A Study of Representative Metabolic Enzymes During Development of the Chick Embryo

by

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Submitted to the Department of
Anatomy and the Faculty of the
Graduate School of the University of Kansas
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy.

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September, 1955

ACKNOWLEDGMENTS

I wish, especially, to thank Dr. B.S. Wenger who originally suggested the problem and followed it critically throughout the actual investigation and the preparation of the thesis.

For the use of the departmental facilities at Kansas, I am greatly indebted to Dr. P.G. Roofe, head of the Anatomy Department at Kansas. I also want to express my appreciation for the helpful suggestions and stimulating conditions provided by Dr. A. Lazarow, head of the Department of Anatomy at Minnesota where a large part of the experimental work was carried out.

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INTRODUCTION

For a more complete understanding of morphogenesis, it is imperative to correlate biochemical, physiological and morphological events occurring during ontogenesis. Investigations along these lines have been undertaken in various species such as guinea pig (Flexner et al, '48, '50, '53), amphibian (Sawyer, '43) and the sea urchin (Gustafson, '54). The following study is one of a series being carried out in our laboratories in an attempt to further analyze the biochemical modifications underlying observable morphological and physiological changes in the chick embryo.

It has been postulated that modification of the activities of specific enzymes in developing embryonic tissue are related to the functional roles characterized by those enzymes (Moog, '52). An enzyme which is limited in location to a certain type of adult tissue would be expected to show a pattern of development in that particular tissue differing from that in other, more general, sites. In contrast, enzymes whose activities are more universal in location would be expected to have similar developmental patterns in all tissues.

Taking the above into consideration, it appeared worthwhile to investigate in a specialized tissue the development of enzymes which might be considered "constitutional." Due to its specialization, relatively large size

and long period of development, the central nervous system of the embryo offered a suitable tissue for such a study. Also, it has been reported that in the central nervous system of amphibian embryos, cholinesterase, an enzyme accepted as characteristic of nervous tissue, accumulates at a rate different from other, less, specific enzymes (Sawyer, '43, Beoll and Shen, '50). Its measurable activity differs with spatial location in the nervous system of chick embryos (Wenger, '51, Nachmansohn, '39). This nonlinearity of accumulation has been accredited to the specialized functional role of cholinesterase in nerve tissue by these authors. It was decided to compare such observations with the results one might obtain from a study of enzymes more generally distributed than cholinesterase. The recent description (Lowry et al, '54b) of a series of microchemical techniques adaptable to the minute quantities of tissue available has made it possible to carry out such biochemical determinations on separate embryonic organs of the chick.

In order to cover more nearly the total metabolic pattern, representative enzymes active in the glycolytic cycle, the tricarboxylic acid cycle and the electron transport system were selected. These were aldolase, succinic dehydrogenase and cytochrome oxidase respectively. Tissues selected for this study were taken from portions of the central nervous system and from the developing heart and liver. It was felt that the results of such a survey would

give both an indication of the basic metabolic patterns in nervous tissue and the manner in which these patterns differ in non-nervous structures.

HISTORICAL REVIEW

I. Early studies on enzyme content of embryonic chick tissue

Early work relating specific enzyme activities to the development of the chick must be accepted with caution. Due to the crude methods of analysis and the selection of poor bases of reference, much of the work reported previous to the past decade appears to be of historical value only. Most of the early observations are merely indications of the absence or presence of a particular enzyme through the use of a negative or positive sign. Quantitative data are missing for the most part and, if present, are related only to the entire egg as a basis for the determination. All in all, the studies made during the early period of enzymatic embryology must be considered severely limited in value.

Contemporary criticism of the era was stated eloquently by Needham in his introduction to enzymatic studies throughout ontogenesis ('24). The following quotation taken from his work, written a quarter of a century ago, exposed the vulnerability of enzyme quantitation and has sounded an admonition to further work in the field.

"A good deal of the work which must be mentioned in this section seems to have been inspired by the idea that, if one could get hold of the original egg-cell, no enzymes would be found to be present at all, and that they all

arise, one after the other, in a kind of ontogenic procession from an ovum completely innocent of any. This idea has only to be stated for its absurdity to be recognized. . . . It seems likely that the embryonic body starts life with an assortment of fundamental enzymes to which, as development goes on, certain others are added, and from which possibly some are subtracted. One has the uncomfortable feeling throughout that a (reported) negative result as often as not may mean no more than the method employed in the case in question was not sensitive enough to discover the presence of the enzyme so early in development. . . . In this way there may be nothing at all final about most of the (negative) data contained in this section. . . ."

One of the first of the early systematic studies of enzymes in the chick embryo was attempted in 1907 by Herlitzka. Her results are expressed solely by an indication of the presence or absence of particular enzymes. According to the observations reported in this paper, lactase, peroxidase and catalase were found in the embryo, but not in the yolk of the infertile egg, whereas amylase was found in both the embryo and infertile egg yolk. Glycolytic enzymes were reported to be entirely absent in both embryo and yolk.

In addition to these general enzymes, Aberhaldene and Steinbeck ('10) demonstrated the presence of pepsin in the ten day chick embryo. Again, only the presence of the enzyme was cited without any evaluation of quantity.

Bone phosphatase in the chick was studied by Kay ('26), in the ossifying cartilage present by the twelfth day of incubation. This was judged to be the earliest stage at which phosphatase determinations could be made. No measure of activity was given, save the observation that the phosphatase content per gram of bone in the embryo was three times that in the bone of the newborn chick.

In 1928, Pryzlecki and Rogalski, studying the enzymes of yolk, detected xanthine oxidase in chick embryos of seven days' incubation. The same article included uricase determinations on these animals. This latter enzyme was detectable after the second day of incubation.

Probably the most complete and enlightening research of the work done prior to 1930 was that of Galvialo ('26) who, by use of micro methods, extracted an amylase, a protease and a lipase from a one day chick embryo. Not only did he use individual samples for each of his determinations, but he also referred the observed activity to a standard nitrogen unit. For the first time one could compare the results of a study on some basis other than that of the whole egg. The activities of the three enzymes prepared by Galvialo from this primitive streak embryo were greater than the corresponding activities of the same enzymes in horse blood. Thus, his work indicated that embryonic cells, however young, do contain certain fundamental enzymes in amounts comparable to adult tissue. This was the first experimental observation of the views expressed by Needham

in the quotation given above.

In an article describing the sulfhydryl dependence of embryonic tissue for growth, Sachs and his collaborators ('33) mentioned in a short note that dehydrogenation of hypoxanthine, succinic acid, citric acid and lactic acid could be observed in embryonic tissues. The preparation used by the author was an extract of an eight day chick embryo prepared for tissue explant studies. However, no data were presented relative to the comparative amounts of the dehydrogenase activities reported.

In 1935, Booth while attempting to prove the identity of xanthine oxidase and the Schardinger aldehyde oxidase enzyme, investigated the time at which each of these enzymes appeared in the chick embryo. He reported the time of appearance to be identical in both cases--on the third day in the yolk sac and by the ninth day in the embryo. Booth tested other substrates to be sure that what he had observed was not a non-specific dehydrogenase. In the process, three other enzymes were described as being present in the eight-day old embryo. These were succinic dehydrogenase, lactic dehydrogenase and glucose dehydrogenase. Once more, no indication as to the amount of individual enzyme activity present was given.

Mystkowski ('36) studied the appearance of cathepsin in chick embryo tissue using gelatin, ovalbumin and lecithovetellin as substrates. The age of the embryo extracts used as an enzyme source varied from four days to

nineteen days of incubation. In the 18-day embryo extracts were made from individual tissues, i.e., liver, kidney and "head parts." Apparently, this was the first reported use of selected portions of the embryo for enzymatic determinations. The results revealed very little modification of cathepsin activity during the entire developmental period.

During 1937 the most complete biochemical work on carbohydrate metabolism of developing chick tissue was published in a series of articles by Needham and his associates ('37a, b, c). Although destined later to be critically reevaluated, this work was monumental in two respects. First, it was primarily an embryological study of intermediary metabolism, rather than an offhand observation of embryonic material as part of a more general report on tissue enzyme content. Secondly, the work involved an organized approach to the entire problem of intermediate carbohydrate metabolism differing from the poorly related determinations employed previously.

In this work, Needham and his co-workers concluded that in the chick embryo there existed four gaps in the phophorylation route of glycolysis. The first of these reported deficits ('37a) was deduced from the fact that glycogen was not converted to lactic acid by the embryo. This was interpreted as indicating a lack of the necessary enzyme(s) for the hydrolysis of glycogen. Also, of a series of carbohydrates tested, only glucose and mannose were metabolized ('37c). Since the embryo resembled brain tissue

in this respect and since the proportion of nervous to non-nervous tissue was exceedingly large in the embryos, the experiments were rerun using only trunk or limb bud material. The results obtained with these selected tissues were similar to those obtained with the whole embryo.

The second major observation ('37b) was that coenzyme 1 (diphosphopyridinenucleotide)* was not demonstrable in the enzyme. Negative results using an enzyme system dependent specifically on DPN as a coenzyme, led to this conclusion. On the other hand, the presence of coenzyme II in the embryo was established through positive experiments with the glucose dehydrogenase system. In the same article ('37b) the quantity of adenyl pyrophosphate found in the phosphorous fraction of the embryo extracts was assumed to be insufficient for phosphorylative glycolysis.

The last of the deficiencies reported was the absence of the enzyme, "dismutase," which would convert the triose phosphates, dihydroxyacetone phosphate and 3-phosphoglyceraldehyde to lactic acid ('37b). Other component enzymes of the cycle up to and including aldolase were found to be present in sufficient quantities for phosphorylative glycolysis.

As will be noted further in this review, these observations must be reappraised in the light of some later studies by Novikoff, Potter and LePage ('48) and Meyerhof

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*In this paper the following abbreviations will be used: DPN = diphosphopyridinenucleotide; ATP = adenosinetriphosphate.

and Perdigon ('40).

In a comprehensive study of succinic oxidase system in some thirty tissues, Elliot and Greig ('38) included material from a seven day chick embryo. The QO_2 given for this embryonic tissue was five to ten times less active than any adult tissue studied. Apparently, since the emphasis in this study was directed toward neoplastic mammalian tissues, no comments concerning this observation were offered by the authors.

Kleinzeller and Weiner also in 1938 studied the changes in catalase activity during the development of the chick embryo. They related the catalase activity to the wet weight of the chick embryo and concluded that the activity of this particular enzyme rose toward the end of development.

II. More recent studies on the enzyme content of embryonic chick tissues

A. Protein metabolism

Levy and Palmer in 1938 reported the distribution of dipeptidase activity in the cephalic region of the three day chick embryo. Embryos were cut on a freezing microtome and alternate sections were utilized for enzyme determination. The intervening sections were used to determine the volume of ectoderm, mesenchyme and cephalic fluid. The dipeptidase content was then related to these components. Ectoderm contained about three times the activity of mesenchyme while cephalic fluid did not have a demonstrable

amount of enzyme.

In 1940, the same authors did a more complete survey of chick embryo dipeptidase content, using material from embryonic chicks incubated for from one and a half days to 18 days. The age of the early embryos was estimated from the beginning of incubation and evaluated to the second decimal point in units of "days." Using this method of evaluating the age of the chicks, Levy and Palmer concluded that there was a period of rapid enzyme accumulation, up to the fourth day followed by a slower accumulation to the fifteenth day.

In a related article, Levy and Palmer ('43) described the development of amino peptidase in chick embryos from 1 1/2 to 19 days of incubation. The age of the embryo in this study was derived from an equation based on the log of the wet weight of the embryo. In this way, the authors felt that they eliminated the variables occurring between embryos of identical incubation age. The aminopeptidase study revealed a relative accumulation of this enzyme up to 4 1/2 days of age followed by a period of stability for three days and a rapid increase in accumulation from that time to the fifteenth day.

Recently, Rudineck and associates ('54) studied the glutamine synthetase and transferase enzymes in a survey attempting to elucidate the relationship of glutamine and glutathione metabolism to protein syntheses. Chick embryo material was selected by the authors because of the assumption that "in embryonic life the anabolic rate of protein

metabolism outweighs by far the katabolic rate." The results indicated that the transferase enzyme is detectable shortly after one day of incubation whereas glutamine synthetase could not be assayed in homogenates of embryonic tissue of any age. Also, interestingly, glutamine transferase activity in the embryo increases during incubation in a manner directly proportional to protein growth of the whole system. The final observation in this paper deals with the amount of glutamine transferase found in individual specialized organs such as liver and brain. In five day liver, the earliest isolated, the specific glutamine transferase activity is already high, but reaches a peak at ten days. In contrast, the brain shows only minimal transferase activity until the eighteenth day. These results point out the need for assays of individual tissues in order to have a more complete appreciation of the fluctuations of enzyme activities within the whole embryo.

B. Carbohydrate metabolism

By 1940 Meyerhof and Perdigon had demonstrated that early chick embryos (three to nine days) readily glycolyze hexose diphosphate forming lactic acid. This was in direct contradiction to Needham's earlier work ('37b) in which a triose phosphate dehydrogenase was postulated to be absent in the chick embryo of this age. Also in the same article and again in contradiction to Needham, Meyerhof concluded that chick embryos from four to nine days old contain at least 1.5 to 3 mg of DPN per gram of dry tissue.

In 1947 Stumpf reported finding certain phosphorylated intermediates in homogenates made from five, eight and ten day chick embryos. The author described the presence of fructose-1-6 diphosphate, phosphoglyceric acid and adenosinediphosphate from these extracts following barium precipitation. In the barium soluble but alcohol insoluble fraction, he found glucose-6-phosphate, fructose-6-phosphate and glucose-1-phosphate. Since these compounds appeared in approximately the same concentration in the three different age groups, it was concluded that the usual phosphorylating pathways do exist in the chick embryo.

Novikoff, Potter and LePage ('48) strengthened the above view by use of two experimental procedures on the embryos: 1) direct chemical analysis for the intermediates, much in the same manner as Stumpf, and 2) use of chick homogenates as enzyme sources in glycolytic systems utilizing glucose, hexose diphosphates, glucose-6-phosphate and fructose-6-phosphate as substrates. The direct chemical investigations which were carried out on three to ten day chick embryos resulted in positive findings of all of the phosphorylated compounds reported by Stumpf, plus certain others not previously found. These other compounds reported were triose phosphate, phosphopyruvic acid, ATP, phosphocreatine and pentose phosphate. In addition, direct chemical evidence was given for the presence of DPN. In those experiments utilizing the embryo as a glycolytic enzyme source, five to eight day embryos were shown to attack all the substrates in

a system properly fortified with DPN and ATP. Since this tissue appears to have the essentials for the normal pathway of phosphorylating glycolysis, Needham's postulate of a non-phosphorylating glucolytic pathway in the early chick would not seem necessary.

In a more recent note, Lenti and Cafiero ('51) have reported somewhat similar findings, with the addition that in their seven and eight day chick extracts, lactate is formed from glucose without the addition of DPN or ATP. Since their description of the preparation of the homogenates is somewhat vague, the question is still open as to whether ATP and DPN may not be present in adequate amounts within the living embryo.

C. Respiratory enzymes

Philips ('41) reported on the total oxygen consumption of early chick embryos. His study was limited to embryos of from four hours to 72 hours of age. Both anterior and posterior portions of these embryos were compared with one another and gave similar QO_2 values. Also, the oxygen consumption of a series of embryos incubated for 4, 15, 24, 36, 48 and 72 hours did not differ even when compared on a nitrogen basis. This rate was the same as that which was found in four, five and six day embryos. The author concluded that the rate of O_2 consumption of the early chick embryo when compared to adult mammalian tissues such as thyroid, testis and kidney was "neither unusually high nor unusually low."

Later, the same investigator ('42) using a Cartesian diver microrespirometer, compared the respiratory rates of different regions of the chick blastoderm incubated up to 37 hours. No correlation could be made between the QO_2 values observed and the regional differences known to exist in the embryo at this age. The rate of oxygen consumption appeared once more to reach a constant level after a short time interval.

Albaum and Worley ('42) studied the pattern of cytochrome oxidase accumulation in the chick embryo. The enzyme activity was assessed by measuring oxygen uptake in the presence of p-phenylenediamine and cytochrome in extracts of chick embryo incubated from 2-12 days. This oxygen consumption was then related to the total nitrogen content of the extract. The results led the authors to conclude that cytochrome oxidase did not appear in the embryo until the fourth day of incubation. However, after the seventh day there was a sharp increase of enzyme activity which continued until hatching.

Moog ('43) took exception to these findings, pointing out that this enzyme had been observed in lower forms at a comparable state in development. She claimed that the failure of Albaum and Worley to find cytochrome oxidase after two to three days incubation was due to the lack of sufficient sensitivity in their methods. Consequently, Moog attempted to demonstrate the presence of this enzyme in the early chick stages. She selected as a suitable

technique, the histochemical detection of the enzyme "indophenol oxidase" which is supposedly quantitatively dependent on cytochrome oxidase (Sumner and Somers, '53). The results of this investigation indicated the presence of the indophenol oxidase in chick embryos of 16 hours' incubation. The enzyme increased in its activity up to the middle of the second day. Addition of sodium azide, which theoretically would halt the action of cytochrome oxidase, did not exert a complete inhibitive affect during the period of study, possibly indicating the coparticipation of non-azide sensitive oxidases during the early stages in the chick.

In 1946, Albaum, Novikoff and Ogur related the development of cytochrome oxidase to the succinoxidase system in the chick. This was done in an attempt to distinguish between the two enzymes, cytochrome oxidase and succinic dehydrogenase, since previously it proved impossible to separate them by centrifugation of cytoplasmic particles. For the study, embryos of from 21 hours' to 21 days' incubation were used. The enzymes were assayed manometrically in Warburg vessels. It is interesting to note that up to 44 embryos were pooled for each run in order to obtain sufficient tissue for an indication of activity. Using this amount of tissue, Albaum et al reported cytochrome oxidase activity in chick embryos incubated for only 25 hours. The succinoxidase system, however, was not evident until the fourth day of incubation. The ratio of cytochrome

oxidase activity to succinoxidase activity was given for selected time periods. The ratio rapidly dropped from a high of 30-1 at four days, leveling off in the final stages to a 6-1 ratio. From this it was concluded that cytochrome oxidase differs from the succinoxidase system both in time of appearance and in rate of development.

Levy and Young ('48) restudied the problem expressing the enzyme activity on a dry weight basis. Using embryonic material of from three to 16 days of incubation, these authors reported that the enzyme activity did not accumulate on a scale directly proportional to dry weight. They chose as a means of expression a double log plot of age versus enzyme activity. This method of evaluation revealed a steady accumulation of the enzyme up to 4 1/2 days, followed by a period of constant activity and a second increase in rate after 11.8 days.

In 1954 Sippel reported on a study of succinoxidase activity in the developing hearts of chick embryos. For his discussion he assumed that succinic dehydrogenase was the restricting factor in the succinoxidase system, since cytochrome oxidase was never found to be limiting. The data given in this paper show two separate increases in enzyme activity; the first from two to five days of incubation and the second from about the ninth day of incubation to termination of embryonic development. Also indicated is the independence of this accumulation of enzyme activity from the rate growth of cardiac tissue. However, in place

of this possible relationship between enzyme activity and growth, Sippel postulated a relationship between enzyme activity and the physiological or functional maturation of the heart, expressed as cardiac effectiveness. From this, he suggested that the role of succinic dehydrogenase activity in the heart should be considered not only as a constitutional one, but also as a specialized one, presumably as a result of the high early physiological activity of the heart.

D. Other enzymes

Moog ('44) described the appearance of alkaline and acid phosphatase during embryogenesis by use of the histochemical technique of Gomori. She found both phosphatases present in the unincubated blastoderm and in all embryonic tissues during the first two or three days of development. From one to five days of incubation the enzyme was found uniformly distributed. From the seventh or eighth day on, the phosphatases was localized according to the adult pattern of its appearance. Quantitation of amount present was estimated by grading the observable precipitate into one of 16 quantitative groups. These ratings were based on the number of hours of incubation necessary to bring forth a "recognizable black deposit." The limited precision of this type of quantitation should be kept in mind, especially since some tissues were incubated up to 16 hours.

In 1950, the same author described the development of alkaline phosphatase in the duodenum of the chick. In

this study, alkaline phosphatase was not only studied histochemically as before, but also by direct biochemical analysis. Thus, the quantiative data expressed in this paper have a more precise meaning. The results given in the paper indicated that the enzyme accumulates slowly in the chick from the ninth to seventeenth day of incubation and then rises to its peak level just after hatching. These results when compared with her 1944 paper, in which a basal level of phosphatase activity was found in all embryonic tissues, led the author to the observation that "enzymological factors are produced in correlation with the function they subserve." A more complete description of this concept of enzyme modification during development is found in a review paper (Moog, '52) dealing with that topic.

In order to evaluate the relationship of early embryonic growth and differentiation to high energy phosphate sources such as ATP, Moog and Steinbach ('45) determined apyrase activity in the chick embryo. For the study, embryos up to 12 1/2 days of incubation were used and the total inorganic phosphate liberated from ATP was estimated as a measure of this enzyme activity. Although the activity was quite variable, the results indicated a low level of apyrase activity up to the sixth day, followed by a three-fold increase between the sixth and the twelfth day. This observation was interpreted as indicating that rapid growth up to the sixth day did not impose heavy demands on the energy-transferring apyrase system, but, rather, that the

onset of function in various organs resulted in the abrupt increase in enzyme activity.

To test this hypothesis further, Moog ('47) determined the apyrase activity in various organs of the chick embryo. Those organs studied included brain, liver and muscle. The time interval chosen for the investigation extended from 12 days of incubation until 14 days after hatching. The results differed in all of the organs studied. For brain, the activity continued rising fairly linearly even during the first week following hatching, after which it leveled off at a constant rate. Heart muscle demonstrated a peak activity at 16 days of incubation followed first by a decline to a low level immediately after hatching and then by a rapid rise to the adult level by 14 days post hatching. The liver demonstrated no change in activity over the entire period of study. On the other hand, skeletal muscle reached a peak at hatching and then declined to a lower, adult level.

Clark ('51) reported on the appearance of carbonic anhydrase activity throughout embryonic development. It was observed that this enzyme made its appearance in the tissues before the 12th day of incubation but not in the blood until after that time. Since only red blood cells of the definitive series are formed from the fifth day on, it was concluded by the author that the site of carbonic anhydrase formation for blood in the chick is limited to the site of the adult formation of the red blood cells, i.e.,

the bone marrow.

Wenger ('51) reported on the cholinesterase activity in different spinal cord levels in the chick embryo. He reported a five-fold rise in the enzyme activity following the onset of nervous function. Also, samples from both limb regions on the spinal cord were stated to have generally higher activity than those from the thoracic and cervical levels. This apparent relationship of enzyme content to physiological function is intriguing.

Lipase activity, as demonstrated by the histochemical method of Gomori, was located in the early chick embryo by Buno and Marino ('52). They described enzyme activity within the embryo after three days of incubation but only in the intestine and derivatives of the gut. Some positive reactions were given by perichordal, endocardial and pre-cartilogenous mesenchyme cells.

MATERIALS AND METHODS

I. Preparation of tissue

Fertile eggs of the New Hampshire Red strain were placed in an incubator maintained at 37.5°C. Embryos taken from these eggs at various time intervals were lifted free of the yolk, placed in isotonic saline and the degree of development evaluated according to the Hamburger-Hamilton stage series ('52). For some of the determinations on the younger stages the entire embryo was used as an enzyme source. However, only the selected organ parts were used for the experiments carried out on the older embryos. The embryonic organs analysed in this survey included brain, liver and heart. These were rapidly dissected with finely sharpened watchmaker's forceps and iridectomy scissors under a dissecting microscope. The brain was freed of dura and divided into the three major divisions, hindbrain, midbrain and forebrain. Samples of liver were not taken before stage 28 since the liver buds up to that time were not readily dissectable. The heart was isolated in toto; no attempt was made to separate atrial from ventricular portions.

Immediately after removal the tissues were suspended in water or phosphate buffer maintained at a temperature less than 5°C. The material was then homogenized in the cold with a glass micro homogenizer of the Ten Brock type

designed by Wenger for this particular type of study. The total final volume of the homogenate was approximately 50 ul. Since preliminary studies indicated no decrease of aldolase activity in frozen homogenates, the material to be used in the aldolase analysis was placed in a deep freeze maintained at -30°C. In contrast, studies on cytochrome oxidase and succinic dehydrogenase activities were run on the fresh tissues within fifteen minutes following their removal from the embryo.

II. Enzyme assays

A. Aldolase

Aldolase activity was assayed by the Lowry ('54b) micro modification of the Sibley and Lehninger method ('49). In our procedure, approximately two to four microliters of the homogenate containing one to five gamma of protein were incubated for the prescribed time in the substrate reagent mixture. The homogenate was delivered by use of a modified Levy ('36) constriction pipette, designed during the course of this investigation. The modification consisted of substituting a rubber diaphragm patterned after the Spemann embryological pipette (see Hamburger, '42) for the usual rubber tubing used for delivery. The pipette design is shown in Figure 1. The substrate reagent and all subsequent reagents were delivered by use of the micro syringe burette described by Lazarow ('50).

Redistilled trichloracetic acid stopped the enzyme reaction after the appropriate time interval. Additions

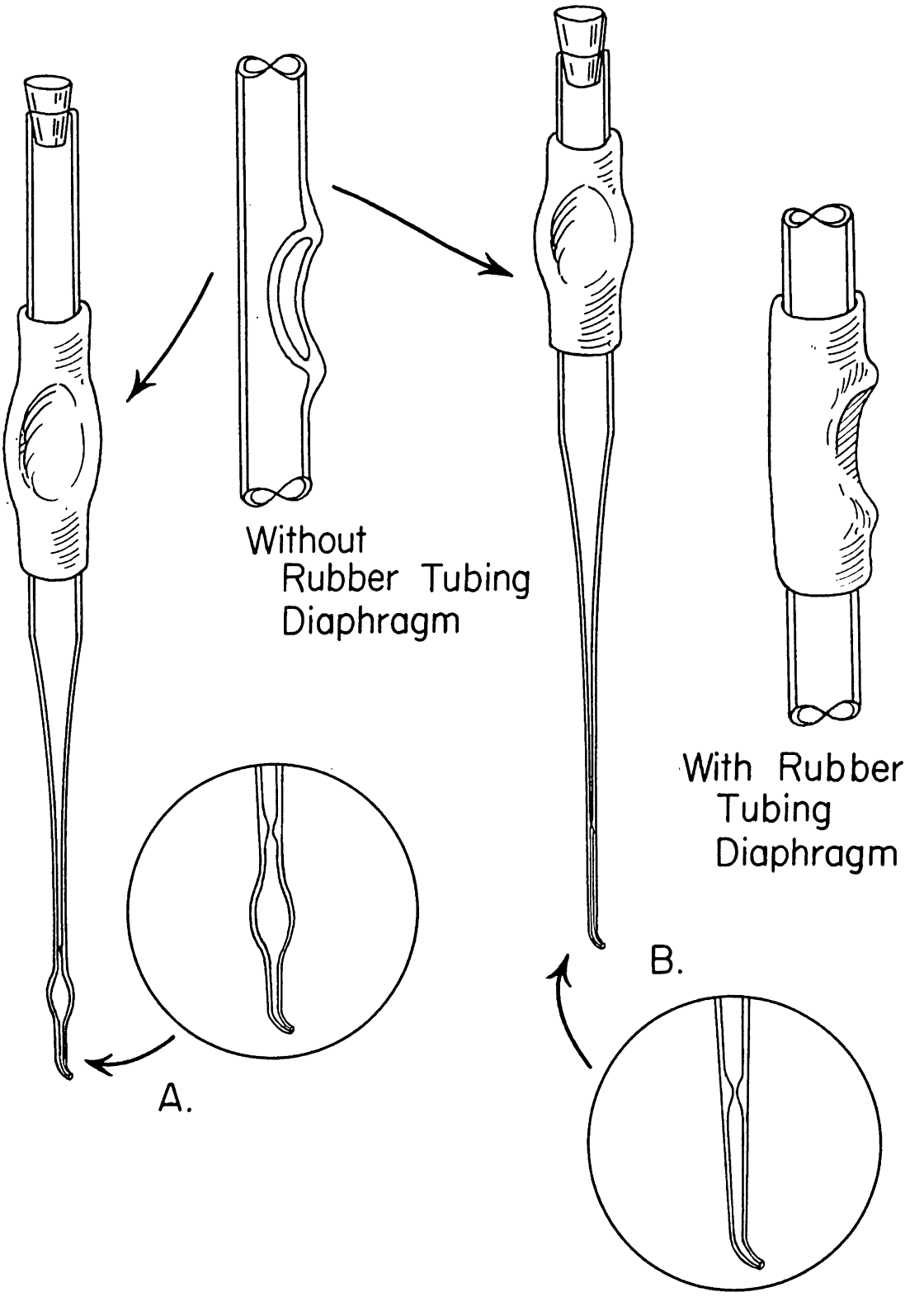
Without
Rubber Tubing
Diaphragm
With Rubber
Tubing
Diaphragm
A.
B.

Figure 1

of NaOH, dinitrophenylhydrazine and methyl cellusolve were made at the designated times and the extinction of mixture was read at wave length 570 mu in a Beckman DU adapted for micro determinations (Lowry and Bessey, '46). All determinations were carried out on triplicate samples of homogenates with the exception of the youngest stages (8-15) in which only duplicate aliquots were compared.

Aldolase activity was expressed in units of "moles of hexoaediphosphate split/hour/gamma protein." This was derived from the molar extinction data given by Lowry et al ('54d). Appropriate changes were made in these calculations for certain differences in homogenate volume.

B. Cytochrome oxidase

In this study the microspectrophotometric method of Cooperstein and Lazarow ('51) was used for the quantitative detection of cytochrome oxidase. This microadaption of Potter's technique permits measurement of the enzyme activity on as little as 1 microgram (protein) of tissue. In addition, the technique has the advantage of utilizing as the reaction indicator the naturally occurring substrate, cytochrome c, rather than a nonphysiological dye.

In this survey the procedure described by Cooperstein and Lazarow was followed exactly as presented in their paper with the theoretically permissably exception that 1.61×10^{-5} M cytochrome c solution was used in place of the 1.70×10^{-5} M cytochrome c solution indicated. It might be mentioned that samples of cytochrome c obtained

commercially proved unsatisfactory for use in the technique, demonstrating an apparent autooxidation following reduction with sodium hydrosulfite. A preparation of cytochrome c from Wyeth Laboratory which did not undergo this autooxidation was generously supplied by Dr. Cooperstein. The standard cytochrome oxidase preparation called for in the procedure was also obtained from the same source. The chick embryo homogenates were prepared as described in the above description for the aldolase determinations.

To the cytochrome c solution previously reduced with sodium hydrosulfite, two to eight microliters of the homogenate were added at zero time. Successive readings were taken throughout the run at thirty second intervals for three minutes at 550 mu on the spectrophotometer. After the addition of a few grains of potassium ferricyanide at the end of the determination, a final reading was taken on the oxidized sample.

The cytochrome oxidase content of the tissues was expressed in terms of "enzyme activity/microgram protein." The standard enzyme activity was calculated according to the original paper. This notation (enzyme activity) is proportional to the slope of a line measuring the drop in concentration of reduced cytochrome c per unit time. To determine this line, optical density of the completely oxidized sample was subtracted from that at any given time and since the reaction is a first order one, the log of this difference was plotted against time.

C. Succinic dehydrogenase

A similar technique was utilized for succinic dehydrogenase determinations (Cooperstein, Kurfess and Lazarow, '50). This procedure also makes use of cytochrome c as an indicator of the reaction. The method, in essence, involves the addition of succinate and oxidized cytochrome c to the enzyme suspension. Addition of sodium cyanide inhibits any cytochrome oxidase present in the sample. Thus, the dehydrogenation of succinate is quantitated by measurement of the resulting reduction of cytochrome c. The cyanide acts to prevent further undesired steps in the succinoxidase system.

The reduction of cytochrome c in this reaction was measured on the Beckman. Readings were taken at thirty second intervals for three minutes, followed by a final reading of the completely reduced sample after the addition of sodium hydrosulfite. The succinic dehydrogenase activity was also expressed as enzyme activity/microgram protein. In this case, the standard unit of enzyme activity was established in a fashion similar to that used for cytochrome oxidase assay. The extinction at any given time was subtracted from that of the completely reduced sample and the logarithm of this difference plotted against time. The slope of this line was a measure of the drop in concentration of oxidized cytochrome c per unit time and is expressed as enzyme activity.

III. Protein determinations

The micromethod described by Lowry et al ('51) was employed for the measurement of protein in these samples. Certain minor changes were made in the original procedure and a non-protein standard was introduced.

The following method was used routinely in this study for the determination of protein. Five milliliters of a 10% copper sulfate solution was brought to 100 milliliters with a 1.05% solution of potassium tartrate. To 0.5 milliliters of this copper tartrate complex, 24.5 milliliters of 2% sodium carbonate and 2.5 milliliters of 1.1N sodium hydroxide were added. One hundred microliters of the resulting reagent were delivered by use of the syringe burette into a micro test tube containing two to four microliters of the unknown protein sample. After 45 minutes, 10 microliters of the Folin and Ciocalteu phenol reagent, prepared according to Lowry were added with immediate mixing. Readings were taken on the Beckman at 750mu after an additional thirty minute wait. Suitable blanks and standards were determined together with the samples. All assays were run in triplicate.

Since it was observed in our laboratory that this method of protein analysis has a somewhat limited range, an approximation of the protein content was rapidly made in the samples by the simple procedure of introducing a selected aliquot of the sample into a tube containing the alkaline copper tartrate complex and immediately adding the proper amount of phenol reagent. The color thus produced

gave a rapid visualization of the approximate protein content of the undiluted aloquot. Modifications of this dilution could then be made before the determination was run in triplicate.

In regard to the standard selected for the protein determination, it was found that standards prepared from human serum albumin and frozen in individual vials did not give reproducable results from day to day. Differences in results were noted both between separate vials and after variable periods of freezing. In order to overcome this difficulty, glutathione, a tripeptide, was used as a standard for the individual runs and then converted to actual protein units at the end of the experiments. With glutathione the exact chemical nature of the standard is known and therefore is readily reproducible. Also, it will keep in the cold without the necessity of freezing and under these conditions does not change in extinction appreciably for time periods exceeding one week. The calibration curve for glutathione resembles precisely that for the protein except that the gram extinction values of the tripeptide is five times less than that of the protein. The one disadvantage to the use of glutathione as a standard might lie in the fact that it is not completely precipitated by TCA. However, since protein measurements were run on separate aliquots of the sample and not on a TCA precipitation fraction, this drawback did not affect our study.

IV. Expression of activities

It is generally agreed that growth in most biological processes does not follow a linear curve. However, most studies of chemical accumulations during the growth of chick embryos have described time of development in a linear fashion. Levy ('52) has taken exception to this method of expression and gives a convincing argument for the abandonment of such procedures. He alternatively estimated age by the weight of the embryo and then converted this to a logarithmic function. While in agreement with this procedure, it was felt that the Hamburger and Hamilton stage series of the chick embryo gave a more reliable indication of the degree of development than wet weight since more than one criterion was used for the determination. The stages in this series were reinterpreted in terms of average hours of development and were converted to days of age (see Table I). Enzyme accumulation was plotted as a function of the log of age thus derived.

TABLE I

NORMAL STAGES OF THE CHICK

Stage	Hours	Stage	Hours
1	0	24	96
2	6 1/2	25	108
3	12 1/2	26	114
4	18 1/2	27	120
5	20 1/2	28	132
6	24	29	144
7	24 1/2	30	156
8	27 1/2	31	168
9	31	32	180
10	35 1/2	33	186
11	42 1/2	34	192
12	47	35	204
13	50	36	240
14	51 1/2	37	264
15	52 1/2	38	288
16	53 1/2	39	312
17	58	40	336
18	67	41	360
19	70	42	384
20	71	43	408
21	84	44	432
22	84	45	492
23	90	46	516

RESULTS

I. Aldolase

Aldolase activity in the organs selected for this study remained at a constant level during the extent of their development. This is graphically shown in Figures 2 and 3. Although the enzyme content of the individual tissues appeared to be constant as noted, there was some variation in amounts of activity when the organs were compared one to another. In order to demonstrate this, the mean and the standard error of the mean for the enzyme activity in each group was determined. Those results are expressed in Table II. From these figures a comparison of significant differences were derived according to Edwards ('54). When tested by this method, heart, liver and all parts of the brain studied, varied significantly from the early whole animal at the 1% level. Similarly, both heart and brain were significantly different from liver at the same level of confidence. On the other hand, all parts of the brain when compared with the heart did not demonstrate a true variation even at the 5% level. Likewise, the individual portions of the brain did not contain significantly different amounts of enzyme activity when tested at that level.

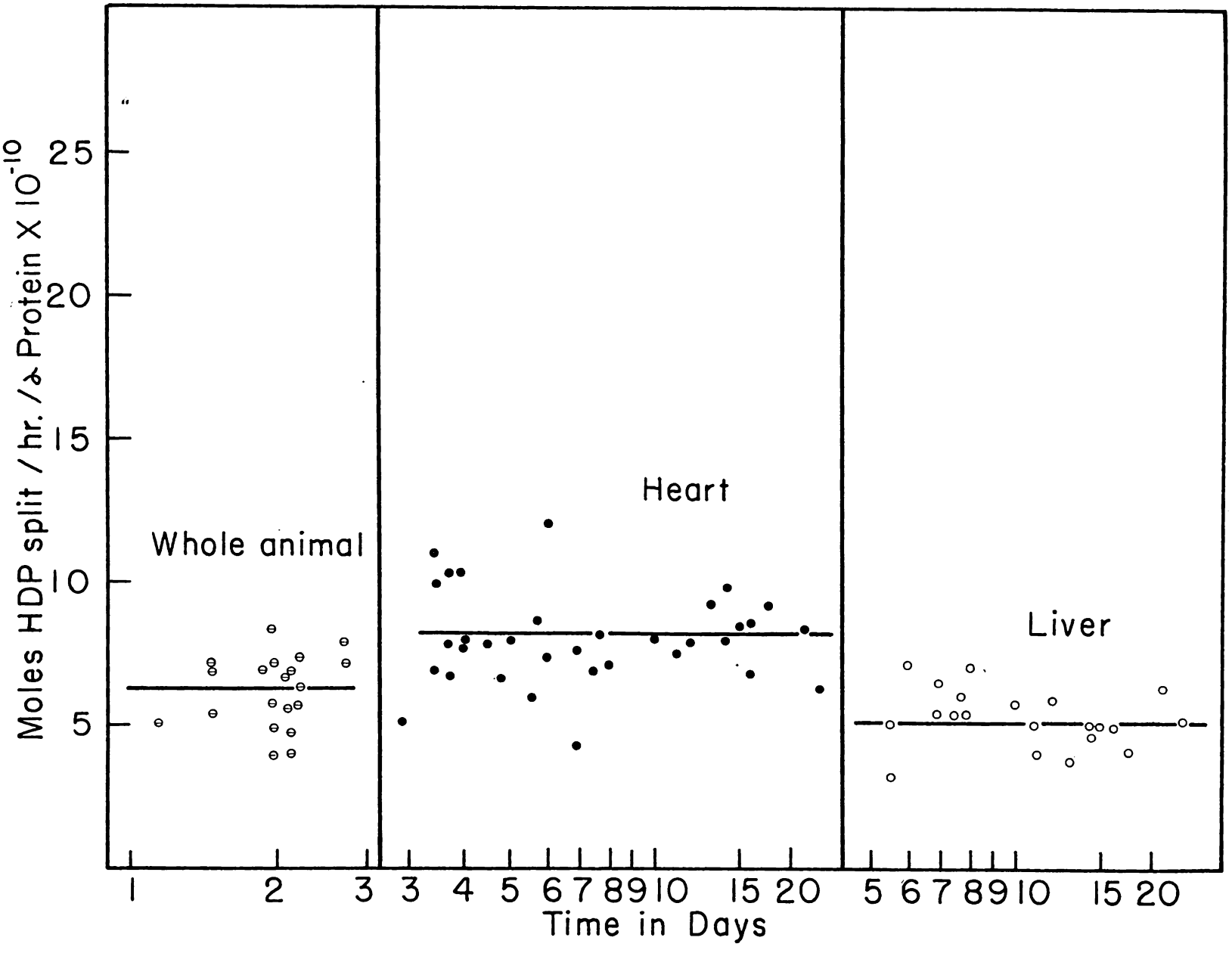
Moles HDP split / hr. / λ Protein X 10^{-10}
25
20
15
10
5
Whole animal
Heart
Liver
1
2
3
3
4
5
6
7
8
9
10
15
20
5
6
7
8
9
10
15
20
Time in Days

Figure 2

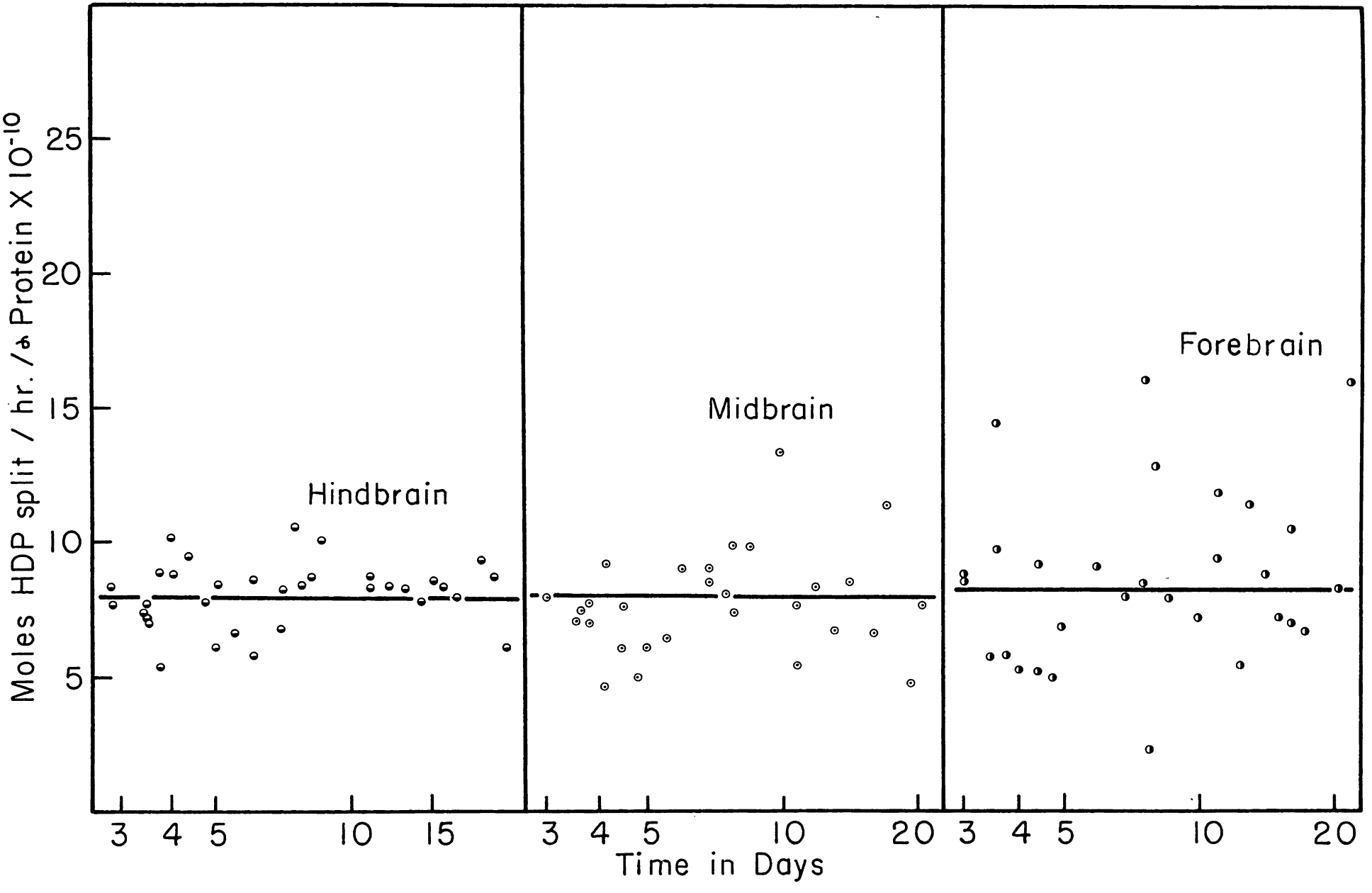
Moles HDP split / hr. / % Protein X 10^{-10}
25
20
15
10
5
Hindbrain
Midbrain
Forebrain
3 4 5 10 15
3 4 5 10 20
3 4 5 10 20
Time in Days

Figure 3

TABLE II

ALDOLASE LEVELS OF INDIVIDUAL EMBRYONIC CHICK TISSUES

	Number of Samples	Mean Value of Activity*	Standard Error of Mean
Early Whole Animal	21	6.30	0.33
Liver	22	5.27	0.22
Heart	33	8.37	0.33
Hindbrain	37	8.03	0.20
Midbrain	35	8.10	0.36
Forebrain	36	8.05	0.42

*Expressed as Moles of Hexose-di-phosphate split/hour/ micrograms protein x 10^{-10}.

II. Cytochrome oxidase

In contrast to the constant level reported in the aldolase study, cytochrome oxidase exhibited a marked increase in activity throughout development in certain tissues. This is immediately apparent from observation of the graphs presented in Figure 4. One should note that the straight lines appearing in these graphs are fitted to the points by sight only and can be interpreted only as an indication of the approximate slope. Keeping this caution in mind it can be seen that cytochrome oxidase activity demonstrated the earliest increase in the embryonic heart, doubling in apparent content in the period from the fifth day to the eleventh day of incubation. This continuous rise in enzyme activity results in a threefold increase at hatching over the level observed at four days. In like fashion, liver cytochrome oxidase activity exhibits a steady increase per unit of protein during the developmental period. Although the rise in the liver activity lags behind that in heart, the rate of accumulation appears surprisingly similar in both liver and heart. The data in Table III indicate such a trend.

In general, brain tissue cytochrome oxidase activity appears to exhibit more of a sluggish accumulation. However, one comment might be made in regard to the individual portions of the brain studied. That is, increases in enzyme activity during development are progressively greater in samples taken from the more rostral portions of the brain.

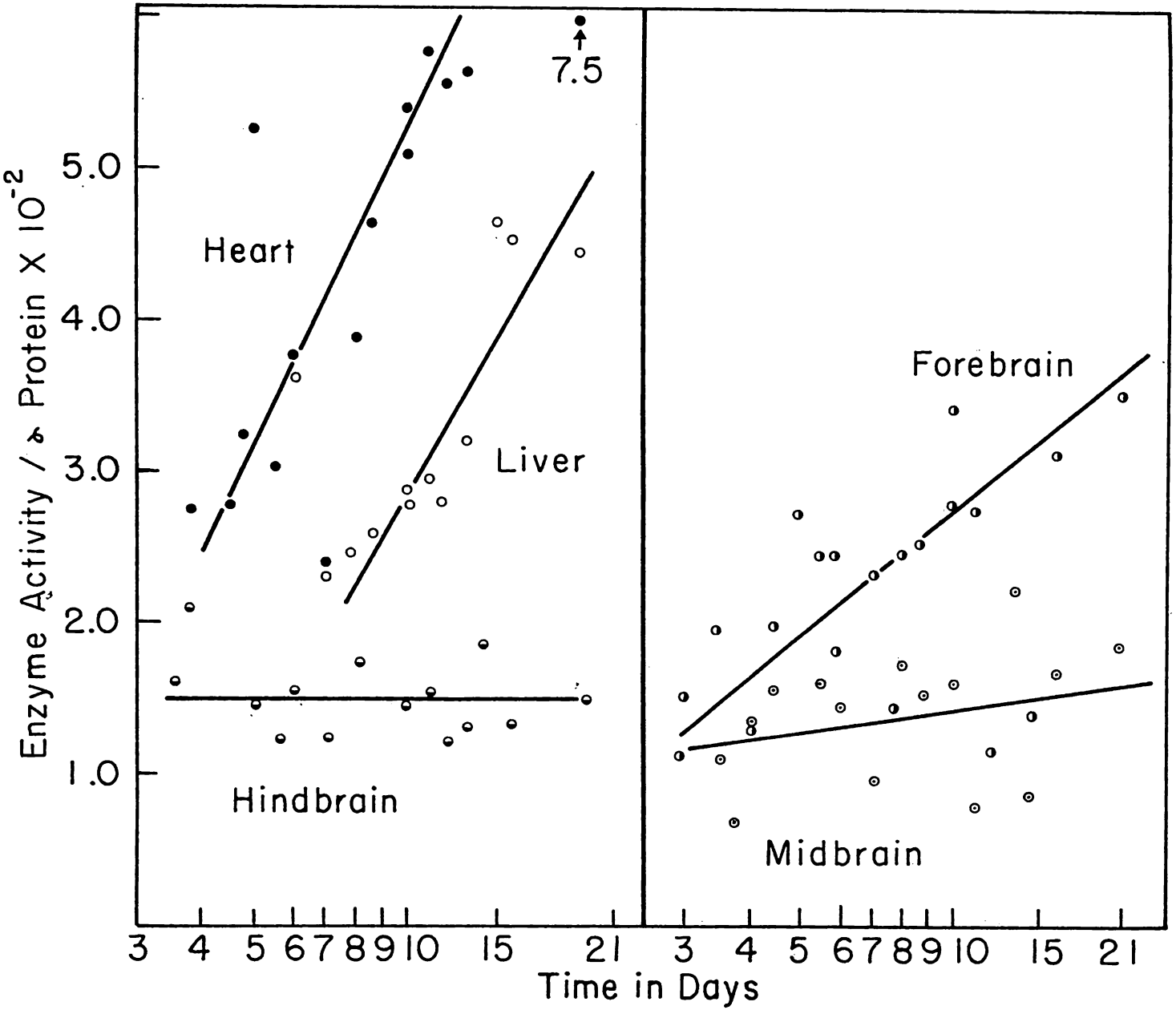
Enzyme Activity / μ Protein X 10^{-2}
5.0
4.0
3.0
2.0
1.0
7.5
Heart
Liver
Hindbrain
Forebrain
Midbrain
3 4 5 6 7 8 9 10 15 21
3 4 5 6 7 8 9 10 15 21
Time in Days

Figure 4

TABLE III

COMPARISON OF CYTOCHROME OXIDASE ACTIVITIES
IN DEVELOPING LIVER AND HEART

Day of Development	Cytochrome Oxidase Activity* of Liver	Cytochrome Oxidase Activity* of Heart	Ratio
7	2.30	4.15	1.8
9	2.60	4.92	1.9
10	2.90	5.39	1.9
13	3.22	5.64	1.8
20	4.51	7.50	1.7

*Expressed as Enzyme activity/microgram protein x 10^{-2}.

This is revealed by comparing the approximate slopes of the plots in Figure 4. Forebrain exhibits a greater rise in enzyme activity than does midbrain while hindbrain displays a relatively constant amount. Due to the amount of variation in the determinations carried out on forebrain and midbrain, this observation can be considered only of a suggestive and preliminary nature.

III. Succinic dehydrogenase

In a qualitative sense, the results of the investigation on succinic dehydrogenase content were much the same as the cytochrome oxidase study (Figure 5). With the exception of the heart, enzyme activity in the individual organs was difficult to determine with any degree of reliability prior to the fifth day of development. Beyond that time the overall pattern of enzyme accumulation is indicated by the graphs.

Liver samples exhibited the greatest amount of variation and due to this fact it was impossible to derive a ratio of heart to liver activity as had been described for cytochrome oxidase. However, it was possible to express a ratio of succinic dehydrogenase activity to cytochrome oxidase activity throughout development in the other tissues studied. Such a series is presented in Table IV. This ratio while demonstrating differences in the individual tissues, does not reveal a general trend in the tissues during development.

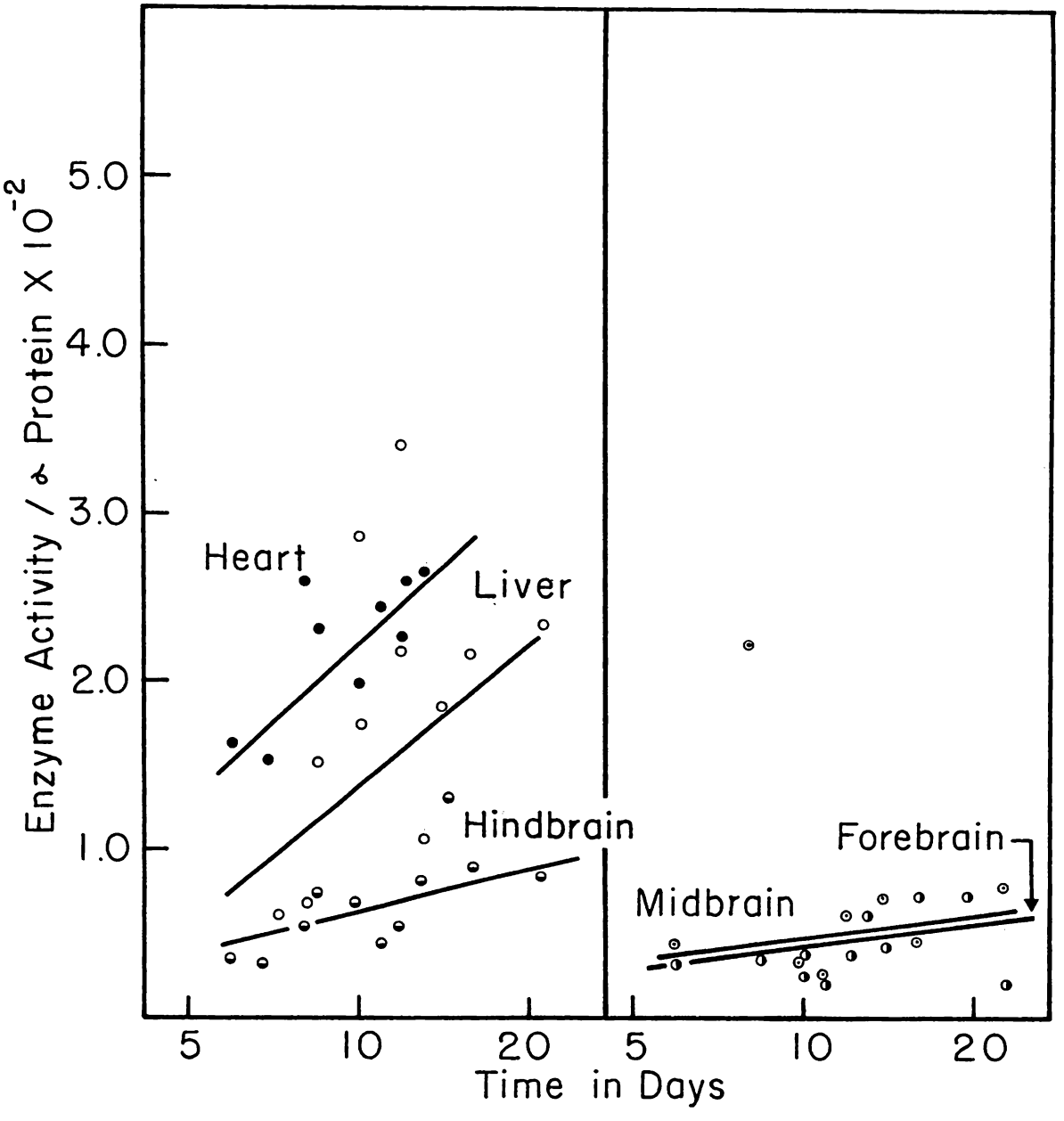
Enzyme Activity / ∝ Protein X 10^{-2}
5.0
4.0
3.0
2.0
1.0
Heart
Liver
Hindbrain
Forebrain
Midbrain
5
10
20
5
10
20
Time in Days

Figure 5

TABLE IV

COMPARISON OF CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE LEVELS IN VARIOUS TISSUES THROUGHOUT DEVELOPMENT

Tissue	Age of Development (Days)	Cytochrome Oxidase Activity*	Succinic Dehydrogenase Activity*	Ratio
Heart	6	3.70	1.62	2.3
	10	5.39	2.00	2.7
	15	6.51	2.78	2.3
Hindbrain	6	1.52	0.40	3.9
	10	1.48	0.70	2.1
	15	1.45	0.70	2.1
	20	1.50	0.89	1.7
Midbrain	6	1.42	0.42	3.4
	10	1.45	0.49	3.0
	15	1.50	0.55	2.7
	20	1.59	0.62	2.6
Forebrain	6	1.71	0.32	5.4
	10	2.72	0.44	6.2
	15	3.25	0.50	6.5
	20	3.40	0.57	5.9

*Expressed as Enzyme activity/microgram protein x 10^{-2}.

DISCUSSION

It is possible to approach the results of this study from two separate points of view. The first involves an evaluation of the data solely with regard to the comparative levels of the different enzymes throughout the development of a given tissue. In this respect, it is obvious that the three enzymes under study have distinct and separate patterns of development in general. The second method of interpreting the results involves the comparison of the selected tissues with respect to a particular enzyme. One should review the results from both considerations for a more complete evaluation of their significance.

I. Comparison of the levels of the various enzymes during development

Reports of aldolase activity in embryos have been limited, for the most part, to demonstrations of its presence without any attempt to follow the pattern of its accumulation (Needham, '37b). The only study other than our own reported in the literature describing the actual levels of aldolase activity throughout development was included in a paper by Gustafson and Hasselberg ('51). In this article, aldolase activity was followed in developing sea urchin eggs up to the pluteus stage. During the entire period covered by this study, the activity of aldolase remained constant. Our data on the chick indicate a similar

unchanging level for this particular enzyme over the entire time of incubation.

When interpreting such results, it is enticing to assume as Lowry ('54b) has suggested that the level of aldolase activity as assayed is proportional to the total amount of glucose metabolized by way of the Embden-Meyerhof scheme for the given sample. The use of this assumption would indicate unmodified level of activity for this major glycolytic cycle throughout development in the chick. Only related, direct studies of the total cycle and similar estimations of other enzymes functioning in the scheme can reveal the validity of this assumption. Such surveys are presently being undertaken in our laboratory.

Succinic dehydrogenase and cytochrome oxidase activities have been estimated during development in many species. Some of the animals in which these enzymes have been studied during the early stages of development include cat (Breush, '37), sea urchin (Deutsche and Gustafson, '52), guinea pig (Flexner, Belknap and Flexner, '53), rat (Sipple, '54) and chick (Levy and Young, '48; Sippel, '54; Albaum, Novikoff and Ogur, '46). Of those reporting on the chick embryo, only Sippel has described the enzyme activity in an individual organ (heart ventricle).

In general, both cytochrome oxidase and succinic dehydrogenase activities in the chick embryo as revealed in our investigation exhibited marked differences from the constant level found in the case of aldolase. In the two

non-nervous tissues studied, these enzymes were observed to follow a distinct gradual rise in activity per unit protein throughout incubation. The activities of both enzymes appeared to be related in all tissues, the ratio between them changing only slightly over the entire time interval of study (Table IV). In contrast to this observation, Albaum et al ('46) had described a changing ratio of the two enzymes in whole chick embryo. However, their work was carried out on the entire succinoxidase system as compared to our assay which was restricted to succinic dehydrogenase.

When one compares our results to those generally encountered in the literature for the adult content of these two enzymes, some differences are noted. Schneider and Potter ('43) for example have reported on the tissue content of succinic dehydrogenase and cytochrome oxidase in fully grown rats. Their results include two of the organs compared in this investigation, i.e., heart and liver. The data given in their paper indicate the ratio for cytochrome oxidase of heart to liver to be 3.5. Other authors have indicated the same ratio to be 3.0 (Elliot and Greig, '38), 2.8 (Shack, '43), 5.7 (Stotz, '39). All of the studies above employed a manometric method for determination of cytochrome oxidase activity and are therefore not strictly comparable to our figures. Using the technique employed in this study, a ratio for heart cytochrome oxidase to liver cytochrome oxidase in the adult rat of 2.7 has been reported (Cooperstein and Lazarow, '51) as well as a ratio of 1.7 in

the adult toadfish (Lazarow and Cooperstein, '51). Our average ratio throughout development in the chick was 1.8 (Table III), a figure which is more in agreement with those of the last papers noted. Unfortunately the cytochrome content of adult chicken heart and liver are not available for a complete comparison.

The absolute activities of all enzymes in this study appear to be slightly lower in the chick embryo than in the adults of other species. This is evident when one compares our results with other studies using the same method of determination. Thus, Lowry et al ('54a) reported higher aldolase activity in adult rabbit brain. Cooperstein and Lazarow likewise indicated that cytochrome oxidase ('51) and succinic dehydrogenase ('50) activity in comparative tissues in adult rat were almost two times higher than described here for chick embryo. On the other hand, Elliot and Greig ('38) reported that the succinoxidase system in embryos was five times less than that found in adult tissue. Likewise, Rudnick, Mela and Waelsch ('54) found limited glutamine transferase activity in embryos. In regard to these observations, some authors (Rudnick et al, '54) have suggested that the lower enzyme activities generally exhibited by embryonic tissue are probably due to the lack of environmental or physiological stresses playing on the organism before birth as compared to those met following hatching. Our studies are too incomplete at the present time to contribute to such an hypothesis.

II. Comparison of the enzyme content of nervous and non-nervous organs during development

The varied pattern of enzyme accumulation observed in each of the embryonic organs studied is of particular interest. Many chemical embryologists have offered the opinion that differences in the enzymatic make-up of developing tissues are directly related to their functional role within the adult organ (Moog, '52; Boell and Shen, '50; and Sippel, '54). The first observations supporting such an assumption were carried out on the nervous system of Amblystoma (Sawyer, '43; Boell and Shen, '50). In these studies, cholinesterase, an enzyme of definite functional significance in nervous tissue, was assayed throughout development. Sawyer correlated the cholinesterase content of the whole animal with the functional stage of the embryo. He described an increase in the development of enzyme activity at the times distinct neuromuscular interplay was noted. Boell and Shen designed their studies to quantitate the cholinesterase activity within various regions of the developing central nervous system. They reported that cholinesterase appeared first in the spinal cords and then progressively in the more anterior regions of the central nervous system. As opposed to this, non-specialized functions, such as respiratory activity, did not vary notably when studied in a similar fashion throughout spinal cord and brain. More recently similar studies on the pattern of development for certain specialized enzymes have been

reported for the chick. In these reports, alkaline phosphatase (Moog, '50), apyrase (Moog, '47), cholinesterase (Wenger, '51), glutamine transferase (Rudnick, Mela and Waelsch, '54) and succinoxidase (Sippel, '54) demonstrated rates of accumulation which varied according to the tissue or particular location studied. Each of these enzymes demonstrated a marked change in distribution among the selected regions studied during development.

Other species investigated in which the same phenomenon of non-parallel accumulation of enzymes is apparent include the sea urchin (Gustafson, '54) and the guinea pig (Flexner et al, '48, '53).

Concerning our observations on the three enzymes reported here, certain comments of a somewhat speculative nature might be made. It is of interest to note that at the earliest dissectable stages, liver possesses lower aldolase activity than does heart or brain. This agrees with the finding of Sibley and Lehninger ('45) and Dounce and Tannhauser-Beyer ('48) for adult tissues. If aldolase is proportional to the level of the Embden-Meyerhof glycolytic cycle as postulated before, this could indicate a relatively low functional state for that cycle in embryonic liver. Since, according to Bloom et al ('55), the Warburg-Dickens hexose-monophosphate shunt is responsible for at least fifty percent of the glucose metabolized in the adult rat liver, the low level of aldolase activity could signify the early development of that system in the

embryonic liver.

The difference in rate of accumulation of the enzymes cytochrome oxidase and succinic dehydrogenase as compared to aldolase particularly in heart and liver is also suggestive. In these tissues, but apparently not in brain, the possibility exists that during later stages there appears an increasing amount of active acetate not derived from the Embden-Meyerhof glycolytic cycle. This might account for the changing respiratory quotients of developing embryos noted by Needham and explained on the basis of successive utilization of carbohydrate, protein and fat as energy sources. This interpretation is further supported by Romanoff's observation ('43) that there is less dependence upon the presence of glucose as a substrate in older embryonic tissues than in young ones.

This increase in cytochrome oxidase and succinic dehydrogenase activity in the heart and liver in contrast to brain tends to exclude the possibility of these enzymes being "constitutive" in all tissues. One might anticipate that since they mediate the final stages in the release of energy, their activities would differ with the varied physiological patterns of unlike tissues. In this respect, Sippel ('54) has correlated the increase in the succinoxidase system of developing heart ventricle with the functional onset of cardiac activity and actual cardiac effectiveness. He describes parallel courses of development for the enzyme activities and the mechanical efficiency of

the organ. Our observations on two components of the succinoxidase system support his contention. Applying the same considerations, our data on liver reveal certain similarities. The rise in activity of liver cytochrome oxidase is apparent beyond the seventh day of incubation. This increase occurs at the time the liver cells are considered to give the first indication of the definitive function (Dalton, '37).

If our conclusions on heart and liver are correct, the presence of a relatively stable pattern for the three enzymes in all parts of the brain might well indicate the existence of a constitutive level of activity for this tissue throughout the greater part of development. A review of the literature failed to reveal any direct studies related to this problem in the chick. However, the work of Flexner, Tyler and Gallant on guinea pigs is most suggestive ('50). They found no indication of organized electrical patterns in the cerebral cortex until the third trimester of development. A similar study in the chick central nervous system would be extremely valuable in relating the metabolic pattern of development to the functional one.

Taking all of the above into consideration, it is apparent that of the three enzymes studied only aldolase maintained a basal level in all developing tissue studied since cytochrome oxidase and succinic dehydrogenase appear to vary with the physiological status of the individual tissue. Thus, neither of the last two enzymes, in contrast

to aldolase, entirely fill Moog's ('52) definition of a truly constitutive enzyme.

SUMMARY

1. The patterns of accumulation for certain enzymes in nervous and non-nervous structures of the developing chick embryo have been studied using microchemical methods.

2. Aldolase activity remains at a constant level throughout development in all organs studied. This level does not differ significantly between developing heart and brain whereas the activity in both is lower than that found in liver.

3. In all the organs studied, aldolase activity was found to differ from the activity found in the young total embryo.

4. In contrast to aldolase, cytochrome oxidase activity shows individual patterns of accumulation in the tissues under study. Heart and liver cytochrome oxidase activity per unit protein increases continuously throughout the period of study whereas all portions of the central nervous system under observation remain at a fairly constant level.

5. In general, changes in succinic dehydrogenase activity during the embryonic period appear to parallel those described for cytochrome oxidase. The level throughout the brain remains constant while heart and liver displayed a progressive rise in activity per unit protein during the course of the investigation.

6. These differences are discussed both with respect to the specific patterns of development for the individual enzymes

and to the variation of enzyme content among the different tissues. In this regard, aldolase has been suggested to be a truly constitutive enzyme, unchanging throughout development whereas both cytochrome oxidase and succinic dehydrogenase appear to vary with the functional status of the embryonic organs.

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