# CERTAIN ASPECTS OF TYROSINE METABOLISM IN THE YOUNG. II. THE TYROSINE OXIDIZING SYSTEM OF FETAL RAT LIVER[1,2]

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A recent study and review (1) of tyrosine metabolism has shown that the overall oxidation of tyrosine to acetoacetate and fumarate requires two moles of oxygen. The first step is the conversion of tyrosine to p-hydroxyphenylpyruvate by a non-oxidative transamination. The p-hydroxyphenylpyruvate is in turn oxidized to homogentisate with the utilization of one mole of oxygen. This reaction requires the presence of a reducing agent such as ascorbic acid (2–5) or dichlorophenolindophenol (6, 7). Recent data (8) indicate that the conversion takes place without the previously postulated (9) formation of an intermediate such as 2,5-dihydroxyphenylpyruvate. The homogentisate is oxidized by one mole of oxygen to maleylacetoacetate in the presence of reduced iron, and the maleylacetoacetate is further acted upon by an isomerase to yield fumarylacetoacetate. Finally, enzymatic hydrolysis of this compound results in the formation of the end products, fumarate and acetoacetate.

This metabolic pathway is of special interest to pediatricians (10–12) since it has been shown (10) that p-hydroxyphenylpyruvic acid and tyrosine are excreted in the urine of premature infants maintained on diets high in proteins but not supplemented with vitamin C. It has also been shown (11) *in vitro*, that there is considerably less tyrosine oxidizing activity in the liver of the premature infant than in the liver of the human adult or full-term infant. This deficiency was ascribed to a decrease in the concentration of the transaminase apoenzyme.

The present investigation indicates a similar deficiency in the liver of the fetal rat. Paucity of tyrosine oxidation in the fetal liver of this animal can also be explained by a lack of specific tyrosine transaminase apoenzyme. The possible relationship of these findings to the so-called "inborn errors of metabolism" will be discussed.

## METHODS

Rats of the Long-Evans strain were used throughout the study. Adult rats were killed by a blow to the head and the liver was immediately removed and chilled. Infant rats were killed by decapitation to facilitate exsanguination. Newborn rats were obtained as they were born. Fetal rats were obtained as near to predicted term as possible. The mother rat was killed by a blow to the head and her gravid uterus was excised and immediately placed on ice in the cold room. The fetuses were separated from the placentae and membranes, decapitated and the livers removed.

Litter mates that were allowed to survive were divided into two groups each comprising two or three rats. One group was nursed by the mother. The other group was taken from the mother, fed only water by tube and kept in cotton batting for warmth. At necropsy all of the nursing animals had milk in their gastro-intestinal tracts.

All procedures were carried out in a cold room (temperature 2° to 4° C). The livers from two to four fetal or infant rats were usually pooled in order to obtain a sample adequate for analysis.

The pooled livers from fetal and infant rats were ground for two minutes in a chilled Teflon-glass homogenizer with the dropwise addition of one to three volumes of ice-cold 0.9 per cent KCl. The livers from adult rats were ground for two minutes in a Waring Blendor with three volumes of the cold salt solution.[3] All of the homogenates were spun at 18,000 × g for 15 minutes in the Sorvall SS-1 centrifuge. The supernatant fluid derived from the high-speed centrifugation (soluble fraction) was

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[3] All the livers were homogenized and analyzed immediately after removal. It was found that quick freezing and storing at − 40° C before analysis resulted in a marked depression in tyrosine oxidizing activity. For example, newborn rat livers analyzed immediately gave a value of 35 µl. $O_2$ per 30′ per mg. N. whereas frozen and stored newborn rat livers gave a value of 8 µl. $O_2$ per 30′ per mg. N.

assayed for tyrosine transaminase and overall tyrosine oxidizing activities.

The manometric procedure as outlined by Knox and LeMay-Knox (2) was followed in the assay of overall tyrosine oxidation. The flask contents were essentially those previously described (11): 2.0 ml. of 0.2 M phosphate buffer pH 7.4 with or without 8 μM. of tyrosine, 0.2 ml. of a solution containing 500 μg. of ascorbic acid neutralized to pH 7.4. The sidearm contained 0.3 ml. of α-ketoglutaric acid (30 μM.) adjusted to pH 7.4 and 0.2 ml. of pyridoxal phosphate in water (60 μg.). The center well had 0.2 ml. of 20 per cent KOH on a strip of filter paper. The total volume was adjusted to 3.7 ml. with glass-distilled water. For assay of tyrosine transaminase the flask contents were the same except that ascorbic acid was omitted and flasks were incubated in an atmosphere of nitrogen to prevent oxidation of the formed p-hydroxyphenylpyruvate.

In all instances, the flasks were equilibrated for a period of ten minutes at 37.3° C. in an atmosphere of either air or nitrogen. Stopcocks were then closed and the contents of the sidearms added. Readings were taken at intervals of ten minutes for a period of sixty minutes. Control flasks containing equivalent amounts of tissue, but no tyrosine, were always run along with the experimental flasks and endogenous oxidative activity was subtracted from values obtained with the experimental flasks.

Nitrogen was determined on the original liver preparation by the Ma-Zuazaga modification of the micro-Kjeldahl method (13). At the end of the incubation period in air separate aliquots were taken from the flasks and added either to trichloroacetic acid or metaphosphoric acid to yield a final concentration of 5 per cent acid. The samples incubated in nitrogen were added only to trichloroacetic acid. The supernatant fluid resulting from the precipitation with trichloroacetic acid was used for the determination of Brigg's reacting material (p-hydroxyphenylpyruvic acid when the samples were incubated in nitrogen). The supernatant fluid from the metaphosphoric acid precipitations was used for the determination of total p-hydroxyphenyl compounds as previously reported (11) in order to check oxygen uptake.

## RESULTS

The data presented in Table I show the activity of the tyrosine oxidizing system as obtained with the soluble fraction of the livers of rats of different ages. The activity of fetal liver was about 12 times less than that of newborn fullterm liver on the basis of either wet weight or nitrogen. The age difference between newborn and fetal rats was no more than one to three days. The liver of the newborn rat averaged 70 per cent of the activity of adult liver on the basis of nitrogen; and 50 per cent on the basis of wet weight. The activities of

TABLE I

*Tyrosine oxidizing activity of the soluble fraction of rat liver*

| | No. | Range μl./30′/mg. N | Ave. | Range μl./30′/gm. wet wt. | Ave. |
|---|---|---|---|---|---|
| Fetal rats | 3* | 2.0–3.6 | 2.7 | 32–40 | 35 |
| Newborn rats | 5* | 19–67 | 35 | 252–812 | 538 |
| Adult rats | 6 | 29–73 | 52 | 760–1,472 | 1,183 |
| Pregnant rats | 2 | 29–60 | 45 | 944–1,424† | 1,225 |

* This refers to the number of separate pools of livers rather than individual livers.

† This range represents the oxidative activity obtained from the livers of three individual rats.

the livers of the pregnant and adult rat were the same.

The effect of postnatal feeding on the activity of the tyrosine oxidizing system was studied. Infant rats were killed at different intervals following birth and activities were determined in the soluble fraction of the livers.

The data in Figure 1 show that the tyrosine oxidizing activity of fetal liver was minimum (3 μl. $O_2$ per 30′ per mg. N.) in comparison to any other age group. The tyrosine oxidizing activity of the livers of newborn rats which had nursed was 43 per cent less than the activity of the livers of rats immediately at birth. The activity in non-nursed litter mates of similar age approximated that of newborn rat liver and was nearly twice as high as that of their nursing litter mates. By 24 to 48 hours of age the activity of the infant rat liver was

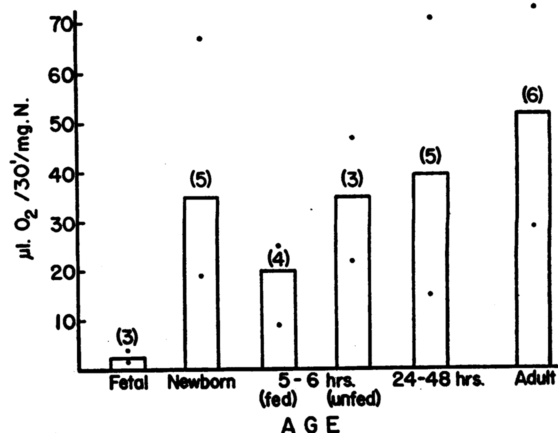


FIG. 1. THE TYROSINE OXIDIZING ACTIVITY OF RAT LIVER IN RELATION TO AGE

The black dots indicate extremes. The numbers in parentheses represent the number of pools of livers. In the case of the adult only individual livers were used.

above the newborn level and averaged 83 per cent of the activity of the adult rat.

Detailed observations on the influence of nursing are shown in Figure 2 where groups of rats from the same litter are compared. The data indicate that the overall tyrosine oxidizing activity in the liver of non-nursed rats reached considerably higher levels than those of the liver of nursed animals.

The activity-time curves presented in Figure 2 would indicate that a value taken at any particular time is less meaningful than an examination of the entire time course of oxygen uptake.

At the end of 30 minutes of incubation the liver of the non-nursed rats took up 28 to 100 per cent more oxygen than did the liver of the nursed rats. After 60 minutes of incubation there was 65 to 143 per cent more oxygen uptake with the liver of the non-nursed rats. However, there was progressive increase in oxygen uptake by the liver of newborn rats with time whereas the oxygen uptake of the liver of rats 5 hours old reached a sustained plateau in 20 minutes or even fell off thereafter.

Since the first and obligatory step in tyrosine oxidation is its non-oxidative transamination to p-hydroxyphenylpyruvic acid, it was postulated in a previous paper (11) that the lesser overall tyrosine oxidizing activity of premature infant and perhaps fetal rat liver might in part be due to deficiency in tyrosine transaminase apoenzyme.

Figure 3 represents the amount of Brigg's reacting material (p-hydroxyphenylpyruvic acid) produced by the soluble fractions of fetal and adult

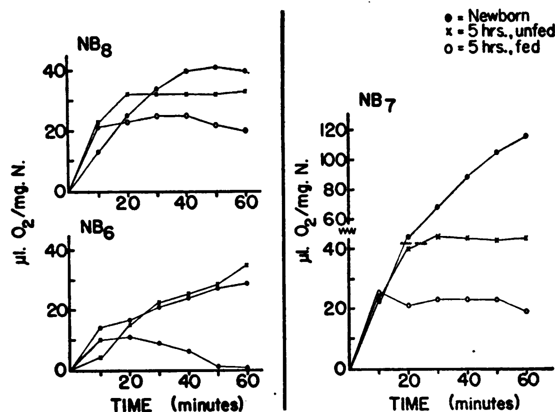


FIG. 2. TYROSINE OXIDIZING ACTIVITY IN THE LIVER OF THE NEWBORN RAT: EFFECT OF FASTING

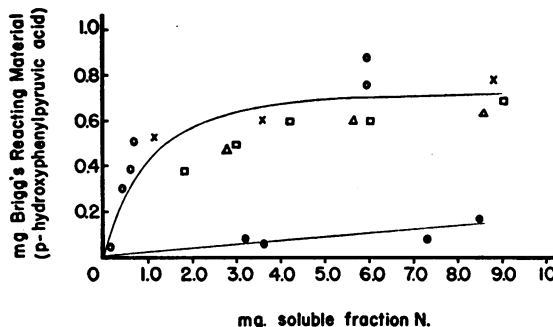


FIG. 3. ACTIVITY OF TYROSINE TRANSAMINASE IN FETAL AND ADULT RAT LIVER

Each different symbol represents separate determinations using the same rat liver or a pool of livers. Adults are represented as ○, ×, △, □; fetuses are represented as ●, ⊗. These lines are drawn for better visualization.

rat livers. Increasing quantities of soluble fraction were incubated with tyrosine for 60 minutes in an atmosphere of nitrogen. In each instance the necessary cofactors, α-ketoglutarate and pyridoxal phosphate, were added in excess. Under these conditions the amount of soluble fraction determines the amount of apoenzyme and the results indicate that the tyrosine transaminase in fetal liver is of the order of 12 times less than that of adult rat liver when 3.0 to 4.0 mg. of soluble fraction nitrogen are used. The remaining enzymes comprising the tyrosine oxidizing system are now being tested in similar fashion.

## DISCUSSION

The data herein presented demonstrate that the activity of the tyrosine oxidizing system is markedly low in fetal rat liver. This minimum activity, measured in the presence of excess cofactors, must be ascribed to a diminished amount or activity of tyrosine transaminase apoenzyme. Under physiologic conditions it is possible that the activity of the transaminase would be even lower than these data indicate since there may be an additional deficiency of the necessary cofactors including pyridoxal phosphate.

Since transamination is the first obligatory reaction, a low concentration or activity of tyrosine transaminase would of necessity result in a low overall tyrosine oxidizing activity. However, data previously presented (11) suggest that all apoenzymes involved in this pathway of tyrosine oxida-

tion may be decreased in amount or activity in the liver of the premature infant.

The activity of newborn rat liver is on the average 74 per cent of the activity of adult rat liver and is 12 times higher than that of fetal rat liver. It is difficult to explain this immediate and marked increase in activity on the basis of age since assays of newborn livers were made immediately at birth and fetal livers were assayed almost at term. This elevation in activity is peculiar to newborn rat liver since the liver from nursing infant rats 5 to 24 hours old showed an activity about one-half that of newborn rat liver. However, deprivation of food from rats of this age resulted in an increase in tyrosine oxidizing activity as measured after 30 minutes of incubation. One possible explanation for these results is that the stress of the birth process led to the release or activation of a hormone or other substance influencing tyrosine oxidation. The persistence of high activity in the livers of infant rats who were not nursed could then be explained by the continuing hormonal stimulation resulting from the additional stress of fasting. This hypothesis receives support from the fact that the induced tyrosyluria of premature infants can be alleviated by the administration of ACTH (14). It is being tested by the administration of pituitary, adrenal cortical and medullary hormones [4] to pregnant rats.

The manifestation or appearance of a particular enzyme activity, at a rate which would be too rapid to result from *de novo* synthesis, could be explained by the activation or unmasking of an inactive form of an enzyme. The special cases of the proenzymes or zymogens of the digestive enzymes are examples of this phenomenon. In addition, other varieties of enzymes exhibiting a similar type of behavior have recently been described, such as the rapid *in vivo* activation of phosphorylase during the course of muscle stimulation (15), the *in vitro* activation of nucleotidases and phosphatases by temperature (16), the hormonal activation of muscle and liver phosphorylase (17) and tryptophan peroxidase (18).

Certain enzyme activities have been reported to be diminished in the organs of the fetus (19–21). Usually, deficiencies in enzyme activity in later life are associated with distinct disease entities. For example, in the liver of patients with glycogen storage disease there is commonly a diminished glucose-6-phosphatase activity (22). Due to this low activity, glycogen can be formed but cannot be converted to glucose. It has also been reported (23) that the liver of a premature infant had a similar low level of glucose-6-phosphatase activity. Nemeth (19) also presented evidence that the glucose-6-phosphatase activity of the livers of guinea pigs increases with the age of the animals. Thus, some of the more clearly identified disorders related to specific enzyme deficiencies or inactivities may stem from a persistence of fetal or embryonic enzymic states resulting from genetic (24) or congenital biochemical influences.

[4] Preliminary experiments in this laboratory have demonstrated that there is no *in vitro* stimulation of tyrosine oxidation by the addition of cortisone, hydrocortisone or ACTH to the liver homogenate or to the soluble fraction of liver.


## SUMMARY

1. The tyrosine oxidizing activity of fetal rat liver is 12 to 15 times lower than that of fullterm or adult rat liver.
2. At birth the tyrosine oxidizing activity of the newborn liver approaches that of adult rat liver.
3. When postnatal rats are unfed, their livers have a tyrosine oxidizing activity approximately equal to that found in the liver of the newborn rat, in contrast to the livers of fed infant rats which show an activity about one-half that of the liver of newborn and unfed rats.
4. The lack of overall tyrosine oxidation by fetal rat liver can be explained by a lack of tyrosine transaminase activity, even in the presence of an excess of pyridoxal phosphate and $\alpha$-ketoglutarate.
5. The relation of these observations to hormonal factors is discussed.



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