formaldehyde did not interrupt the normal estrous cycle. There was no change in the onset of puberty as demonstrated by the age at opening of the vagina. On the basis of these results and of earlier work in cycling rats (2), it is suggestive that the increased gestation length is due to prolonged progesterone secretion as a result of the hypothalamic blockage of PIF. Causes of decreased fetal and placental weights were not determined.

Summary. Daily subcutaneous injections of 20 mg of yohimbine-HCl per kg of body weight from the 15th day of gestation until parturition increases the length of pregnancy in the rat by 1 day. A single injection of yohimbine on day 20 of pregnancy was without effect on gestation length. Rats which were treated with a daily dose of 20 mg of yohimbine per kg (started on day 15 and sacrified on day 21 of gestation) had significant increased pituitary and adrenal weights as well as smaller fetuses and placentas. Cold stress did not affect the weight of the fetuses or placentas. No difference in growth rate was produced by either yohimbine-HCl or formalin injections.

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Free and Protein-Bound Lysine Flux in Developing Rabbit Brain*† (32703)

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Protein concentration increases with cerebral maturation (1,2). *In vitro* studies of separated cells (3) reveal greater capability of immature brain cells to incorporate amino acid into protein. Other *in vitro* studies show that pH 5 enzyme activity of rat brain is greater in the immature animal and also that there is a greater rate of ribosomal protein synthesis in the developing brain than in mature brain (4). Few *in vivo* studies of rate of protein synthesis in immature brain are available. Comparison of flux of lysine into cerebral protein of intact mice reveal that 10-day-old mice manifest a higher rate of flux of lysine into brain protein than mature mice (5). Recent studies have pointed out that rate of incorporation of phenylalanine-^{14}C into piglet brain protein is primarily a function of postnatal age and not of gestational age (6). Although *in vitro* studies demonstrate the potentiality for greater lysine incorporation into protein in neonatal brain, factors present in the intact animals may significantly modify the actual rate of synthesis. The present studies were undertaken to demonstrate *in vivo* changes in rate of lysine flux into rabbit brain protein during the first 12 days of life. This span coincides with a period of profound neurophysiological, behavioral, and neurochemical change.

Materials and Methods. The general scheme of the experiments and methods of calculation are essentially those used by Lajtha *et al.* (5). Rabbits of ages 1, 4, 8, and 12 days were injected intraperitoneally with U-^{14}C-L-lysine[1] (198 mC/mmole), 80 μC/kg. The brains of the animals were removed at 10,20,40, and 60 min after injection and processed as discussed below. Two rabbits of each age were studied for each elapsed time period. Hepa-

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[1] Purchased from Volk Radiochemical Company, Burbank, California.

TABLE I. Lysine Concentrations.[a]

	Age (days)			
	1	4	8	12
Plasma conc. (μmoles/ml of plasma)	.22 ± .06	.22 ± .07	.29 ± .06	.49 ± .05
NPN conc. [μmoles/gm brain (wet wt.)]	.21 ± .04	.11 ± .02	.15 ± .03	.22 ± .02
Protein conc. [mμmoles/mg of protein (dry wt.)]	660 ± 34	691 ± 48	589 ± 25	599 ± 69

[a] Each figure represents the analysis of 8 rabbit brains.

rinized blood samples were collected simultaneously.

After centrifugation of the whole blood at 1200*g* for 20 min the plasma protein was precipitated with 3% sulfosalicylic acid. The precipitate was twice washed and recentrifuged. The supernatants were combined, flash evaporated and freeze-dried. They were then resuspended in pH 2.2 sodium citrate buffer, 0.20 *N*, and utilized for determination of lysine concentration.

After decapitation, the brain was rapidly removed and the area below the midbrain was sectioned and discarded. Surface blood vessels and blood were removed by gentle blotting with gauze squares and the cerebrum was placed into cold 5% perchloric acid (PCA). The brain was homogenized in a homogenizer tube fitted with a motor-driven teflon pestle with 10 strokes of 5- sec duration each. The homogenizer tube was kept in ice during the procedure. The homogenized tissue was then centrifuged at 0° for 40 min at 10,000*g*. The precipitate was twice washed and recentrifuged. The supernatants were combined and the PCA precipitated with 5 *N* KOH. The material was centrifuged for 20 min at 0° at 10,000*g*. The precipitate was washed and recentrifuged. The resulting combined supernatants were flash evaporated and were freeze-dried. They were then resuspended in pH 2.2 sodium citrate buffer, 0.20 *N*, and utilized for the determination of the nonprotein nitrogen fraction (NPN) lysine concentration.

The precipitate was washed twice with 0.5 *N* HCl and recentrifuged for 20 min at 10,000*g*. The pellet was suspended in distilled water and heated in a boiling water bath for 5 min to remove nucleic acids (7). After cooling the precipitate was subsequently washed and centrifuged in chloroform twice, acetone twice, and ether twice. The precipitate was dried to constant weight. Hydrolysis was performed in 6*N* HCl for 24 hours in an autoclave at 115°. The resulting solution was evaporated to dryness and resuspended in pH 2.2 sodium citrate buffer, 0.20 *N*, for analysis of lysine concentration and radioactivity.

Lysine concentration was determined by means of an automatic amino acid analyzer. A 0.9 cm diameter chromatography tube was packed with a 16 cm column of Aminex A-5 resin.[2] After the sample was loaded on the column the 7-cm head space was filled with pH 3.230, 0.20 *N*, sodium citrate buffer. The sample was eluted from the column with pH 5.280, 0.35 *N* sodium citrate buffer, 50°, at 60 ml/hour. Duplicate samples were eluted in a similar manner and collected for determination of radioactivity.

Radioactivity was determined by means of a Nuclear-Chicago Liquid Scintillation Spectrometer. A 1.0-ml portion of the pH 5.280 eluate was placed into vials containing 19.0 ml of standard scintillation solution. Corrections for quenching were made either by internal standard or by the channels-ratio procedure.

Results. Lysine concentrations are depicted in Table I. The plasma concentration rose with maturation and was over twice as high in the 12-day-old animal as in the newborn animals. The NPN lysine concentration fell after the first day, but climbed to its neonatal values by day 12 of life. The lysine concentration of protein appeared to decrease with development.

The rate of flux of lysine from the plasma to the NPN fraction of brain is shown in

[2] Purchased from Bio-Rad Laboratories, Richmond, California.

TABLE II. Flux of Lysine from Plasma to NPN Fraction of Developing Rabbit Brain.[a]

Min	Age (days)			
	1	4	8	12
10	24.7; 33.6	24.7; 26.7	17.8; 19.7	6.0; 8.2
20	14.4; 12.3	8.2; 6.3	5.4; 4.8	4.7; 5.0
40	6.2; 6.7	6.0; 6.3	4.9; 4.9	4.0; 3.7
60	3.2; 3.0	3.9; 4.4	3.1; 3.3	1.6; 1.5

[a] Results expressed in mμmoles per gm brain (wet wt.) per min.

Table II. The well-known greater net uptake of small molecules across the blood-brain barrier system in immature brain is well documented here. The radioactivity of the plasma lysine had reached its peak before the initial 10-min samples were obtained and the data for lysine radioactivity all fell on the descending portion of the curve. The range of lysine specific activity in plasma noted in these experiments was 15,850 dpm/μmole of lysine to 159,500 dpm/μmole of lysine. The rate of flux of lysine into brain in the 1- and 4-day-old animal is fourfold greater at 10 min than in the 12-day-old animal. This differential diminished with time but the rate was still significantly faster in the younger animal after 60 min had elapsed. The radioactivity of the NPN lysine reached its peak between 20 and 40 min after injection. The range of lysine specific activity in the NPN fraction was 5700 dpm/μmole of lysine to 105,600 dpm/μmole.

The flux of lysine from the brain NPN to protein is seen in Table III. The rate of flux is significantly greater in the 1-day-old rabbit brain than the 4-day-old rabbit brain and there is a decreasing rate of flux with maturation at all times studied. The comparative changes in the rate of flux of lysine to protein at different times are nowhere near as great as the changes in the rate of flux of lysine from plasma to brain. The radioactivity of the protein-bound lysine did not reach a peak during these 60-min experiments. The radioactivity was rising linearly by 60 min and other studies demonstrate a plateau at about 5 hours followed by a slow decrease. The range of lysine specific activity in the protein fraction was 207 dpm/μmole to 1824 dpm/μmole.

TABLE III. Flux of Lysine from NPN to Protein of Developing Rabbit Brain.[a]

Min	Age (days)			
	1	4	8	12
10	165; 160	139; 152	152; 144	120; 125
20	126; 132	124; 118	120; 116	100; 104
40	74; 69	68; 63	64; 59	60; 51
60	53; 50	56; 52	50; 47	30; 33

[a] Results expressed in mμmoles of lysine per mg protein (dry wt.) per min.

Although in general there is a gradual decrease in rate of flux of lysine into brain protein, the changes in rate between 8- and 12-day-old animals at 10 and 20 min suggest a more abrupt drop which coincides with the appearance of spontaneous electroencephalographic potentials (8) and great increase in capability of cerebral *in vitro* oxygen consumption (9).

Study of the recovered ^{14}C radioactivity in the brain NPN demonstrated that a portion of radioactivity initially injected intraperitoneally in the form of lysine is no longer found in lysine in brain. The percentage of injected radioactivity found outside lysine in the eluate from the ion-exchange resin column varied with the age of the animal and with the duration of the experimental period. In the 1-day-old rabbit, 10 min after injection, 9% of radioactivity was not in lysine and after 60 min fully 35% of recovered radioactivity was not in lysine. In the 12-day-old animal, 10 min after injection, 8% of recovered radioactivity in the NPN fraction was no longer found in lysine and after 60 min 28% of radioactivity was not in lysine. Other preliminary studies suggest that the ^{14}C radioactivity present in glutamate and glutamine is 3–5% that found in the recovered lysine. This finding suggests that lysine is undergoing breakdown via pathways leading to the tricarboxylic acid cycle.

Discussion. There is no reason to believe that the incorporated lysine is bound in any other form than by an α-peptide linkage (5). The rapid transformation of the labeled lysine into other compounds found in the NPN, especially in the younger animals,

presents certain problems in assessing results of experiments of this type. If specific activity of lysine in the free amino acid pool is to be calculated, then the lysine must be isolated and its radioactivity determined. The assumption that all radioactivity in the NPN is present in lysine, even after only 10 min is unwarranted. The greater rate of transformation in the neonatal animals suggests a greater rate of metabolism of lysine which parallels the apparent greater rate of flux.

Calculations of flux of amino acids into protein are based on the assumption that the incorporating protein population is either truly homogenous or has a homogenous turnover rate. The presence of a variety of proteins with varying rates of synthesis and breakdown has important bearing on the results of experiments of this type. Results of short-term experiments emphasize the flux of lysine into the more rapidly metabolized proteins whereas results of experiments of longer duration disproportionately reflect the rate of flux into proteins of longer turnover time. This phenomenon is most likely the basis of the computed reduction in flux of lysine with greater elapsed time. The results of these experiments suggest that faster metabolizing proteins are more prevalent in the more immature rabbit brain.

Previous studies in this laboratory (2) have demonstrated that although there is an increase in the albumin/globulin ratio during maturation, there is an acceleration of this rate of increase between the fifth and tenth day of life. It is of interest that the greatest changes of rate of flux occur when relatively greater amounts of albumin than globulin are being synthesized.

There has been speculation that certain metabolic diseases, including phenylketonuria, alter the normal growth of brain by interfering with protein synthesis (10). The results of these studies intimate that if this line of thought is correct that the most likely time of greatest interference might be expected during the neonatal period when incorporation of lysine and likely other amino acids is proceeding at a comparatively rapid rate.

Summary. The rate of flux of lysine from plasma to the nonprotein nitrogen fraction of brain and the rate of flux of lysine from the nonprotein nitrogen fraction to brain proteins is greater in newborn rabbits than in rabbits 12 days old. These *in vivo* findings parallel the *in vitro* findings of other investigators that cerebral tissue in the newborn has greater ability to incorporate amino acids into brain protein than cerebral tissue removed from slightly older animals. The greater rate of flux of lysine into protein of the more immature brain most likely is a reflection of the dominance of the synthesis of rapidly metabolized brain proteins at this age.

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