1. Madin, S. H., York, C. J., and McKercher, D. G., *Science*, 1956, v124, 721.
2. York, C. J., Schwarz, A. J. F., and Estela, L. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 740.
3. Miller, N. J., *J.A.V.M.A.*, 1955, v126, 463.
4. McKercher, D. G., Moulton, J. E., and Jasper, D. E., *Proc. U. S. Livestock Sanitary Assn.* 58th Meeting, 1954, 260.
5. Jensen, R., Griner, L. A., Chow, T. L., and Brown, W. W., *ibid.*, 1955, 189.
6. Kendrick, J. W., York, C. J., and McKercher, D. G., *ibid.*, 1956, 155.
7. Schroeder, R. J., and Moys, M. D., *J.A.V.M.A.*, 1954, v125, 471.

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Development of Phenylalanine Hydroxylase in Liver of the Rat.* (23506)

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Inability to convert phenylalanine to tyrosine, a reaction catalyzed by the enzyme system phenylalanine hydroxylase, is known to occur as an hereditary metabolic abnormality. Jervis(1) and Udenfriend and Bessman(2) have reported that individuals with phenylpyruvic oligophrenia almost completely lack the ability to oxidize phenylalanine, while Jervis(3) has shown that postmortem specimens of livers of two patients with this disease were inactive in *in vitro* assays for phenylalanine hydroxylase. Recently, detailed biochemical observations on liver biopsy(4) and autopsy (5) material have confirmed and extended his findings.

Since other enzymes whose deficiencies in postnatal life are associated with disorders of incomplete metabolism are known to be essentially inactive in fetal life(6)—glucose-6-phosphatase(7) and the enzymes of tyrosine oxidation(8)—it seemed worthwhile to determine if the enzyme system, phenylalanine hydroxylase, demonstrates a similar developmental pattern.

Methods. Fetal, newborn, and adult rats of the Long-Evans strain were used. Fetal animals were studied as close as possible to predicted term. They and newborn rats were killed by decapitation; adult rats by a blow to the head. Livers were removed in the cold room (temperature 2 to 4°C) and immediately placed on cracked ice. Fetal as well as newborn livers were pooled to obtain adequate samples. The livers were homogenized in the cold with a Teflon homogenizer in 2 volumes of ice-cold 0.15 M KCl. Homogenates were spun in a Spinco Model L centrifuge for 30 min. at 100,000 x gravity. The soluble fractions were assayed directly or after 3 hours of dialysis against 0.15 M KCl in 0.01 M phosphate buffer at pH 7.0. Freezing at −15°C for several days resulted in no loss of activity. The soluble fractions of livers (1.0 ml) were shaken at 35°C in air in a reaction mixture containing 2 μM of L-phenylalanine, 150 μM of phosphate buffer, pH 7.0, 5 μM of nicotinamide, and 2 μM of reduced diphosphopyridine nucleotide; final volumes were 1.75 ml. The reaction was stopped after 20 to 60 minutes incubation by addition of 1.75 ml of 10% trichloroacetic acid. Following centrifugation, formed tyrosine was measured colorimetrically(9) on suitable aliquots of the deproteinized solution. Control values obtained with reaction mixtures incubated without phenylalanine were subtracted in each case. Preparations of known activity were concurrently assayed to ensure satisfactory experimental conditions. Nitrogen was determined on aliquots of the liver soluble fractions by the Ma-Zuazaga modification of the micro-Kjeldahl method(10).

Results. Phenylalanine hydroxylase activity in dialyzed soluble fractions of livers of

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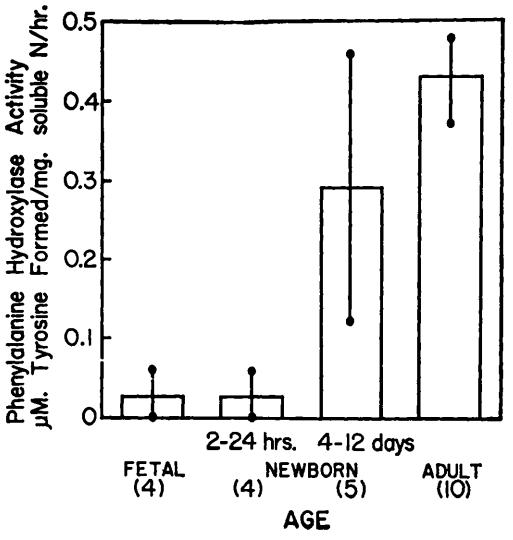

FIG. 1. Phenylalanine hydroxylase activity of rat liver in relation to age. Dialyzed soluble fractions of liver were assayed as described in the text. Height of bars represents the mean; lines drawn through points represent range of experimental values obtained. Figures in parentheses denote number of experiments performed. Values greater than 0.4 μM tyrosine/mg N correspond to complete conversion of the 2 μM of phenylalanine added to the reaction mixtures.

fetal, newborn, and adult rats are summarized graphically in Fig. 1.

Fetal rats. Experiments with dialyzed soluble fractions of fetal liver indicated that conversion of phenylalanine to tyrosine was absent or negligible at this stage of development. Samples were incubated for one hour; under these conditions 8% or less of the added phenylalanine was hydroxylated.

Newborn rats. Enzyme activity of dialyzed preparations of livers of rats less than 24 hours old was within the range of fetal preparations; not more than 8% of added phenylalanine was converted to tyrosine. These essentially inactive soluble fractions could not be stimulated by addition of larger amounts (up to 6.0 μM) of pyridine nucleotides in either oxidized or reduced form. In the dialyzed soluble fractions of livers of rats 4 to 12 days old, complete conversion of the added phenylalanine to tyrosine was observed in one of the assays. The other 4 converted 35 to 55% of the phenylalanine.

Adult rats. Phenylalanine hydroxylase activity of dialyzed soluble fractions of livers obtained from adult animals of both sexes, following periods of incubation of 20 to 60 minutes, showed a conversion capacity of 50 to 90% of added phenylalanine in 20 minutes. Within one hour conversion was complete in 8 of 10 observations. In the remaining 2, conversion was 80% and 95% complete.

TABLE I. Effect of Dialysis on Activity of Phenylalanine Hydroxylase in Liver Soluble Fractions of Rats.*

Age	μM tyrosine/ml sol. frac. Before dialysis	After dialysis for 3 hr	% activity lost
Adult	1.84	1.34	27
"	2.37	1.24	48
"	2.02	1.68	17
"	2.11	1.23	42
Fetus	0	0	
"	.15	.05	67
Newborn (23 hr)	.85	.16	81
" (4 days)	2.18	1.14	48

* Incubation time for liver soluble fractions of adult rats—20 min.; of fetal and newborn rats—60 min.

Effect of Dialysis. Recent studies by Kaufman(11,12) on the mechanism of this reaction in liver extracts have indicated the presence of a hitherto unrecognized cofactor. Since this cofactor is presumably dialyzable, the influence of dialysis on the activity of the soluble fraction was studied (Table I). The activity of adult liver preparations was decreased 17 to 48% after 3 hours of dialysis. Soluble fractions of livers of fetal and newborn rats were also tested before and after dialysis. Loss of activity in these preparations was considerably more marked, ranging from 48 to 81%. Nevertheless, the activity of undialyzed preparations of fetal livers never exceeded a conversion of 8%, and in one experiment, no activity was found prior to or following dialysis. Thus the low activity of fetal and newborn liver preparations is not due primarily to inactivation by dialysis, but the more marked lability of these preparations does suggest that they contain less of a dialyzable component (or a component more readily inactivated during dialysis) than those of adult animals.

Discussion. The results indicate that phenylalanine hydroxylase activity is absent or negligible in the livers of fetal rats near term

and in newborn rats less than 24 hours old. Within a few days following birth the enzyme system approaches the level of activity observed in adult animals.

It has recently been established that conversion of phenylalanine to tyrosine in liver extracts requires participation of 2 discrete enzymes(13), as well as reduced pyridine nucleotide(14) and the unidentified cofactor mentioned previously. One of the components of the enzyme system can be found in tissues which do not hydroxylate phenylalanine, while the second enzyme, which is specific, has been found only in liver. This second specific enzyme is now known to be the component which is lacking in the livers of individuals with phenylketonuria(4,5). Recent experiments in this laboratory have shown† that the non-specific components of the overall reaction are present in fetal liver, and that inactivity of these preparations can also be traced to a deficiency in the specific enzymic component of liver. The study of phenylalanine hydroxylase provides a striking example of a situation wherein enzymic deficiency encountered in an hereditary metabolic disease, phenylpyruvic oligophrenia, occurs as the normal pattern in the fetal mammal. Further studies of this enzyme in the fetal animal may contribute information on the pathogenesis of the enzymic defect of phenylpyruvic oligophrenia.

Summary. Development of phenylalanine hydroxylase in livers of fetal, newborn, and adult rats has been studied. The results indicate that the enzyme system develops shortly after birth. Activity of the enzyme is negligible in livers of fetal rats near term and rats less than 24 hours old; it approaches the adult level in livers of animals only a few days old. The virtually inactive preparations obtained from livers of fetal and newborn animals could not be activated by adding increasing amounts of pyridine nucleotide to the reaction mixtures in either oxidized or reduced form. The activity of soluble fractions of adult liver was partially lost during short dialysis; the initially low activity of preparations of livers from fetal and newborn animals was particularly sensitive to this procedure.

† F. T. Kenney, *et al.*, in preparation.

1. Jervis, G. A., *J. Biol. Chem.*, 1947, v169, 651.
2. Udenfriend, S., and Bessman, S. P., *ibid.*, 1953, v203, 961.
3. Jervis, G. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 514.
4. Wallace, H. W., Moldave, K., and Meister, A., *ibid.*, 1957, v94, 632.
5. Mitoma, C., Auld, R. M., and Udenfriend, S., *ibid.*, 1957, v94, 634.
6. Kretchmer, N., and Etzwiler, D. D., to be published in *Pediatrics*, 1957.
7. Nemeth, A. M., *J. Biol. Chem.*, 1954, v208, 773.
8. Kretchmer, N., and McNamara, H., *J. Clin. Invest.*, 1956, v35, 1089.
9. Udenfriend, S., and Cooper, J. R., *J. Biol. Chem.*, 1952, v196, 227.
10. Ma, T. S., and Zuazaga, G., *Indust. and Engin. Chem. Anal. Ed.*, 1952, v14, 280.
11. Kaufman, S., *Fed. Proc.*, 1957, v16, 871.
12. ———, *J. Biol. Chem.*, 1957, v226, 511.
13. Udenfriend, S., and Mitoma, C., *in Amino Acid Metabolism*, W. D. McElroy and B. Glass, eds. 1955, Johns Hopkins Press, Baltimore.
14. Mitoma, C., *Arch. Biochem. and Biophys.*, 1956, v60, 476.

Received August 1, 1957. P.S.E.B.M., 1957, v96.