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Role of Protein Degradation in the Growth of Livers after a Nutritional Shift

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Fractional rates of synthesis and degradation of liver proteins were estimated during the rapid restoration of liver mass observed in protein-depleted mice when they are fed with an adequate diet. 1. Net protein gain was fastest 12h after the nutritional shift, when it reached a rate of 48 % per day. 2. The RNA/protein ratio in livers of protein-depleted animals was essentially the same as in normal livers; it increased by a maximum of 13 % 12h after the nutritional shift. 3. Rates of protein synthesis *in vivo* were measured by the incorporation into liver protein of massive amounts of L-[1-^{14}C]leucine. In protein-depleted animals, the rate of synthesis per mg of RNA was 72 % of that in normal livers. Normal rates were recovered within 12h of the nutritional shift. 4. The fraction of newly synthesized protein retained by the liver was studied after they were pulse-labelled by the intravenous injection of radioactive leucine, and, 5min later, pactamycin (an inhibitor of the initiation of protein synthesis); 3h later the livers in both experimental situations retained 58 % of the newly synthesized protein. 5. Fractional rates of protein degradation were estimated either from the difference between the synthesis of stable liver proteins and the net protein increase, or by the disappearance of radioactivity from the liver protein previously labelled by the administration to the mice of $NaH^{14}CO_3$. Both procedures demonstrated a large decrease in the rate of protein degradation during liver growth.

Adult mouse liver synthesizes the equivalent of its own protein content in 1 day (Scornik, 1974*a*). One-half of this protein is either exported as plasma proteins or degraded within 3h, and the rest is retained by the cells and turned over at different rates (Scornik & Botbol, 1976). With such an active protein metabolism, changes in protein content may result from relatively subtle modifications in the rates of synthesis or degradation, or in the proportion of the newly synthesized protein retained by the liver. A detailed study of these parameters *in vivo* (Scornik, 1974*a*,*b*, 1975*a*,*b*; Scornik & Botbol, 1976) convinced us that the net protein gain during liver regeneration is due predominantly to a decreased rate of protein degradation. Because we were interested to know whether this was also true for other types of liver growth, we focused our attention on the rapid restoration of liver mass observed in protein-depleted animals when they are fed with an adequate diet (Addis *et al.*, 1936; Leduc, 1949; Munro, 1964; Short *et al.*, 1974). Changes in liver-protein degradation as a result of starvation or protein depletion have been described by Waterlow & Stephen (1968), Millward (1970) and Garlick *et al.* (1973, 1975). In the present paper we give evidence that the dramatic growth of the liver in protein-depleted animals, in response to an adequate diet, is predominantly the result of a slower degradation of liver protein.

Experimental

Diets

Purina Rat Chow (pellets), containing 23% (w/w) protein, was from Purina Foods Co., St. Louis, MO, U.S.A. The Protein-Depletion Diet (U.S.P. XV; powder), was from ICN Pharmaceuticals, Cleveland, OH, U.S.A.

Chemicals

L-[1-^{14}C]Leucine (53μCi/μmol) and $NaH^{14}CO_3$ (4.9μCi/μmol) were from New England Nuclear Corp., Boston, MA, U.S.A. Non-radioactive L-leucine was from Calbiochem, San Diego, CA, U.S.A. Pactamycin was a gift from 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

CD-1 mice (male, 6 weeks old), were from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. On arrival, the animals were housed (five per cage) in an air-conditioned room illuminated from 06:00 to 18:00h, and given Purina Rat Chow and water *ad libitum*. They were kept for at least 1 week under these conditions. Protein deprivation was started at 18:00h, by providing the Protein-

Depletion Diet in special holders, in an amount sufficient to maintain *ad libitum* feeding; 6 days later, some of the animals were again fed with Purina Rat Chow. The present paper is primarily concerned with the restoration of liver mass after this second nutritional shift.

Analytical procedures

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

Injected solutions

L-[1-^{14}C]Leucine was injected either in trace amounts or in massive amounts. In the first case, the radioactive amino acid was diluted to 6.6 μCi/ml in 0.9 % NaCl, and adjusted to pH 7.4. In the second case, enough radioactive leucine was added to a 0.1 M solution of non-radioactive leucine in 0.9 % NaCl to give a final specific radioactivity of 0.01 μCi/μmol. $NaH^{14}CO_3$ was dissolved in previously boiled 0.9 % NaCl to give a final radioactivity of 15.7 μCi/ml. Pactamycin was dissolved at a concentration of 1.5 mg/ml, at pH 4.5–5.0, as described by Scornik (1974*b*).

Results

Liver growth after nutritional changes

Mice were kept on Purina Rat Chow for at least 1 week. They were then transferred to the Protein-Depletion Diet for 6 days. At the end of this period, their livers had lost 48 % of the protein (Fig. 1*a*) and 22 % of the DNA (Fig. 1*c*), resulting in a decrease of the protein/DNA ratio to 67 % of the control value (Fig. 1*d*). The loss of RNA paralleled that of protein, so that the RNA/protein ratio was the same as that of normal animals (Fig. 1*e*).

When the protein-depleted mice were re-fed with Purina Rat Chow, a dramatic growth of the liver was observed. The initial liver protein content was restored after 36 h, and surpassed by 20 % 1 day later (Fig. 1*a*). The rate of protein gain reached a maximum of 48 % per day 12 h after the shift, and decreased thereafter (Fig. 1*b*). By the end of the

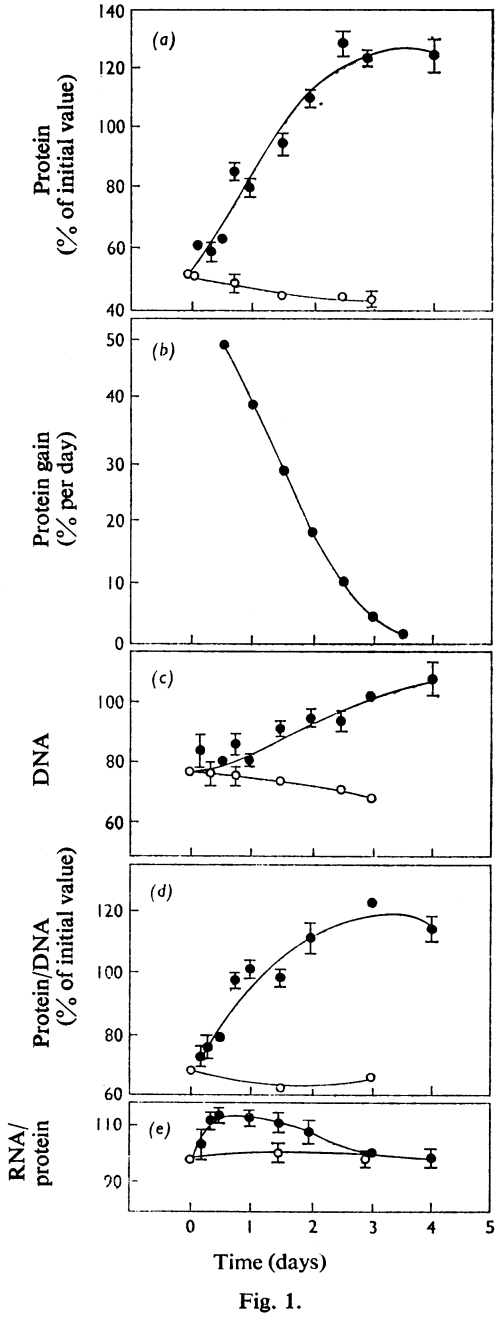

Fig. 1.

Fig. 1. *Time-course of liver growth*

Mice were fed on the Protein-Depletion Diet (U.S.P. XV) for 6 days. At zero time, some animals were returned to Purina Rat Chow (●), whereas others were kept on the same diet (○). The values for total liver protein (*a*), total liver DNA (*c*), protein/DNA (*d*) and RNA/protein ratio (*e*), were expressed as percentages of the values in control animals, killed at the time at which Protein-Depletion Diet was first administered; 100% values were: (*a*) 1188 mg/100 g initial body wt., (*c*) 14.3 mg/100 g initial body wt., (*d*) 83.1 and (*e*) 0.041. Each point represents at least nine animals; the S.E.M. is indicated by the bars, unless too small to be represented. The rate of protein gain after the nutritional shift, (*b*), was calculated as follows: the increment in total protein over a 1 day period was divided by the total amount of protein in the middle of that period, and expressed as a percentage. For further details see the Experimental section.

second day the process had been essentially completed. The lost DNA was also rapidly restored (Fig. 1*c*). The gain in liver RNA was somewhat faster than that of protein, resulting in a transient increase in the RNA/protein ratio (Fig. 1*e*), which was small, but consistent. At 12h after the shift, it reached a maximum of 13 % above the value at zero time. All these changes are in agreement with previous observations in the rat (see the introduction).

Protein synthesis

Rates of protein synthesis *in vivo* have often been estimated from the incorporation into protein of trace amounts of a radioactive amino acid, and the simultaneous measurement of the specific radioactivity of the free amino acid extracted from the tissue. The latter measurement is cumbersome, and its results are subject to criticism, because the measured value is at best an average, and the accuracy with which it is characteristic of the actual precursor pool is controversial (Hider *et al.*, 1971; Fern & Garlick, 1973). In a previous study of normal and regenerating livers, 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 acid in the precursor pool (Scornik, 1974*a*,*b*). The simplest procedure, both technically and conceptually, proved to be the incorporation of radioactive leucine injected intravenously in massive amounts. The animals were flooded with radioactive leucine of known specific radioactivity; the pool of free leucine was thus expanded to the point where endogenous sources became negligible. In the short time required for the measurement, this procedure did not interfere with protein synthesis, as measured by the simultaneous incorporation of trace amounts of radioactive lysine (Scornik, 1974*a*). The incorporation of massive amounts of leucine was studied in several groups of mice, representing the different nutritional conditions discussed in the present paper. The results, shown in Table 1, are expressed as the incorporation of radioactivity into protein per mg of liver RNA, and represent the efficiency with which RNA is used for protein synthesis. Absolute rates of protein synthesis were estimated from these values as explained in the legend of Table 1, and are presented in the last column of that Table. In order to calculate the fractional rate of protein synthesis (g of protein/day per g of liver protein), this value must be multiplied by the RNA/protein ratio (Fig. 1*e*), as shown below. The efficiency with which liver RNA was used for protein synthesis was almost 30 % lower in the protein-depleted animals than in the normal ones. At 12h after re-feeding them with Purina Rat Chow, the normal efficiency was restored.

Proportion of the newly synthesized protein retained by the livers

It has been previously shown (Scornik & Botbol, 1976) that one-half of the newly synthesized protein disappears from normal livers within 3h; 30 % was found to be exported as plasma proteins, whereas the remaining 20 % remained unaccounted for (we assumed it represented rapidly turning-over protein). The proportion of pulse-labelled protein retained by the livers was studied in depleted and re-fed mice (Table 2). For this purpose, radioactive leucine was administered, and its incorporation into liver protein was interrupted 5min later by the injection of pactamycin, an inhibitor of the initiation of protein synthesis. A group of animals was killed 2 min later. A second group was killed 3h after the injection of leucine, and the proportion of pulse-labelled protein retained by their livers was calculated by comparison with the first group (Scornik & Botbol, 1976). The

Table 1. *Incorporation of massive doses of radioactive leucine into liver protein*

Protein-depleted mice were either re-fed with Purina Rat Chow before the experiment, or maintained on the Protein-Depletion Diet, as indicated. A group of control animals, fed on their normal diet throughout, is also included for comparison. Each animal received the intravenous injection of a massive dose of L-[1-^{14}C]leucine (286μmol/100g body wt.), and was killed 5 min later. The radioactivity of liver protein and the RNA content of the livers were determined as described in the Experimental section; results are means ± S.E.M. 'Liver-protein radioactivity' summarizes the incorporation of leucine in the 5min period per mg of liver RNA. Protein synthesis was estimated from this value, the specific radioactivity of the amino acid (2.22×10^4 d.p.m./μmol), the extent to which it saturates the precursor pool at this dose (86%, determined previously from the extrapolation to infinity of varying doses of leucine; Scornik, 1974*a*), the mol.wt. of leucine (131), and the molar proportion of leucine in liver protein (10.5%; Richmond *et al.*, 1963), and expressed as mg of protein/day per mg of RNA (final column).

Condition	No. of mice per group	Liver-protein radioactivity (d.p.m./mg of RNA)	Protein synthesis (mg of protein/day per mg of RNA)
Normal diet throughout	15	875±18	16.5
Protein-Depletion Diet, 6days	17	632±15	11.9
Re-fed with normal diet, 12h	17	898±12	16.9
Re-fed with normal diet, 24h	13	924±13	17.4

Table 2. *Proportion of the newly synthesized protein retained by the liver after 3 h*

Protein-depleted mice were either re-fed with Purina Rat Chow 16h before the experiment (re-fed), or maintained on the Protein-Depletion Diet (depleted). At zero time, each animal received the intravenous injection of a trace amount of L-[1-^{14}C]leucine (1.3 μCi, 53 μCi/μmol), followed by pactamycin (0.3 mg) 5 min later. The animals were killed either 7 min or 3 h after injection of leucine and the radioactivity in protein in their whole livers was determined (see the Experimental section). Results, expressed as d.p.m./μCi injected, are summarized as means ± S.E.M. (the number of mice per group is indicated in parentheses).

		10^{-4} × Radioactivity in total liver protein (d.p.m./μCi injected)		
Expt. no.	Condition	7 min	3 h	% Retained
1	Depleted	16.3 ± 0.4 (10)	8.5 ± 0.3 (10)	52
	Re-fed	16.2 ± 0.7 (10)	9.4 ± 0.3 (10)	58
2	Depleted	14.4 ± 0.6 (7)	8.4 ± 0.5 (6)	58
	Re-fed	13.7 ± 0.7 (7)	7.5 ± 0.5 (6)	58
3	Depleted	17.2 ± 0.5 (9)	10.5 ± 0.3 (10)	61
	Re-fed	17.0 ± 0.7 (7)	10.3 ± 0.3 (10)	60

Table 3. *Estimation of the rates of protein degradation from those of synthesis and net growth*

Explanations are given in the text.

	Fractional rates (g of protein/day per g of protein)			
Condition	Total synthesis	Stable liver components	Net protein gain	Degradation
Depleted	0.49	0.28	−0.08	0.36
Re-fed, 12 h	0.78	0.45	0.48	0.00
Re-fed, 24 h	0.80	0.46	0.39	0.07
Re-fed, 36 h	0.77	0.44	0.28	0.16
Re-fed, 48 h	0.75	0.43	0.18	0.25

experiment was repeated on three occasions and the results are presented in Table 2: the proportion was the same under both conditions. The average of these determinations, 58 %, will be used in the calculations below.

Protein degradation

The fractional rates of liver protein degradation in depleted and re-fed mice were determined in two different ways. In the first, they were calculated as the difference between the rate of synthesis of stable liver components and net protein gain (Table 3). First, the fractional rates of protein synthesis were estimated (second column) as the product of the RNA/protein ratio for each situation (from Fig. 1*e*) and the efficiency with which RNA was utilized for protein synthesis (from Table 1; it was assumed to remain unchanged after 24h of re-feeding). These values were next multiplied by the fraction of the newly synthesized protein retained by the liver, which as explained in the previous paragraph was 0.58 in both depleted and re-fed mice; the product was taken as the fractional rate of synthesis of stable liver proteins (third column). The net protein gain (fourth column) was obtained from Fig. 1(*b*). The fractional rates of degradation (last column) were then calculated as the difference between the values in the third and fourth columns.

In the second procedure, the rates of degradation were estimated through the disappearance of radioactivity from liver protein labelled by the administration of $NaH^{14}CO_3$. The advantages of this precursor in experiments of this kind have been discussed by Swick & Ip (1974). As shown in Fig. 2, during the first 36h after the nutritional shift, protein radioactivity disappeared from the livers of re-fed mice only half as fast as from the livers of depleted animals.

Discussion

The purpose of this study was to investigate whether changes in rates of protein degradation are as important in determining the growth of the liver after a nutritional shift as they are after a partial hepatectomy (Scornik & Botbol, 1976). Indeed, the rapid increase in protein content in response to a change in diet was accompanied by a large change in the rates of protein degradation. The conditions were selected so as to provide a dramatic and reproducible liver growth with a simple experimental design. The use of commercial diets and their administration *ad libitum* served this purpose.

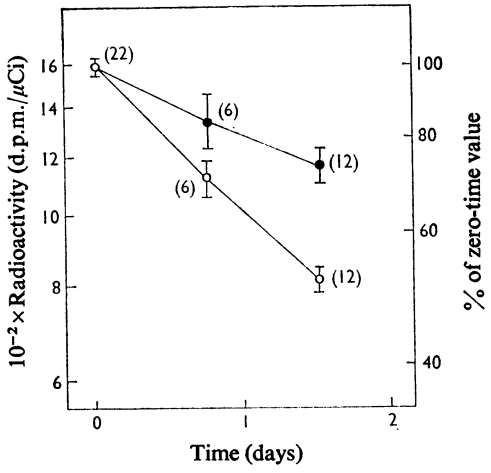

Fig. 2. *Disappearance of protein radioactivity from livers labelled by the administration of* $NaH^{14}CO_3$

At zero time, protein-depleted mice were either re-fed with Purina Rat Chow (●), or maintained on the Protein-Depletion Diet (○). Each animal received $NaH^{14}CO_3$ intraperitoneally 30h before zero time. At the times indicated on the abscissa, groups of animals were killed and the total liver-protein radioactivity was determined (see the Experimental section). Results were expressed either as d.p.m. in protein/μCi injected (left-hand ordinate), or as percentage of the value at zero time (right-hand ordinate). The bars indicate S.E.M.; the number of animals in each group is indicated in parentheses. The apparent rates of degradation during the first 1.5 days were calculated on the assumption that the process follows first-order kinetics (see the text). For re-fed mice $k = 0.21\,day^{-1}$; for protein-depleted mice $k = 0.43\,day^{-1}$.

We have shown that the efficiency with which liver RNA was used for protein synthesis in protein-depleted animals was lower than normal; it recovered within 12h of re-feeding the normal diet. This change can be explained by the observations of Wunner *et al.* (1966) that polyribosomes disaggregate during tryptophan depletion, and reform on re-feeding with an adequate diet. Similar results have been reported by Wilson & Hoagland (1967) in starved and re-fed rats. This conclusion differs from the findings of Garlick *et al.* (1975), who observed that the livers of protein-depleted rats incorporated more radioactive tyrosine than did those of control animals; in their study the incorporation was estimated after a continuous infusion of the labelled precursor, in trace amounts, for 6h.

We have measured the fractional rates of protein degradation either from the difference between the synthesis of stable liver proteins and the net protein increase, or by the disappearance of radioactivity from previously labelled liver proteins. The balance procedure is indirect: we conclude that degradation must have been slower, because the increase in the rate of synthesis is too small to account for the net protein gain. In the second procedure, the apparent rate constants are calculated on the assumption that the disappearance follows first-order kinetics. However, the label represents a mixture of proteins, which are turning over at different rates; therefore, the constants depend on the time elapsed since the injection of the precursor (Scornik & Botbol, 1976). The assumption of first-order kinetics is further complicated in the re-fed animals by the transient nature of the growth process (restoration of liver mass is largely attained in 36h, whereas after partial hepatectomy growth continues for several days). A better description of the disappearance of the liver protein radioactivity would require its measurement at several times during the first day after the nutritional shift. Because in this interval the total liver protein radioactivity has been decreased by 20% of the initial value, and the coefficient of variation of these determinations was on average 15%, an accurate description would require an unreasonably large number of animals. In spite of these limitations, both procedures clearly indicate that liver growth is accompanied by a large change in the overall rate of protein breakdown.

An inverse relationship between growth and rates of protein degradation has been proposed in bacteria (Mandelstam, 1962) and mammalian cells (Hershko *et al.*, 1971). Other examples have been discussed (Scornik & Botbol, 1976). This inverse relationship may not be universal. Protein degradation was calculated to be faster in skeletal muscle after re-feeding an adequate diet to protein-depleted animals (Millward *et al.*, 1975), based on the difference between synthesis (measured by the incorporation of trace amounts of radioactive tyrosine) and net growth. However, the opposite conclusion has been reached in a similar situation, where protein degradation was estimated from the loss of radioactivity from muscle protein labelled with [4-^{14}C]aspartate (Young *et al.*, 1971), or from the urinary excretion of N^{τ}-methylhistidine (Haverberg *et al.*, 1975).

Dramatic effects of changes in protein degradation on total cell-protein content can only be expected in tissues with a high protein turnover, such as liver. Owing to the relative ease with which large effects can be obtained experimentally, the liver is an ideal model for the study of protein degradation in the regulation of cell growth. In this respect the nutritional shift offers several distinct advantages over the partial hepatectomy. The growth occurs earlier and is faster; also, surgery is avoided. Further, whereas the identity of humoral factors involved in the stimulation of liver regeneration remains obscure (Lesch & Reutter, 1975), there is a more immediate opportunity, in nutritional shifts, to

define the mechanisms involved in the control of protein degradation. The rate of this process has been shown in the isolated liver to be sensitive to changes in the concentration of amino acids (Woodside & Mortimore, 1972). It has also been demonstrated that amino acids injected *in vivo* (together with other substances) can elicit a growth response in the normal liver (Short *et al.*, 1972).

Results from our laboratory, presented in this and previous papers (e.g. Scornik & Botbol, 1976), have provided conclusive evidence of the predominant role of protein degradation in the regulation of liver mass.

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