purified xanthine dehydrogenase. Purified XDH was cut out of 9% polyacrylamide gel as described in the methods and the slices placed on top of stacking gels of wider internal diameter. Electrophoresis was then performed at pH 4.3 (right) and pH 9.3 (left) for 3 and 2 hours respectively, into $7\frac{1}{2}$ % separating gels at 3 mamp/tube. Gels were stained for protein.

disc gel electrophoresis, the final specific activity (see Table X) and overall degree of purification are very close to those obtained by Murison (72). In addition, the incorporation of L-valine-$^{14}C(u)$ into XDH (see Table XIII) was very nearly the same as that obtained by Murison (72), who used a completely different technique of selective antibody precipitation.

Synthesis of Chicken Liver Xanthine Dehydrogenase

In the experiments designed to determine the rate of synthesis of XDH, the radioactivity incorporated into protein at time t is proportional to the area under the specific radioactivity-time curve at time t (43).

Results can be treated mathematically as follows:

(1) $$\frac{P_t^* - P_0^*}{\int_0^t a_t^* dt} = k_1$$

since at time 0, $P_0^* = 0$ then

(2) $$\frac{P_t^*}{\int_0^t a_t^* dt} = k_1$$

since

(3) $$P_t^* = \frac{(P_t)(S)(P^*_{obs,t})}{(\lambda)(N)(m)}$$

and

(4) $$\int_0^t a_t^* dt = \frac{r}{(A_t)} \frac{\int_0^t A_t^* dt}{(\lambda)(N)}$$

then

(5) $$\frac{\frac{(P_t)(S)(P^*_{obs,t})}{(\lambda)(N)(m)}}{\frac{(r)}{(A_t)} \frac{\int_0^t A_t^* dt}{(\lambda)(N)}} = k_1$$

where

P_t = units of XDH at time t per gram of liver

S = specific absorption of XDH in gels at 280 mμ, expressed as area under the absorption peak of XDH per unit of XDH and is 20.8 cm^2/unit of XDH activity

$P^*_{obs,t}$ = radioactivity incorporated into XDH at time t per cm^2 of area under the XDH absorption peak at 280 mμ

$\int_0^t A_t^* dt$ = integral of the radioactive intracellular amino acid pool to time t

λ = time constant for carbon-14 is $\frac{2.37 \times 10^{-10}}{\text{minute}}$

N = 6.023×10^{23} atoms/mole

m = moles of arginine/mole of XDH or moles of valine/mole of XDH (72)

A_t = concentration of unlabeled intracellular amino acid per gram of liver present at time t

r = ratio of the intracellular liver amino acid dpm/gm liver at time t, for chicks used in the XDH synthesis study, to the intracellular liver amino acid dpm/gm liver at time t found on the standard amino acid vs. time curves in Figures 1, 3, 4 and 5.

Definitions of other symbols can be found on page 7 of this thesis.

Since $P^*_{obs,t}$ was obtained by measuring the radioactivity incorporated into XDH and dividing by the area under the absorption peak of XDH obtained from the polyacrylamide gel separation, the results are expressed in terms of dpm of XDH per cm^2 of area. Because of this, P had to be converted into cm^2 of XDH per gram of liver. Since 1 unit of XDH gave an absorption peak with an area of 20.8 cm^2, then the conversion of units of XDH per gram of liver into cm^2 of area per gram of liver was accomplished by the following multiplication:

(units of XDH/g liver) x (20.8 cm^2/unit of XDH) = XDH cm^2/g liver

Knowing the amino acid content of XDH (72), $P^*_{obs,t}$ can be converted into moles of labeled XDH/cm^2 of absorption at 280 mμ,

as follows:

$$P^{*}_{obs,\,t} = \frac{\text{dpm of XDH/cm}^2 \text{ @ 280 m}\mu}{(\lambda)(N)\,\dfrac{(6.76 \text{ mole arginine})}{(\text{mole of XDH})}}$$

A_t represents the moles of intracellular amino acid per gram of liver present in the chick liver. This varies during development (see Table IV).

The values for the $\int_0^t A_t^{*} dt$ (see Table XI) were obtained by integrating the intracellular amino acid dpm vs. time curves shown in Figures 1, 3, 4 and 5. The integrations were done by cutting out the areas under the curves and weighing the paper.

Since chicks used for studying the rates of synthesis of XDH received different amounts of radioactive label than the chicks used for studying the kinetics of the intracellular radioactive amino acid pool, it was necessary to correct for this. The following ratio was used to correct for these variations:

$$\text{ratio (r)} = \frac{\text{dpm per gram of liver in the intracellular amino acid pool of the chicks used in the XDH synthesis study at time t}}{\text{dpm per gram of liver in the intracellular amino acid pool of the chicks used in the study of the kinetics of the intracellular radioactive amino acid pool at time t}}$$

By multiplying these ratios by the values obtained for the $\int_0^t A_t^{*} dt$ (see Table XI), the time integrated radioactive amino acid pool for each chick used in the study of XDH synthesis was determined.

FOOTNOTES FOR TABLE XI

[a] The specific radioactivity was determined by measuring the amount of radioactivity of XDH in polyacrylamide gels and measuring its absorbance at 280 mμ, using a scanner and a Beckman monochromator with a Gilford attachment. The cm^2 refers to the area under the absorbance peak of XDH at 0.5 optical density full scale.

[b] This is the area under the XDH absorption peak per unit of enzyme activity applied to the polyacrylamide gel. Units are in μmoles NADH formed per minute per mg protein. Area is in cm^2.

[c] Units are in terms of μmoles NADH formed per minute. The values were obtained from Table VIII this thesis.

[d] Values were obtained from Table IV this thesis.

[e] These radioactive amino acid pool values were found in those chicks used in the study of the synthesis of XDH, and they represent the size of the radioactive amino acid pool at a time t after the injection of isotope.

[f] These radioactive amino acid pool values were obtained from Figures 1,3,4,5 this thesis, which were studies of the kinetics of radioactivity in the liver amino acid pools of the developing chick. These values represent the size of the pool at time t, after injection of isotope.

[g] The integration of the radioactivity versus time curve for Figures 1,3,4,5 was done by cutting out the curve from a piece of graph paper and weighing the paper. Each value represents the integration from time zero to time t.

[h] These values were obtained from Murison (72).

[i] For an explanation of the calculation of the rate of synthesis see pages 58-63 this thesis.

Table XI. Rate of synthesis of xanthine dehydrogenase during development.

Age of chick	C-14 labeled amino acid used	Incubation time (min)	$P^*_{obs,t}$ Specific radio-activity of XDH[a] (dpm/cm^2)	Absorbance of XDH at 280 mμ[b] (cm^2/unit)	P_t Pool size of XDH[c] (units/g liver)	A_t Intracellular amino acid pool size[d] (μmoles/g liver)
8 day chick	Arginine	120	3.308	20.8	0.84	0.596
		60	2.406	20.8	0.84	0.596
1 day chick	Arginine	120	36.7	20.8	0.177	0.92
		60	31.5	20.8	0.177	0.92
1 day chick	Valine	240	37.8	20.8	0.177	0.69
14 day embryo	Arginine	240	200	20.8	0.016	0.68
		120	226	20.8	0.016	0.68
		60	151	20.8	0.016	0.68

Intracellular amino acid radioactivity (dpm/g liver)			$\int_0^t A_t^* dt$		
XDH synthesis study[e]	Values from Figures 1,3,4,5[f]	r ratio	Integral of intracellular amino acid pool[g] from Figures 1,3,4,5 (Disintegrations)	m Molar amino acid content of XDH[h]	k_1 Rate constant for synthesis of XDH[i] (moles/minute g liver)
5,492	4,350	1.26	1.07×10^6	6.76	3.8×10^{-12}
7,752	5,870	1.32	0.754×10^6	6.76	3.7×10^{-12}
34,762	10,000	3.48	1.97×10^6	6.76	2.7×10^{-12}
52,114	12,500	4.17	1.30×10^6	6.76	2.9×10^{-12}
4,057	3,060	1.33	1.32×10^6	7.79	7.0×10^{-12}
26,728	1,060	25.2	3.68×10^5	6.76	7.2×10^{-13}
36,408	1,220	29.8	3.02×10^5	6.76	8.4×10^{-13}
33,572	1,430	23.4	2.25×10^5	6.76	9.6×10^{-13}

In order to convert the dpms in the intracellular liver amino acids into moles of labeled amino acids, the dpms in the radioactive intracellular liver amino acids are divided by λ and N, which have the same values as given previously.

The values for the rate constant of synthesis of XDH during chick liver development are given in Table XI. When arginine is the labeled amino acid, the rate constant for XDH synthesis is low in the embryo, increases about 4 fold immediately upon hatching and reaches a maximum by the eighth day after hatch. The rate constant when valine was the substrate is 2.5 times greater in the 1 day chick than the value obtained with arginine.

Rate of Degradation of Xanthine Dehydrogenase and Soluble Protein

According to Koch (56), total radioactivity will decrease exponentially in a growing system and the rate constant calculated will be a measure of the degradative rate. The total radioactivity of XDH can be obtained in the following manner:

$$P_t^* = \text{total dpm in XDH} = (P^*_{obs,\,t})(S)(P_t)(W_t)$$

where

$P^*_{obs,\,t}$ = dpm in XDH at time t/cm^2 @ 280 mμ

S = specific absorption of XDH in gels at 280 mμ and is 20.8 cm^2/unit of XDH activity

P_t = units of XDH at time t per gram of liver

W = wet weight of liver at time t.

The total radioactivity for the soluble liver proteins is obtained by the following formula:

$$P^*_{total} = \frac{\text{dpm in the total soluble protein}}{\text{gram of liver}} \times \text{wet weight of liver in grams}$$

The results of a plot of the $\log_{10}$ (dpm) vs. time are shown in Figures 11-16 and Table XII.

The rise in the value of $\log_{10}$ (dpm) with time in Figures 11 and 13 for the 1 day chick and 14 day embryo can be explained on the basis of no degradation of XDH occurring, accompanied by a continued synthesis of labeled XDH from label that remains in the amino acid pool. The label in the amino acid pool may have arisen from protein breakdown followed by re-utilization of these amino acids for further protein synthesis. Because of this result, i.e., re-utilization of L-(guanido-^{14}C)-arginine, the values for the rate of degradation of XDH and the total soluble proteins are to a certain degree underestimated. This is in contrast to results with rat liver, where little or no re-utilization occurs (102). These differences may be due to the fact that chickens are uricotolic animals, which excrete mainly uric acid instead of urea, and by the findings (31, 84) that levels of arginase are

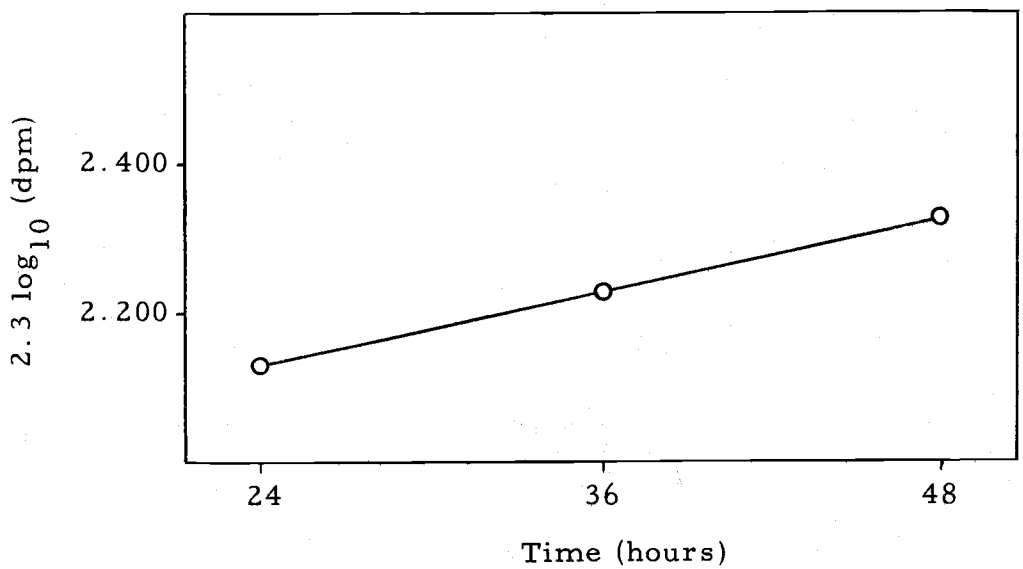

Figure 11. Degradation of liver xanthine dehydrogenase in the 14 day old embryonic chicken.

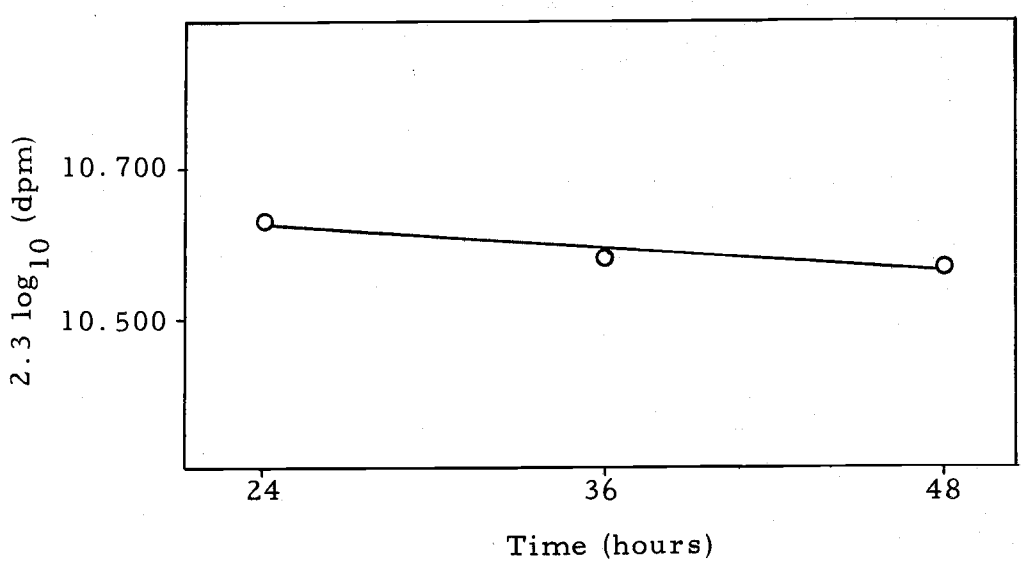

Figure 12. Degradation of liver total soluble protein in the 14 day old embryonic chicken.

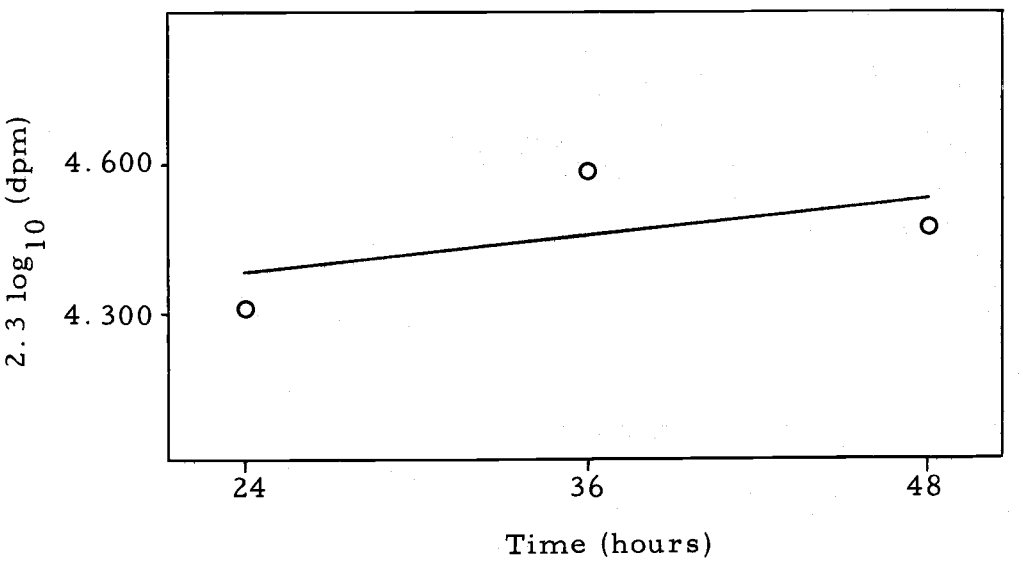

Figure 13. Degradation of liver xanthine dehydrogenase in the 1 day old hatched chicken.

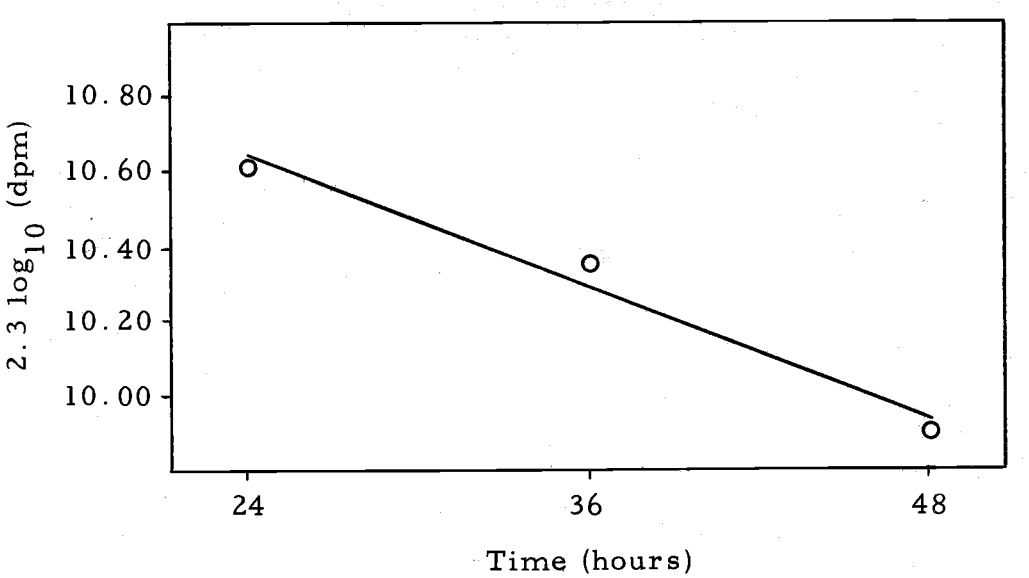

Figure 14. Degradation of liver total soluble protein in the 1 day old hatched chicken.

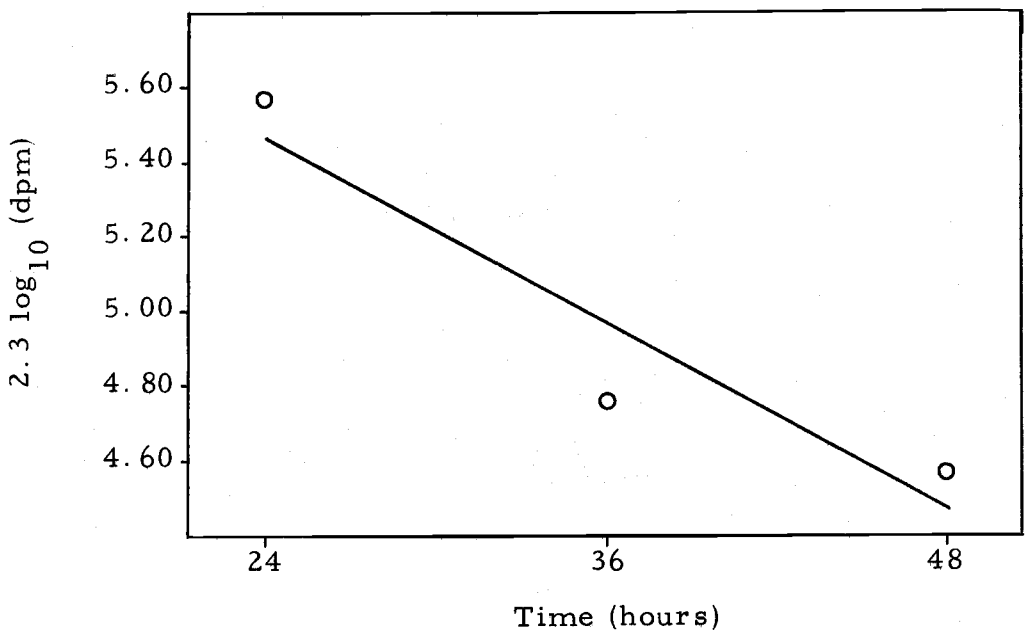

Figure 15. Degradation of liver xanthine dehydrogenase in the 8 day old hatched chicken.

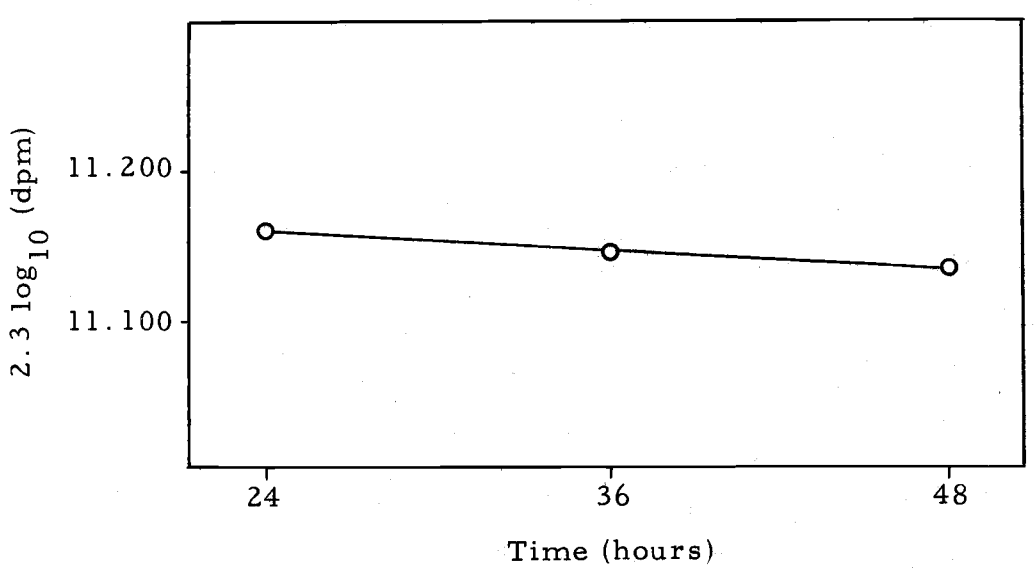

Figure 16. Degradation of liver total soluble protein in the 8 day old hatched chicken.

Table XII. Rate of degradation of xanthine dehydrogenase and total soluble protein.

		XDH						Total Soluble Protein		
Age of chick	Time after Injection (hours)	Specific absorbance of XDH in gels[a] (cm^2/unit)	Liver Wet Weight (gram)	Specific Radioactivity of XDH[b] (dpm/cm^2)	Pool Size of XDH[c] (units/g liver)	Rate Constant for Degradation[d] ($hour^{-1}$)	$t_{\frac{1}{2}}$ (hours)	Specific Radioactivity (dpm/g liver)	Rate Constant for Degradation[d] ($hour^{-1}$)	$t_{\frac{1}{2}}$ (hours)
8 day chick	48	20.8	1.78	3.14	0.84	4.14×10^{-2}	17	38,993	1.15×10^{-3}	600
	36	20.8	1.73	3.91	0.84			40,566		
	24	20.8	1.68	9.00	0.84			42,448		
1 day chick	48	20.8	1.147	8.337	0.44	0	∞	17,567	2.97×10^{-2}	23
	36	20.8	1.024	14.90	0.31			31,017		
	24	20.8	0.902	22.19	0.18			45,606		
14 day embryo	48	20.8	0.220	140	0.016	0	∞	179,372	2.58×10^{-3}	270
	36	20.8	0.194	146	0.016			206,265		
	24	20.8	0.168	151	0.016			250,210		

[a] One unit of XDH in polyacrylamide gel has an area under its absorption peak of 20.8 cm^2 at 280 mu and 0.5 O.D. full scale using a linear scanner.

[b] The specific radioactivity was determined by measuring the amount of radioactivity of XDH in polyacrylamide gels and measuring the absorbance at 280 mu. The cm^2 refers to the area under the absorbance peak of XDH at full scale 0.5 O.D. Isotope used was L-(guanido-^{14}C)-arginine.

[c] Units are in terms of umoles NADH/g liver.

[d] Derived from a least squares fit of the data.

very low in the adult chicken liver and whole embryo.

Even though there is no measurable turnover of XDH in the embryo, turnover of proteins is taking place in the embryonic liver, since there is a significant degradation of total soluble proteins with a $t_{\frac{1}{2}}$ of 270 hours. In the 1 day chick, the $t_{\frac{1}{2}}$ for soluble protein is 23 hours, indicating that the rate of degradation of liver proteins is greatly increased upon hatching. It is quite significant that XDH still has no measurable turnover rate, even in the presence of extensive degradation of the soluble protein pool where XDH is found. By the eighth day, there is degradation of XDH with a $t_{\frac{1}{2}}$ of 17 hours. The total soluble proteins in contrast, turns over less than was found in the 1 day chicks, the $t_{\frac{1}{2}}$ being equal to 600 hours.

It is quite clear, that although degradation is a random first order process, its controls make it selective for certain proteins and the controls change during development.

The Rate of Synthesis of Xanthine Dehydrogenase Using Labeled Arginine and Valine as Precursors

An attempt was made to compare the results of the procedure used in this thesis to the results published by Murison (72). As shown in Table XIII, the values obtained for the relative incorporation of L-valine-^{14}C(u) into XDH for the 1 day chick are nearly the same using either the technique of selective antibody precipitation or

Table XIII. Comparison of the rate of synthesis of xanthine dehydrogenase using L-(guanido-^{14}C)-arginine and L-valine-^{14}C(u) as isotopes.[a]

Age of Chick	Isotope	Disintegrations per Minute per Gram of Liver		Relative Rate of Synthesis[b]	Absolute Rate of Synthesis[c] ($\frac{\text{moles}}{\text{min-gram liver}}$)
		XDH	Soluble Protein		
1 day chick	L-(guanido-^{14}C)-arginine	118	133,000	0.9	2.8×10^{-12}
1 day chick	L-valine-^{14}C(u)	142	81,129	1.7	7.0×10^{-12}
1 day chick[d]	L-valine-^{14}C(u)	500	245,000	2.0-2.5	

[a] Blank space in the table indicates that no determination was made.

[b] This ratio is in terms of XDH dpm $\times 10^{+3}$/dpm of soluble protein.

[c] This data is from Table XI of this thesis and is in terms of moles XDH/minute-gram liver.

[d] This data was obtained from Murison (72).

enzyme purification.

It was of interest to determine if the relative rate of incorporation of L-(guanido-^{14}C)-arginine would agree with that obtained with L-valine-^{14}C(u), since arginine and valine and found in roughly the same quantities in XDH being 6.76 and 7.79 respectively (72). The results are shown in Table XIII. Considering that the relative rate of incorporation is 0.9 for arginine and 1.7 for valine, it is obvious that they do not agree.

In order to compare the absolute rate of synthesis of XDH using L-valine-^{14}C(u) as substrate with the rate obtained using L-(guanido-^{14}C)-arginine as substrate, the kinetics of the radioactive intracellular liver valine pool was studied for the 1 day chicken (Figure 4). The rate constants were calculated and are shown in Tables XI and XIII. These results also show that the rate constant for synthesis of XDH obtained with arginine is 1/2 that obtained with valine.

Thus, on the basis of two independent measurements, one based on the time integrated radioactive amino acid pool and the other on incorporation of isotope relative to total soluble protein, the rate of synthesis of XDH is different when studied with L-(guanido-^{14}C)-arginine and L-valine-^{14}C(u).

Since the results should be the same, either the technique is not accurate enough to measure precisely the actual rate of synthesis or some of the assumptions made in setting up the mathematical model

are invalid.

One obvious point is that the system is not in a steady state and that the results are at best only approximate values. Another possibility is that the amino acids from the total free pool are not utilized for protein synthesis. Separate compartments of amino acids may exist (75) which could possibly complicate the determination of the rate of synthesis based on measurements of the total specific radioactivity pool. In addition, it has been shown (43) that amino acids may be utilized directly from the extracellular amino acid pool. Gan (25) has shown that amino acids derived from protein breakdown are re-utilized for protein synthesis. In this thesis (Table VI), it was shown that the labeled amino acids may be metabolized. If the products of the catabolism are other labeled amino acids, then the true rate of synthesis can not be easily obtained, since the determination of the rate of synthesis is based on a knowledge of the specific radioactivity of one amino acid.

DISCUSSION

Rates of Synthesis and Degradation of Xanthine Dehydrogenase and Soluble Protein During Chicken Liver Development

It is of interest to know why the levels of chicken liver XDH change during development. The results of this thesis show that the low level of XDH in the embryo is a result of a low rate of synthesis of XDH. The dramatic increase in XDH upon hatching is due to an increase in the rate of synthesis with little or no accompanying degradation, thereby achieving a rapid accumulation of enzyme in a short period of time. Although synthesis is maintained at this high level, the constant level of XDH at 8 days is accomplished by turning on of the degradative process. These results are in agreement with those obtained by Murison (72).

The total soluble liver proteins follow, however, a different pattern of controls than does XDH. When the level of synthesis of XDH is low in the embryo, there is a very high rate of soluble protein synthesis (Table VII). There is a small but significant turnover of soluble protein in the embryo but little or no turnover of XDH. Upon hatching, the rate of synthesis of XDH increases while the synthesis of soluble protein remains high. There is a dramatic increase in the turnover of the total soluble proteins upon hatching, while XDH exhibits little or no turnover. After 8 days when the XDH level is high,

the total soluble proteins have the same high rate of synthesis and a decreased rate of turnover from the 1 day chick, while XDH shows its highest rate of synthesis and its highest rate of turnover. It is, therefore, apparent that synthesis and turnover of the total soluble proteins and XDH are subject to developmental controls.

Previous studies have shown that the turnover of chick liver XDH is, also, subject to nutritional controls (18, 93, 100). Chicks starved for 24 hours showed no loss of activity, indicating that XDH was selectively spared from degradation. High protein diets lead to an increase in XDH activity (93). Inosine stimulation of XDH activity is prevented by puromycin and actinomycin D, indicating that inosine stimulates XDH synthesis (18, 100). Xanthine does not stimulate synthesis of XDH (1). Curtis (14) has shown that starvation causes a decreased rate of synthesis of pancreatic XDH and subsequent refeeding causes a rapid increase in the rate of synthesis of pancreatic XDH.

All these studies show that degradation as well as synthesis play an important role in determining the levels of enzymes.

Control Mechanism at the Level of Synthesis

This thesis has shown the importance of synthesis in determining the levels of XDH in the chicken liver. It is of interest to know how a cell can control the changes in the rate of synthesis of proteins

during development.

There are many possible mechanisms for controlling the rate of synthesis of proteins. Of particular interest, for this study, is the role of hormones. Fisher (22) showed that pituitary extracts can induce an increase in embryonic chicken liver XDH. Presumably then, a hormonal signal causes the increase in the synthesis of liver XDH upon hatching. Greengard (32) has suggested that hormones are probably the natural stimuli for the induction of enzymes immediately following birth.

Hormones have been shown to increase the rate of synthesis of many enzymes. For example, glucocorticoids stimulate the level of tyrosine transaminase (61), hydrocortisone, insulin and glucagon may stimulate different isozymes of tyrosine transaminase (44), glucocorticoid hormones stimulate synthesis of glutamic-alanine transaminase, glutamine-tyrosine transaminase and tryptophan pyrrolase (55).

Recent studies have elucidated possible sites of hormonal action. Plasma membrane sacs of isolated rat fat cells possess an adenyl cyclase system which is activated by adrenocorticotropin, glucagon and epinephrine. Hormone stimulation depends on hormonal selectivity factors which are coupled to adenyl cyclase in the membrane (11). Diethylstilbesterol binds to a nucleoprotein component in the calf uterus (69), estradiol binds to specific proteins in the rat uterus (96).

Steroids bind to histones in vivo by non-covalent binding to hydrophobic groups which are located on C-terminal regions of arginine-rich histones (97). Oestrogen complexes with a specific 9S receptor in the cytoplasm but is rapidly translocated to the nucleus. The 9S receptor is composed of a 4S oestrophilic binding unit and a non-oestrogen binding unit (71). Dexamethasone stimulates the increase in the rate of synthesis of tyrosine aminotransferase in rat hepatoma cells. Steroids antagonize a repressor which, inhibit m-RNA translation and promotes m-RNA degradation (105). A soluble cytoplasmic macromolecular steroid-binding fraction was identified from a line of rat hepatoma cells in which tryosine transaminase is induced by adrenal steroid hormones (27). Histones and acidic nuclear proteins may interact with DNA to promote the synthesis of tissue specific RNA (28). Cyclic-AMP stimulates histone phosphorylation in a cell free liver preparation (59). Cyclic-AMP is known to mediate the synthesis of tyrosine transaminase, serine dehydratase and phosphoenolpyruvate carboxykinase.

In addition, there are many other mechanisms by which a cell can regulate the rate of synthesis of proteins. Only a few of these will be mentioned.

Extensive gene duplication may cause enzyme induction (12). Rapid intranuclear RNA turnover within the nucleus (5, 86, 94) implies that regulation depends on transporting a nuclear m-RNA into the

cytoplasm. Synthesis of a specific m-RNA in response to a substrate has been shown to regulate the rate of synthesis of particular proteins in bacteria (47). Regulation of protein synthesis may occur by selective stabilization of m-RNA, since m-RNA turnover has been found to be heterogeneous (80, 83). Translation of m-RNA may yet be another site of control (24). Amphibian eggs prior to fertilization synthesize long lived m-RNA (19). Additional post transcriptional controls may include specific regulatory proteins involved in the association of m-RNA to polyribosomes and alterations in the function of specific factors involved in the initiation or termination of protein synthesis (105). For a more complete review of this subject, the reader is referred to the review article written by Schimke (90).

Control Mechanisms at the Level of Degradation

Little is known about the process of degradation and its control mechanisms. There exists a wide variety of degradation rate constants among proteins. Although degradation is a random process, except in the case of hemoglobin, its rate changes through developmental, dietary and hormonal changes (This Thesis, 41, 93, 100).

Several mechanisms are thought to be involved. One may involve the conformational state of the protein. Interactions with cofactors, substrates etc. confer to the protein either stability or lability toward degradative enzymes. Some conformations may

expose labile peptide bonds (35).

A number of proteolytic enzymes found inside cells can hydrolyze proteins to amino acids without an energy requirement. Conformational changes and/or denaturation could make proteins susceptible for metabolism by these proteolytic enzymes (2). In perfusion experiments in rat liver (29), it was found that heat denatured bovine serum albumin was degraded faster than undenatured albumin. Tryptophan pyrrolase is degraded rapidly in vivo. Its substrate, however, stabilizes the enzyme and prevents its degradation. L-tryptophan stabilizes the purified enzyme. Two binding sites have been proposed, one at the active site and the other at a site which affects the changes in the protein conformation (91). Changes exerted by oxygen on the conformation of hemoglobin affects its resistance to attack by proteolytic enzymes (110). Glucose protects hexokinase against inactivation by trypsin (7). Co-factor stabilizes serine dehydratase against degradation (54).

In many tissues, proteolytic enzymes are found not only in the lysosomes but also in the supernatant. β-glucuronidase is found in both the lysosomes and in the endoplasmic reticulum (106). Areas of the endoplasmic reticulum situated in the golgi region may be the site of intracellular digestive processes (17). Acid phosphatase, cathepsin, β-glucuronidase and acid ribonuclease activity were found in both the sediment and supernatant in a high-speed centrifugation of Müllerian

duct homogenates from chick embryos (85). The degradation of proteins may, therefore, be controlled by the presence of effectors which act to protect or labilize the protein in the presence of these proteolytic enzymes. Grisolia and Schimke have reviewed this aspect of degradation (35, 90).

Another mechanism for degradation may envolve the maintenance of the degradative system through energy requirements, synthesis of molecules for the degradative process, transport through the cell and maintenance of the structure of organelles such as lysosomes (90).

Anaerobic conditions during incubation of liver slices diminished the degradation of total protein (91). The degradation of albumin by a rat liver particulate system requires CoA and ATP (78). Turnover of cellular constituents might be controlled by the release of lysosomal enzymes (74). Androgen liberates several catabolic enzymes during the regression of the oviducts of the male chick embryo (85). The appearance of the activity of hydrolytic enzymes may depend upon their release from the lysosomes. Vitamin A deficiency in pregnant rats causes rat embryos to have incomplete regression of aortic arches, and thyroxin initiates tissue regression during metamorphosis, perhaps by lowering the stability of the lysosomes and promoting the release of hydrolytic enzymes (41). Vitamin A, pregnanolone and progesterone labilize lysosomes, while corticosteroids stabilize

them (107).

Catabolism may be dependent on a structural component of the degradative system (13). This structural component required in protein catabolism may require energy for its maintenance. Protein catabolism requires energy (13, 57) and it is linked to protein synthesis (53).

In addition to structural and energy requirements, there have been found proteins which inactivate or inhibit specific enzymes including rat liver ribonuclease (8) and glucose-6-P dehydrogenase (9).

There are then many levels of control in the process of degradation of proteins. The rate constants for degradation of proteins indicate that the metabolism of new as well as old molecules occurs with equal probability. It is evident, however, from previous studies, (54, 91, 93) that certain proteins can be protected while other proteins are rapidly degraded, even though the process of degradation envolves random destruction of macromolecules. Proteases found in the cytoplasm (85, 106) could cause protein destruction by either hydrolyzing the proteins down to its constituent amino acids or by nicking them so that they are denatured and could be selectively taken into the lysosomes for complete hydrolysis. As discussed previously and reviewed by Grisolia (35), selectivity could be attained by altering conformational states of proteins by substrates, co-factors, hormones, etc. making certain conformations more or less susceptible to proteolytic

attack than others.

The energy requirement for proteolysis (13, 57, 78, 91) could be explained by an energy requirement for the transport of proteins destined for degradation into the lysosomal particle, the maintenance of the structure of the particle and the synthesis of proteolytic enzymes (13). The overall rate of degradation may be dependent on the activity of the degrading system (90).

There must be a physiological need for a proteolytic system, such as the lysosome, otherwise, the system would have been lost through evolution. DeDuve (17) has an excellent review on the function of lysosomes. Among these are functions that depend on heterophagy, autophagy, cellular breakdown, as found in fertilization and metamorphosis, food processing, breakdown of secreted macromolecules, immunity, detoxification, scavenging and thrombolysis. One of the most interesting functions is that used to maintain long lived cells. Turnover maintains homeostasis in cells by replacing cellular decay products thus eliminating the need for the cell to design "immortal molecules" that are not subject to denaturation or breakdown.

Critique of Methodology

Re-utilization of isotopes is the major limitation of methods based on the decay of radioactivity after a pulse label of a radioactive amino acid (90). Gan (25) showed that amino acids in rat liver may be

derived from intracellular protein breakdown and may result in over-estimated rates of degradation. The degree of error will be a function of the extent of re-utilization and will be greater for those proteins with a rapid rate of degradation (90). L-(guanido-^{14}C)-arginine was found to be re-utilized to a certain extent in this thesis and in the measurement of the rate of degradation of catalase (81).

Implicit in the determination of the true rate of synthesis of an enzyme is a knowledge of the specific radioactivity of the amino acid pool. If the products of catabolism of the labeled amino acid are other amino acids, then the true rate of synthesis cannot be easily obtained.

The possibility, that L-valine-^{14}C(u) and L-(guanido-^{14}C)-arginine are metabolized in the chicken, is shown in Table VI. The pathway for valine degradation was described by Mahler (66, p. 690). Being a glycogenic amino acid, it has as an end product succinyl-CoA, which via the tricarboxylic acid cycle can be catabolized to pyruvate and then to glucose. The products of glucose can then become substrates for other amino acids. The metabolism of the guanidine portion of arginine is not well established in avian species. They are uricotolic animals which excrete mainly uric acid with only a small proportion being urea (84, p. 12-14). Only very low arginase levels have been measured in chickens (84, p. 36; 31).

Separate compartments of amino acids may also complicate the determination of the rate of synthesis of proteins based on specific

radioactivity measurements of the total intracellular amino acid pools. Separate compartments were found that isolate metabolites participating in competing metabolic sequences (75) and another study showed that amino acids from the extracellular amino acid pool may even be utilized for protein synthesis (43). There may then exist several different protein precursor amino acid pools.

Because of the above and the fact that the developing chick is not in a steady state, the rates of synthesis and degradation can be considered only approximate values and not true absolute rates.

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APPENDIX

APPENDIX

Solutions for Disc-gel Electrophoresis at pH 4.3.

A)	1 N KOH	48 ml	B)	1 N KOH	48.0 ml
	Glacial acetic acid	17.2 ml		Glacial acetic acid	2.87 ml
	Temed	4.0 ml		Temed	0.46 ml
	H_2O to 100 ml pH 4.3			H_2O to 100 ml pH 5.7	
C)	Acrylamide	30.0 gm	D)	Acrylamide	10.0 gm
	Bisacrylamide	0.8 gm		Bisacrylamide	2.5 gm
	H_2O to 100 ml			H_2O to 100 ml	
E)	Riboflavin	4 mg	F)	Ammonium persulphate	0.28 gm
	H_2O to 100 ml			H_2O to 100 ml Made fresh weekly	

Lower gel	Upper gel
1 part A	1 part B
2 part C	2 part D
1 part H_2O	1 part E
4 part F	4 part H_2O

Buffer 10X

B-alanine	31.2 gm
Glacial acetic acid	8.0 ml
H_2O to 1000 ml pH 5.0	

0.1% Methyl green (previously cleaned with chloroform) was used as an indicator.

Anode -- top reservoir
Cathode-- bottom reservoir