ENZYMATIC PATTERNS DURING DEVELOPMENT
An Approach to a Biochemical Definition of Immaturity
E. Mead Johnson Award Address

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I would like to express my gratitude to you, the American Academy of Pediatrics, for selecting me for an E. Mead Johnson Award. I accept the honor with humility because no scientific work is accomplished without inspiration from teachers and other investigators, and without actual participation of many colleagues. I appreciate this opportunity to acknowledge publicly those individuals who have been and are particularly influential in my progress.

Dr. Jean Oliver, the man to whom I owe my entry into medicine and pediatrics, with great effort instilled in me an everlasting appreciation for the inseparable relationship between structure and function. Fortunately, I have remained in close contact with Dr. Oliver throughout the years, continually reaping benefits from his advice and influence.

I conceived of function as only cellular and molecular until Dr. Henry L. Barnett introduced me to organ function, especially in relation to the young individual. In addition, he fostered in me an interest in the broader aspects of pediatrics, and since that time has been available constantly for intimate exchange and crystallization of ideas.

I am indebted particularly to Dr. S. Z. Levine for leading me into the field of biologic development and insisting that my time be divided between clinical pediatrics and the laboratory. Dr. Levine has always emphasized that ideas, philosophies and problems originating in the ward or clinic can and should be considered in the laboratory. His subtle direction has guided me towards the integration of clinical and investigative pediatrics, and his conceptual knowledge of medicine has been an invaluable stimulus to me.

Finally, without the loyal, unselfish and untiring help of Miss Helen McNamara, it would have been exceedingly difficult to have accomplished these studies.

THE FOLLOWING is a review of the various steps we have taken in our attempt to arrive at a biochemical interpretation of immaturity. About 6 years ago, Dr. Levine and I discussed his extensive observations concerning the role of vitamin C in the complete metabolism of tyrosine in premature infants. Originally, Dr. Levine observed that if the diet of the premature infant was not supplemented with ascorbic acid, tyrosine and p-hydroxyphenylpyruvate appeared in the urine, and addition of ascorbic acid caused these substances to disappear. Later it was shown in vitro that ascorbic acid was required for complete metabolism of tyrosine. We have since found that large amounts of ascorbic acid will activate the enzyme, p-hydroxyphenylpyruvate oxidase, from liver of premature infants or fetal animals. This observation is analogous to the findings in vivo that premature infants required as much as 50 mg of vitamin C for alleviation of the tyrosyluria. These data provided an impetus for investigations into the mechanism of action of vitamin C in tyrosyluria. From the initial studies our work has followed a variety of
pathways, some of which will be discussed. A program considerably broader than originally intended has developed from these observations. As yet the results do not comprise a complete story but they do permit formulation of some conclusions.

The discussion may begin with a diagrammatic description of the pathways for metabolism of phenylalanine and tyrosine, as shown in Figure 1. Every reaction in this scheme involves at least one enzyme and the name and the position of each is specified. The tyrosine oxidizing system, designated as 1, includes all enzymes essential to the degradation of tyrosine. The triple arrow between homogentisate and fumarate-acetoacetate is designed to indicate the probability of at least three intermediate steps. Each enzyme requires cofactors, i.e., coenzymes or activators for optimum activity. These will be discussed as required for elucidation of the present report. The biochemical intricacies of this pathway have been recently reviewed extensively.

Each enzyme noted has been studied in the livers of both fetal and adult animals and the results are shown in Figure 2. Under standard conditions the individual enzyme shows considerably less activity in fetal than in adult tissue. Livers from premature infants were obtained at necropsy and showed activities comparable to those with tissue from fetal rats.

The tyrosine oxidizing system, tyrosine transaminase, and phenylalanine hydroxylase show minimal activities in fetal liver but p-hydroxyphenylpyruvate oxidase from tissue of fetal animals has an activity about 30% of the level in adult livers. The lack of activity observed could be due to the presence of an enzymatic inhibitor or absence of an activator in the livers of premature infants and fetal rats; consequently experiments were designed to test this hypothesis, utilizing the tyrosine oxidizing system. Thus homogenate of a liver from a premature infant was added to that from an adult and there was no depression or elevation of activity observed. A preparation of adult liver was boiled and ultrafiltered and addition of these nonprotein extracts failed to activate the preparation from liver of the premature infant. Similar extracts from a liver of a premature infant did not inhibit activity of enzyme from adult liver. Finally, large amounts of ascor-
bic acid did not stimulate the tyrosine oxidizing system.

From these data it became apparent that slight causal information could be gained from studies of the over-all system, and so the investigation was extended to a survey of individual enzymatic reactions. These observations demonstrated that three different types of deficiency of enzymatic activity may occur in perinatal life: 1) absence of enzyme; 2) necessity for an activator; and 3) absence of one enzyme in a multi-enzyme system.

An example of deficiency of enzymatic activity due to the absence of enzyme is shown by the results with tyrosine transaminase, an enzyme which has specificity solely for tyrosine. This enzyme requires 2-ketoglutarate as the amino-group acceptor and a coenzyme, pyridoxal phosphate, for maximum activity. Activity of the enzyme was ascertained from the amount of p-hydroxyphenylpyruvate produced under controlled conditions. Figure 3 is derived from a sample experiment showing the effect of increasing concentration of pyridoxal phosphate on the activity of tyrosine transaminase in livers from a 2-hour-old and a 10-hour-old rat. The difference in activity with age is apparent, but increase in coenzyme did not result in a proportionate increase in enzyme activity in liver from the younger animal. In addition, excessive amounts of the amino-group acceptor, 2-ketoglutarate, failed to increase activity in very young animals. However, the addition of much larger amounts of tissue preparations did result in demonstrable enzyme activity, showing that some enzyme was present in the liver of the younger animal. This result was interpreted to indicate the presence in tissue from very young animals of an almost negligible amount of apoenzyme (protein portion of the enzyme) and therefore excessive addition of non-protein components of the system could not influence activity of the enzyme. Thus, lack of production of the protein portion of a specific enzyme is one possible mechanism that can lead to lack of enzymatic activity in tissues of the very young organism.

p-Hydroxyphenylpyruvate oxidase of fetal liver illustrates a contrasting situation in which there is a requirement for large amounts of activator (Fig. 4). The activity of this enzyme is measured by utilization of p-hydroxyphenylpyruvate and also by consumption of molecular oxygen. The reaction is aerobic and requires ascorbic acid or a suitable substitute, such as dichlorophenolindophenol. When 1 mg of ascorbic acid (an amount sufficient to give maximum activity with the preparation from the child) is added to enzyme from liver of a premature infant, 30% of the activity found
in liver of the child is obtained. When either preparation is boiled or when no enzyme is added, there is no utilization of p-hydroxyphenylpyruvate. Homogentisate was determined in selected experiments and an amount was detected equivalent to the p-hydroxyphenylpyruvate utilized. As shown in Figure 4, increasing concentration of ascorbic acid stimulated activity of the enzyme until maximum activity was finally reached. Dichlorophenolindophenol could activate the enzyme but activity was not maximal. Results similar to these obtained with liver of premature infant were observed with the enzyme derived from livers of fetal rats or rabbits. It was possible that young tissues, in particular, destroyed ascorbic acid but further investigations indicated that ascorbic acid was not inactivated either by oxidation or enzymatic action by liver from a premature infant. As it had been shown that catalase was also probably required for the conversion of p-hydroxyphenylpyruvate to homogentisate, catalase was added to enzyme from liver of a premature infant, but no increase in p-hydroxyphenylpyruvate utilization resulted. These data probably indicate that the enzyme, p-hydroxyphenylpyruvate oxidase, is present in fetal tissue but that very large amounts of ascorbic acid are required for activation. The ascorbic acid may act to remove an inhibitor of enzymatic activity.

The third mechanism which could be
TABLE I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Tyrosine Formed (μmole/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult liver</td>
<td>0.43</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>0.07</td>
</tr>
<tr>
<td>Fetal liver and Fraction I</td>
<td>0.42</td>
</tr>
<tr>
<td>Fetal liver and Fraction II</td>
<td>0.05</td>
</tr>
<tr>
<td>Fraction I</td>
<td>0.08</td>
</tr>
<tr>
<td>Fraction II</td>
<td>0</td>
</tr>
</tbody>
</table>

\[ \text{TPN}^+ + \text{H}_2\text{O} + \text{tyrosine,} \]

\[ \text{I+II} \]

...responsible for deficient activity of an enzyme, that of lack of one enzyme in a two-enzyme system, is exemplified by phenylalanine hydroxylase,\textsuperscript{16, 17} the enzyme required for conversion of phenylalanine to tyrosine. The data in Table I were obtained with livers from rabbits, but results were similar with preparations from rat, pig and man. As shown in Table I, phenylalanine hydroxylase consists of at least two enzymes:\textsuperscript{14} Fraction I, found only in liver, and Fraction II, found in all tissues examined. Fraction I is extremely labile and very difficult to work with; Fraction II is quite stable. Separately Fraction I or Fraction II shows only slight ability for hydroxylation of phenylalanine, but when added together the mixture is very active. Phenylalanine hydroxylase from the liver of the fetal rabbit will form only minimal quantities of tyrosine. When Fraction I isolated from adult rat liver is added to fetal liver, there is an elevation in activity equal to that of the adult liver system, but
the addition of Fraction II has no effect. Thus, Fraction I is probably lacking in fetal liver but Fraction II is present.

In summary, three different causes for lack of specific enzymatic activities in fetal tissue have been discussed: First, an actual lack of enzyme protein, as with tyrosine transaminase; second, the presence of seemingly adequate concentrations of the protein portion of an enzyme, but an inordinate and unexplained requirement for nonprotein activators, as in the case of p-hydroxyphenylpyruvate oxidase; and finally, the lack of one or more of the individual enzymes in a multi-enzyme system, as in the instance of phenylalanine hydroxylase.

These experiments provided an understanding of the basis for some deficiencies of enzymatic activity at a particular stage in early life. Information was still lacking as to the unique biochemical events leading to the complete development of enzymatic activity. As a first step in this direction, we proceeded to investigate patterns of enzymatic activity associated with postnatal development of the organism.

Preparations were made from livers of animals of different ages, starting during fetal life (Fig. 5). Fetal age was determined with a maximum error of 1 day by means of a vaginal smear from the female the morning after the rats were mated. One outstanding feature of these developmental patterns is that the activity of one enzyme, phenylalanine transaminase, reaches the adult level 1 day before birth, then exceeds that level, and gradually returns to the activity of the adult between 10 to 20 days after birth.

In contrast, activity of phenylalanine hydroxylase remains at a low level for 2 to 3 days following birth and then, during the next few days, slowly reaches the activity found in the adult.

Most striking is the pattern of development of tyrosine transaminase, which suddenly exhibits activity 2 hours postnaturally and at 12 hours shows maximum

![Graph showing enzymatic activity development](image_url)
activity, which is 2 to 10 times that characteristic of the adult liver. Sixteen to twenty hours postnatally, the activity of this enzyme returns to that found with liver from the adult animal.

Thus, there are real differences in the developmental pattern among three metabolically related enzymes. The physiologic significance of these differences is unknown, but the unpredictability of developmental pattern for any one enzymatic activity becomes strikingly apparent.

Incidentally, these data help to clarify the observations of Levine et al.\textsuperscript{a}—that p-hydroxyphenylpyruvate and tyrosine are excreted in the urine of the premature infant who does not receive a dietary supplement of vitamin C. The developmental pattern for tyrosine transaminase indicates that it is active soon after birth and tyrosine could be converted to p-hydroxyphenylpyruvate. However, without ascorbic acid, p-hydroxyphenylpyruvate oxidase would be relatively inactive, accounting for the accumulation of p-hydroxyphenylpyruvate and, consequently, tyrosine.

To return to the main discussion, difficulties multiply when one considers the development in different organs of enzymes which apparently serve the same function. Results illustrating this point with an enzymatic activity unrelated to tyrosine metabolism are shown in Figure 6.\textsuperscript{b} Glucose-6-phosphatase activity in kidney shows a steady rise until the activity characteristic of the adult is attained. In liver, activity of glucose-6-phosphatase shows considerable change. The initial elevation in activity at birth is followed within 24 hours by a further considerable increase and ultimately approaches adult activity after 10 days of life. When these data are plotted on the basis of nitrogen content, the curves maintain their general shapes and relationships. Correlation between these enzymatic changes and the physiology of these two organs is now being investigated.

It would appear that each enzyme may develop its activity in a different manner, related in part to its immediate environment, the organ. Specific changes within the organ and peripheral to it must occur for initiation of an increase in enzymatic activity.

What are the possible "trigger" mechanisms for development of enzymatic activity? A few years ago, we observed\textsuperscript{10} that there was an immediate postnatal spurt of activity of the tyrosine oxidizing system in the rat, which very shortly reached the activity of the adult. It was postulated that this elevation resulted from the release of

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure6.png}
\caption{Activity of glucose-6-phosphatase in liver and kidney during development of the rat.}
\end{figure}
hormones by the mother or the newborn, attendant to stress at birth. Lin and Knox recently showed that activity of tyrosine transaminase is considerably increased by parenteral administration of adrenal hormones and/or tyrosine to the adult rat.

On the basis of these data we undertook to study the relationship of adrenal hormones to development of activity of tyrosine transaminase; the findings are shown in Figure 7. Newborn rats were adrenalectomized immediately after birth, and the activity of tyrosine transaminase in their livers was measured at intervals until 12 hours of age, the time of peak activity. The liver from the adrenalectomized animal, 12 hours of age, had an activity similar to that of newborn animals and 20% of the activity of the adult liver. When hydrocortisone was administered subcutaneously to the animal at the time of adrenalectomy, the activity in the liver at 12 hours was similar to that of liver of the intact animal of the same age. When adrenalectomy was delayed until 2 hours after birth, results were erratic but there was a tendency for the activity to be that in the liver of the intact animal.

These data indicate that there is an immediate secretion of the adrenal glands, which acts in some unknown manner to stimulate formation of enzyme. When adrenalectomy is delayed, the mechanism for enzyme formation apparently has already been initiated and is self-perpetuating. It is of interest that the adaptive phenomenon of increased enzyme activity reported by Lin and Knox, as a consequence of administration of tyrosine and/or adrenal hormone to adult animals, does not occur in animals less than 16 hours of age. Nemeth mentioned similar results with tryptophan peroxidase in fetal guinea pigs.

Further evidence for the relationship of hormonal function to enzymatic activity is shown in Figure 8. These are data gathered in our laboratory and are a repetition of work reported by Jost et al. The open bars represent the concentration of glycogen in liver during late fetal life in rabbits. Soon after the twenty-eighth day of gestation the concentration of glycogen normally decreases rapidly. If the fetal rabbit is decapitated in utero on day 22 or 23 of gestation, the amount of glycogen in the liver on the twenty-eighth day is negligible. But Jost and Jacquot showed that when the fetus is decapitated on day 25 or 26, glycogen continues to accumulate as in the intact fetus. If at the time of decapitation
Fetus decapitated on 22-23 day of gestation

Fig. 8. Effect of intrauterine decapitation on glycogen storage in the liver of the fetal rabbit.

(day 22 or 23) adrenocorticotropic (ACTH) is administered, then at 28 days, there is a normal amount of glycogen.

Thus, glycogen storage in fetal liver is dependent upon activity of the hypophysis at a specific time. This endogenous secretion can be adequately replaced by exogenous ACTH. Joint studies are in progress with Professor Jost and his group to determine the exact locus of action of hypophyseal hormones on glycogen metabolism in fetal life. Preliminary data indicate that there may be increased degradation of glycogen subsequent to decapitation.

DISCUSSION

It is apparent that certain enzymes are inactive during fetal life and even in early postnatal life. The developmental pattern for an individual enzyme may be unique and depend upon stimulation by specific hormones at a critical time in the life of the organism.

I have been primarily interested in two major aspects of this problem: 1) basic mechanisms for the development of activity of an enzyme; and 2) relationship of these phenomena to an understanding of immaturity.

In Figure 9 a sequence is proposed for mechanisms governing the appearance and regulation of enzymatic activity in an organism. For proven examples of these events we are forced to borrow information from microbial geneticists, for they have contributed invaluable observations necessary for understanding interrelations of heredity and development.

In a normal animal it can be assumed that all genetic information necessary for synthesis of a required quota of enzymes is present in the embryo. Following from this assumption there are three possibilities that could be responsible for lack of enzyme activity in the young animal: 1) enzyme is not produced by virtue of some substance repressing gene action; 2) an incomplete enzyme is produced that is inactive because it is in the form of a proenzyme; and 3) enzyme is produced in its complete form but is inactive as a result of an inhibitor which must be removed. Figure 9 attempts to depict these relationships.

It was postulated that the lack of activity of tyrosine transaminase was a consequence of lack of enzyme formation. Final proof necessitates an actual demonstration of a deficiency in enzyme synthesis early in
life, or an increase in formation of enzyme at a time immediately preceding the appearance of activity. These studies are now in progress in our laboratory. As absence of adrenocortical secretions has been shown to result in deficiency of activity of tyrosine transaminase, it can be assumed that adrenocortical hormones are required for formation of the enzyme in a way as yet not understood. In contrast, an enzyme can be formed, but inactive, as shown with p-hydroxyphenylpyruvate oxidase which requires addition of ascorbic acid to activate it.

However, there are many controls and pacemakers which are required for development to proceed in an orderly and organized fashion. Even after an enzyme is active, the product formed may in turn inhibit further action of the enzyme. Product inhibition is a well known biochemical phenomenon; recently Gorini and Maas, as well as others, have shown, with bacteria, that a product of enzymatic action can inhibit directly at the gene site to prevent further formation of enzyme. In this manner the synthesis of the enzyme is controlled. In some of the inborn errors of metabolism in which the enzymatic defect has been identified, it is probable that the disturbance is a result of a genetic aberration leading to inactivity or absence of a particular enzyme. It is readily apparent that a great many observations must be made before these processes are fully comprehended.

The intact organism presents a different group of problems not entirely genetic. Levine et al. showed that when ACTH is given to a premature infant whose diet is not supplemented with vitamin C the tyrosyluria is alleviated. Preliminary data indicated that the probable site of action of the ACTH is the renal tubule, causing a decrease in tubular reabsorption of tyrosine and p-hydroxyphenylpyruvate. Still another factor in the intact organism is the actual amount of protoplasm available for any particular function. Observations from our laboratory indicate that cells from the kidney of a fetal rabbit can transport p-aminohippurate as adequately as cells...
from the kidney of an adult animal. However, there are fewer cells in the fetal kidney and total function of the organ, when compared with the adult, is diminished.

Superimposed on these problems is the possibility that a deficiency in enzyme activity could lead to a lack of a metabolically important product. For example, from our data, phenylalanine hydroxylase is inactive in liver of the premature infant, making tyrosine a dietary essential amino acid. Holt recently reported that histidine is an essential amino acid for the infant but not the adult. It is these potential nutritional aspects which may be important in the care of infants but are less important in utero, where many nutritional substances deficient in the fetus are supplied via the placenta.

What then is immaturity? It is a word defined as a lack of development, but inherent in the concept of immaturity must be the factor of time and the relationships of genes, molecules, cells and organs, symphonically arranged in an intact organism. It is possible that these wide differences in enzymatic development could contribute to the variable vitality of premature infants and their ability to adjust to a changing environment. Immaturity can be explained and tested for on any or all of the bases discussed. Many of these metabolic deficiencies may be related to the fact that the infant channels most of his effort towards protein synthesis and ultimately growth.

REFERENCES


27. Cohn, M.: Contributions of studies on the β-galactosidase of Escherichia coli to our understanding of enzyme synthesis.


Physicians should be aware that the accidental ingestion of Clinitest® tablets, now in common use for urine analysis, can cause severe stricture of the esophagus because of their content of sodium hydroxide; perhaps the copper sulfate contained in the tablets may play a secondary role. The author adds a report of a case to 4 cases previously recorded. It is important that both physicians and patients realize this hazard and keep Clinitest® tablets beyond the reach of small children.
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Pediatrics 1959;23:606

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