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Faster Synthesis and Slower Degradation of Liver Protein during Developmental Growth

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A study is presented of the liver protein gain during the early stages of postnatal development. Fractional rates of protein synthesis and degradation were determined *in vivo* in livers of 4-day-old mice. At this age, liver protein accumulated at a rate of 18% per day. Synthesis was measured after the injection of massive amounts of radioactive leucine. Degradation was estimated as the balance between synthesis and accumulation of stable liver proteins, or from the disappearance of radioactivity from liver protein previously labelled by the administration of $NaH^{14}CO_3$. We found that the neonatal livers: (1) synthesize 139% as much protein per unit time and unit mass as adult tissue, which is accounted for by a higher ribosome concentration (synthesis per mg of RNA was the same); (2) retain 39% of the newly synthesized protein as stable liver components (compared with 48% in adult mice); (3) degrade protein at 56% of the rate in the adult liver. This lower rate of degradation is quantitatively the most significant difference between the growing and non-growing liver.

Parenchymal liver cells of adult mice synthesize each day the equivalent of their own protein content (Scornik, 1974*a*). Half of the newly synthesized protein is either exported or degraded within 3h; the portion retained represents a mixture of stable liver proteins which turn over at an average rate of 2% per h (Scornik & Botbol, 1976). This is one of the highest rates of protein turnover in mammalian cells; for this reason, relatively rapid changes in liver protein content may result from moderate variation in protein synthesis or degradation, or in the proportion of newly synthesized protein retained by the organ. Previous work from this laboratory has established that changes in the rates of protein degradation are the single most important factor determining the net protein gain during the rapid liver growth after partial hepatectomy (Scornik, 1974*a*,*b*, 1975*a*,*b*; Scornik & Botbol, 1976) or the re-feeding of a normal diet to protein-depleted mice (Conde & Scornik, 1976). In these experimental situations, the liver underwent a transient period of growth, until the normal mass of the organ was restored. We wished to know whether slower rates of protein degradation would also be found under conditions of steady growth, and turned our attention to the liver during postnatal development.

At the time of birth, the hepatic tissue undergoes drastic physiological changes, as it takes over many functions that were performed up to then by the maternal liver. After this initial qualitative change and while haemopoietic cells (very abundant in the foetal liver) disappear from the organ, parenchymal cells proliferate, resulting in a relatively rapid increase in liver mass, accompanying the growth of the other organs in the newborn animal (Widdowson & McCance, 1960; Enesco & Leblond, 1962; Winick & Noble, 1965; Miller, 1969).

Studies of the neonatal liver have included determinations of the incorporation of trace amounts of labelled amino acid into liver protein *in vivo* (Schreier *et al.*, 1960, 1961; Vavrousek-Jakuba & Miller, 1975). The work of Schreier *et al.* (1960, 1961) appeared to indicate that liver protein synthesis is slow shortly after birth, and it was speculated that these low rates are related to the transition from placental nurturing to milk feeding (Schreier *et al.*, 1961; Miller, 1969). These results could also be due to the unusual lability of the immature animals to inadequate nutrition (Vavrousek-Jakuba & Miller, 1975) or temperature changes during manipulation (see the Results section). Also, rates of protein synthesis could not be deduced from measurements of this kind owing to the lack of reliable information on the specific radioactivity of the precursor pool. Even when the specific radioactivity of the amino acid extracted from the tissue was known (Vavrousek-Jakuba & Miller, 1975) this value was at best an average, and probably did not represent the radioactivity of the actual precursor: increasing evidence indicates a compartmentalization of intracellular amino acids (Khairallah & Mortimore, 1976; Hod

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& Hershko, 1976). For this reason, we have explored alternative ways of measuring rates of protein synthesis which do not depend on the estimation of the specific radioactivity of the amino acids in the precursor pool (Scornik, 1974*a*,*b*). The simplest procedure, both technically and conceptually, proved to be the measurement of the incorporation of radioactive leucine injected in massive amounts. The animals were thus flooded with a precursor of known specific radioactivity, and the pool of free leucine was expanded to a point where endogenous sources became negligible. This procedure was used in the present study.

Protein degradation in the neonatal liver has received only marginal attention. Schreier *et al.* (1960) measured liver protein radioactivity 0.5 and 3h after the injection of [^{14}C]glycine, and observed during this interval a larger loss of radioactivity shortly after birth than at later stages of development. They interpreted this loss to represent protein turnover, but they did not determine to what extent the results were affected by continuing incorporation of the tracer during this period, and the observed interval was too short to represent the turnover of stable liver proteins (Scornik & Botbol, 1976). In the experiments described here, average rates of protein degradation were measured, as in previous studies (Scornik & Botbol, 1976; Conde & Scornik, 1976), either as the balance between the rates of synthesis and accumulation of liver protein, or from the disappearance of radioactivity from the protein of livers that have been labelled *in vivo* by the administration of $NaH^{14}CO_3$ (the advantages and limitations of this precursor are discussed in the Results section).

In this paper we demonstrate that the protein gain in growing livers of newborn mice is due to faster synthesis and slower degradation of protein than in livers of adult animals.

Experimental

Diet

Purina Rat Chow (pellets) was purchased from Purina Foods Co., St. Louis, MO, U.S.A.

Chemicals

L-Leucine was bought from Calbiochem, San Diego, CA, U.S.A. L-[1-^{14}C]Leucine (53μCi/μmol), L-[4,5-^{3}H]lysine (55mCi/μmol) and $NaH^{14}CO_3$ (4.9μCi/μmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Pactamycin was a gift of the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. All other reagents were of the highest purity available.

Animals

Male CD-1 mice (6 weeks old) and 15-day-pregnant mice of the same strain were purchased from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. The animals were kept at 25°C in a room illuminated from 06:00 to 18:00h, and given Purina Rat Chow and water *ad libitum*. After delivery the number of young was adjusted to eight per litter. Adult animals were used at 8 weeks of age.

Analytical procedures

The collection of the livers, the determination of protein, RNA, rRNA, DNA, and the measurement of the radioactivity in liver protein were as previously described (Scornik, 1974*a*).

Injected solutions

Pactamycin (1mg/ml) was prepared as described previously (Scornik, 1974*b*). All tracers were dissolved in 0.9% (w/v) NaCl and adjusted to pH7.4.

L-[1-^{14}C]Leucine was prepared in a solution of 4μCi/ml for its injection in trace amounts. For the administration of massive doses, non-radioactive L-leucine (0.143M) was mixed with L-[^{14}C]leucine to give a specific radioactivity of 0.01μCi/μmol. This solution was further diluted with 0.9% NaCl, when necessary, to inject in all cases the same volume per animal (4ml/100g body wt.).

L-[4,5-^{3}H]Lysine (2.5μCi/ml) was injected in trace amounts. When indicated, it was mixed with non-radioactive L-leucine (0.143M).

$NaH^{14}CO_3$ was dissolved to 10μCi/ml.

Unless otherwise indicated, all the injections were given subcutaneously in the back through a 27½-gauge needle.

Histological examination

Livers were fixed with Bouins fluid [10% (w/v) formaldehyde, 1% (w/v) picric acid, 5% (v/v) acetic acid] and embedded in paraffin. Slices (3μm thick) were obtained and stained with Haematoxylin/Eosin. Preparations were observed at 40× magnification, and representative areas photographed. In each condition 70–130 cells were examined and the proportion of parenchymal cells was determined.

Results and Discussion

Neonatal liver growth

Some characteristics of the liver at an early stage of neonatal development are presented in Table 1. Between 3.75 and 10.5 days after birth, liver protein and RNA content increased at an approximately linear rate by a factor of 2.2. It should be noted that a constant growth rate would have produced a logarithmic increase; instead, the fractional rate of growth (determined graphically as described

Table 1. *Changes in body weight, parenchymal-cell content, liver protein, RNA and DNA during neonatal development*
Each value represents the average±S.E.M. of 16 animals, except for the first group (immediately after birth), which represents the average of four pools of three animals each.

		Liver				
Age (days)	Body wt. (g)	Parenchymal cells (%)	Protein (mg)	RNA (mg)	DNA (mg)	RNA/protein
0	1.45	19	9.5	0.72	0.40	0.076
3.75	2.25±0.04	46	14.2±0.2	0.81±0.02	0.47±0.02	0.057
4.75	2.60±0.07	54	17.5±0.4	0.95±0.02	0.51±0.02	0.054
6.25	3.46±0.11	—	22.6±0.5	1.18±0.02	0.52±0.03	0.052
7.75	4.23±0.07	—	24.0±0.3	1.43±0.02	0.50±0.03	0.060
10.5	5.92±0.06	—	31.2±1.4	1.78±0.04	0.60±0.04	0.057
60	36.7 ±1.5	58	441.0±1.0	18.10±0.10	5.40±0.1	0.041

before; Scornik, 1974*a*) decreased from 0.18 per day on day 4 to 0.09 per day on day 9 *post partum*. These rates are similar to those reported in the rat by Rosso *et al.* (1973). Other studies (Schreier *et al.*, 1960; Oliver *et al.*, 1962) have indicated a lag in rat liver growth for the first 5–10 days after birth, which we have not observed.

Haemopoietic cells are very abundant in the foetal liver. Shortly before birth, they comprise half of the liver volume (Rohr *et al.*, 1971) and synthesize an amount of haemoglobin that represents 20% of the total protein synthesis of the organ (Mathews & Haschemeyer, 1976). This situation changes rapidly after birth (Rohr *et al.*, 1971); haemopoietic cells disappear and parenchymal cells proliferate very actively. As shown in Table 1, at birth parenchymal cells represent 19% of the cell population. At the time of our study, 4 days later, this proportion increased to 46%. Because parenchymal cells are much larger (Rohr *et al.*, 1971) and synthesize protein more actively than the other cell types (Scornik, 1975*b*), the contribution of non-parenchymal cells to total protein synthesis by the organ, in day 4 *post partum*, is probably negligible. On the other hand, DNA content per cell is the same in both cell types (newborn liver parenchymal cells are all diploid; Enesco & Leblond, 1962), so that non-parenchymal DNA represents a large portion of the total liver DNA. The RNA content is discussed in the next section.

Protein synthesis

The rate of liver protein synthesis was measured *in vivo* after the injection of massive amounts of radioactive leucine. The procedure was as described before (Scornik, 1974*a*), except that the precursor was administered subcutaneously, rather than intravenously (owing to the size of the animals). This change in the route of administration had two significant consequences. (*a*) Incorporation proceeded with an apparent lag of approx. 2min; it was linear with time between 5 and 15min after the tracer injection (Fig. 1*a*). (*b*) Presumably as a result of the slower absorption of the precursor, it was necessary to inject doses of leucine approx. 3 times as large to attain a comparable expansion of the leucine pool.

The incorporation of different doses of leucine, expressed per mg of liver RNA, was the same in newborn and adult mice (Fig. 1*a*). The rate of incorporation was extrapolated to infinity (Scornik, 1974*a*) in a reciprocal plot (Fig. 1*c*). From this extrapolated value (200d.p.m./min per mg of RNA) the specific radioactivity of the precursor (2.22×10^4d.p.m./μmol), the mol.wt. of leucine (131) and the molar proportion of leucine in liver protein (10.5%; Richmond *et al.*, 1963), we calculated a rate of synthesis of 16.2mg of protein/day per mg of liver RNA in both conditions. This value was essentially identical with that calculated for normal adult animals after an intravenous injection of massive amounts of leucine (Conde & Scornik, 1976); it indicates that RNA is used for protein synthesis with the same efficiency in the livers of newborn and adult mice. This result is consistent with the observation that the proportion of ribosomes in polyribosomes in the livers of newborn rats is as high as that in adult animals (Otten, 1968). The proportion of rRNA in the livers of six newborn animals was 75±1% of the total liver RNA, a value similar to that (80%) reported previously for adult livers (Scornik, 1974*a*).

The validity of the above calculations rests on the assumption that the injection of massive amounts of leucine does not interfere with protein synthesis. This assumption was supported in adult animals by the finding that massive amounts of leucine did not affect the incorporation of trace amounts of radioactive lysine (Scornik, 1974*a*). This control experiment was performed in newborn mice with the same results (Table 2). In all these studies the young were kept at 36°C. This is an important precaution,

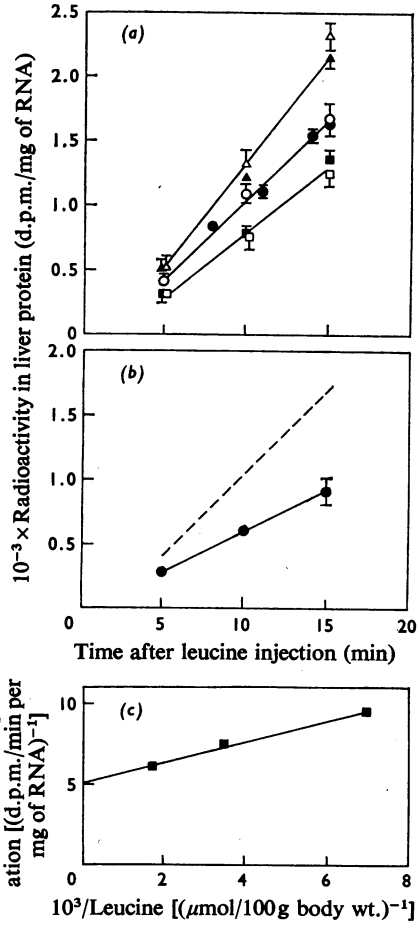

Fig. 1. *Incorporation of massive amounts of* L-[1-^{14}C]-*leucine*

Each point represents at least five mice, and S.E.M. values are shown by bars unless they were too small to be represented. (*a*) 143 (■, □), 286 (●, ○), or 570 (▲, △) μmol of L-[1-^{14}C]leucine/100g body wt. was injected subcutaneously to 4-day-old (closed symbols) and adult (open symbols) mice. The young were kept at 36°C for 16min before the injection, as well as during the incorporation period. Liver protein radioactivity is expressed as d.p.m./mg of RNA as a function of the time elapsed after the injection. (*b*) Newborn mice were handled at 20°C and injected with 286μmol of leucine/100g body wt. (●). ----, Incorporation for the same dose in animals maintained at 36°C, as shown in (*a*). (*c*) ■, Reciprocal value of the rate of incorporation as a function of the inverse of the amount of leucine injected.

because immature animals are unable to maintain their body temperature (Hahn & Koldovsky, 1966) and protein synthesis is very sensitive to changes in temperature (Conconi *et al.*, 1966). The importance

Table 2. *Effect of massive amounts of leucine on the incorporation of trace amounts of lysine*

Newborn animals (4 days old) received a subcutaneous injection of 10μCi of [^{3}H]lysine/100g body wt., with or without non-radioactive leucine, as indicated. They were killed 10min later. The radioactivity in liver protein and the liver RNA were determined as explained in the Experimental section. At 15min before the injection the young were kept at 36°C in an incubator and maintained at this temperature until the moment of killing. Values are expressed as d.p.m./mg of RNA (average ±S.E.M. for seven animals).

L-Leucine (μmol/100g body wt.)	Radioactivity in protein (d.p.m./mg of RNA)
0	3173±88
570	3404±95

of this factor is illustrated by an experiment in which the animals were kept at room temperature (20°C). The incorporation of leucine was severely inhibited (Fig. 1*b*). This could provide an explanation to the apparent decrease in the incorporation of [^{14}C]-glycine observed by Schreier *et al.* (1960, 1961) in rats and rabbits shortly after birth; these authors kept their animals at 27°C (Schreier *et al.*, 1961). To calculate the fractional rate of protein synthesis (g of protein/day per g of liver protein), the value of 16.2mg of protein/day per mg of RNA must be multiplied by the RNA/protein ratio, which, as shown in Table 1, was 39% higher in the newborn than in the adult mice. This difference reflected a higher concentration of ribosomes in the growing livers. As a result, the fractional rate of synthesis was also 39% higher on day 4 *post partum* than in adult livers (see Table 4*c*).

Proportion of the newly synthesized protein retained by the liver

In adult animals, half of the newly synthesized protein disappeared from the livers within 3h; 30% was exported as plasma proteins, whereas the other 20% presumably represented rapidly turning-over liver proteins (Scornik & Botbol, 1976). The proportion of pulse-labelled liver protein retained by the neonatal liver was studied after the injection of L-[1-^{14}C]leucine, followed by pactamycin 4min later. At 3h the livers retained 39% of the total protein radioactivity measured at 7min (Table 3). This value was multiplied by the rate of total liver protein synthesis (Table 4*c*) to obtain the rate of synthesis of stable liver proteins: 0.36mg of protein/day per mg of liver protein (Table 4*e*).

Thus although the neonatal liver synthesized 39% more protein per unit time than the adult liver (because of a higher ribosome concentration), it

retained less of the newly synthesized protein. The synthesis of stable liver proteins was only 12% higher in the growing than in the non-growing livers (Table 4*e*).

It seems puzzling that the growing liver retained less of the newly synthesized protein than in adult mice. The fraction disappearing in 3h comprised exported plasma proteins and rapidly turning-over proteins (Scornik & Botbol, 1976). We did not measure, in the present study, the relative proportion of these components. It seems likely that plasma protein synthesis was proportionally higher in newborn animals, where it should have met the demand for a rapidly expanding plasma protein pool (Oliver *et al.*, 1962). Interestingly, the fraction retained by neonatal livers was the same as in regenerating livers, a situation in which the supply of plasma had to be maintained by a drastically decreased liver mass (Scornik & Botbol, 1976).

Table 3. *Proportion of the newly synthesized protein retained by the liver*

Mice (4 days old) received a subcutaneous injection of 0.2μCi of L-[1-^{14}C]leucine, followed 4min later by 0.03mg of pactamycin intraperitoneally. At 15min before the tracer injection, the young were kept in an incubator at 36°C. One group was maintained at this temperature and killed 7min after the tracer injection. The other was kept in the incubator for 30min and returned to the mother for an additional 2.5h before they were killed. The total liver protein radioactivity was determined as explained in the Experimental section and expressed as d.p.m./μCi injected. Values represent average ±S.E.M. for seven mice.

Time after tracer injection (min)	Liver protein radioactivity (d.p.m./μCi)	(%)
7	21050±1400	100
180	8240± 200	39

Protein degradation

The average rate of protein degradation was estimated, as in our previous work (Scornik & Botbol, 1976; Conde & Scornik, 1976) in two ways. First, fractional rates of degradation were measured as the difference between the synthesis of stable liver proteins and the net protein gain (Table 4*g*). The livers of 4-day-old animals degrade their proteins at 56% of the rate calculated for adult livers by the same procedure.

Rates of degradation were also studied by the disappearance of radioactivity from the liver protein of animals previously labelled by the injection of $NaH^{14}CO_3$ (Fig. 2). The decreasing rate of disappearance was due to the fact that one observed the degradation of a mixture of proteins with different half-lives: as the rapidly turning-over components were depleted, the average rate of disappearance of the remaining proteins slowed down. The advantages of $NaH^{14}CO_3$ as a precursor for the measurement of liver protein degradation have been discussed by Swick & Ip (1974). They depend in part on the high activity of the urea cycle characteristic of the liver, which permits incorporation of $^{14}CO_2$ into the guanidino C of arginine, and decreases the re-incorporation of the radioactivity once the arginine is returned to the amino acid pool. Because in newborn mammals the activity of the urea cycle is lower than in the adult (Illnerová, 1966; Räihä & Suihkonen, 1968), and because both exogenous and endogenous radioactive arginine are incorporated into liver protein more efficiently (Drotman & Campbell, 1972), one should view these results with caution. However, $^{14}CO_2$ is also incorporated into aspartate and glutamate, and these amino acids share with arginine the advantage of low re-utilization (Swick & Ip, 1974). In any case, the differences in the rate of disappearance of protein radioactivity from the livers of newborn and adult mice (Fig. 2) are con-

Table 4. *Estimation of the rates of protein degradation as the difference between the synthesis of stable liver proteins and the net protein gain*

RNA/protein ratio (*a*) and net protein increase (*f*) for newborn animals were taken from Table 1. Synthesis (*b*) was from Fig. 1(*c*), and the fraction retained (*d*) was determined as described in Table 3. Values reported previously for adult animals are included for comparison: (*a*) Conde & Scornik (1976), and (*d*) Scornik & Botbol (1976). Key for calculations: $c = a \times b$; $e = c \times d$; $g = e - f$.

Measurement	Units	4-day-old	Adult
(*a*) RNA/protein ratio	—	0.057	0.041
(*b*) Synthesis	mg of protein/day per mg of RNA	16.2	16.2
(*c*) Total synthesis	mg of protein/day per mg of protein	0.92	0.66
(*d*) Fraction retained	—	0.39	0.48
(*e*) Synthesis of stable liver proteins	mg of protein/day per mg of protein	0.36	0.32
(*f*) Net protein gain		0.18	0.00
(*g*) Degradation		0.18	0.32

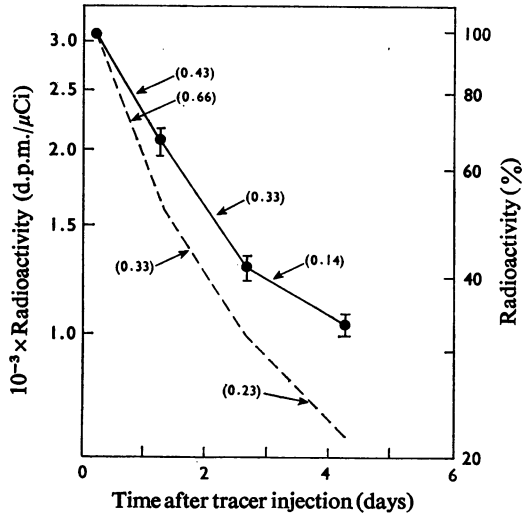

Fig. 2. *Disappearance of protein radioactivity from livers labelled with* $NaH^{14}CO_3$

Animals 3.5 days old were injected with 0.5 μCi of $NaH^{14}CO_3$, subcutaneously; 15 min before and after the injection, they were kept at 36°C. Groups of 16 animals were killed at the time intervals indicated on the abscissa and total protein radioactivity was determined. Results were expressed as d.p.m. per injected μCi (left ordinate), or as a percentage of the value at 0.25 day after the injection (right ordinate). The S.E.M. is indicated by the bars, and the apparent rates of degradation (k)/day (values in parentheses) were calculated at each interval on the assumption that the process follows first-order kinetics. ----, Disappearance of liver protein radioactivity reported by this laboratory for adult animals (Scornik & Botbol, 1976).

sistent with the results obtained by the balance procedure.

As explained earlier in this section, during the period of observation in the experiment of Fig. 2 the rate of liver growth decreased appreciably. In comparing the results of both procedures, it should be noted that the calculations in Table 4 referred to day 4 *post partum*, when the fractional rate of protein gain was 0.18/day, and that of degradation 0.18/day (56% of the adult rate). These rates would have been respectively: on day 5, 0.15 and 0.21 (66%); on day 6, 0.13 and 0.23 (72%); and day 7 *post partum*, 0.11 and 0.25 (78%).

Both procedures estimate average rates. Thus we do not know whether the observed differences are due to a lower activity of the mechanism(s) of protein degradation after birth, or if they reflect a difference in the protein composition of the livers in both conditions. An answer to this question must await the study of individually defined proteins.

In conclusion, the growing livers of newborn animals synthesize protein faster and degrade it more slowly than do adult livers. The lower rate of degradation is quantitatively more important in explaining why the organ gains protein in one condition, whereas it does not grow in the other. These observations add further weight, and a new dimension, to our previous conclusion (Scornik & Botbol, 1976; Conde & Scornik, 1976) that changes in rate of protein degradation are the single most important regulatory factor determining the protein content of the liver.

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