Effect of long-term refeeding on protein metabolism in patients with cirrhosis of the liver

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Patients with cirrhosis of the liver require an increased amount of protein to achieve N balance. However, the utilization of protein with increased protein intake, i.e. the slope from regression analysis of N balance *v.* intake, is highly efficient (Nielsen *et al.* 1995). In the present study, protein requirement and protein utilization were investigated further by measuring protein synthesis and degradation. In two separate studies, five or six patients with cirrhosis of the liver were refed on a balanced diet for an average of 2 or 4 weeks. Protein and energy intakes were doubled in both studies. Initial and final whole-body protein metabolism was measured in the fed state by primed continous [^{15}N]glycine infusion. Refeeding caused a statistically significant increase of about 30 % in protein synthesis in both studies while protein degradation was only slightly affected. The increase in protein synthesis was associated with significant increases in plasma concentrations of total amino acids (25 %), leucine (58 %), isoleucine (82 %), valine (72 %), proline (48 %) and triiodothyronine (27 %) while insulin, growth hormone, insulin-like growth factor (IGF)-I and IGF-binding protein-3 were not changed significantly. The results indicate that the efficient protein utilization is due to increased protein synthesis, rather than decreased protein degradation, and suggest that increases in plasma amino acids may be responsible for the increased protein synthesis. A comparison of the patients who had a normal protein requirement with the patients who had an increased protein requirement suggests that the increased protein requirement is due to a primary increase in protein degradation. It is speculated that this is due to low levels of IGF-I secondary to impaired liver function, since initial plasma concentration of IGF-I was about 25 % of control values and remained low during refeeding.

Nitrogen retention: Protein metabolism: Alcoholic cirrhosis

A large number of patients with liver cirrhosis are malnourished (Lautz *et al.* 1992) and a review of controlled studies suggested that nutritional therapy improves liver function and may improve the clinical course including mortality (Kondrup *et al.* 1992). However, the use of artificial nutrition was necessary in all these studies since it is often difficult to increase oral intake sufficiently in patients with cirrhosis. Even in a clinically-stable situation, protein intake is often inadequate among patients with liver cirrhosis, especially since the requirement for protein to achieve N balance is increased (Swart *et al.* 1989; Nielsen *et al.* 1993, 1995). In contrast, the utilization of protein with increased protein intake (i.e. the slope of balance *v.* intake) is highly efficient and considered to be similar to that of underweight individuals without organ disease (Nielsen *et al.* 1995).

In order to investigate regulation of protein metabolism during repletion, we performed this study in the fed state before and after refeeding. This was considered necessary because regulatory mechanisms involved in repletion after weight loss probably

differ from the regulatory mechanisms involved in the diurnal fasting–fed state transition. Repletion is thought to be characterized by sustained increases in plasma concentrations of substrates and hormones (e.g. amino acids, insulin, triiodothyronine (T_3), insulin-like growth factor (IGF)-I) that lead to net anabolism within the diurnal cycling of nutrient storage and loss (Millward, 1990).

The effect of refeeding on protein metabolism may be time dependent and, therefore, the patients were refed for an average of 2 or 4 weeks in two separate studies. Protein and energy intakes were doubled in both studies. Initial and final whole-body protein metabolism was measured by the primed continuous [^{15}N]glycine dual-endproducts method (Picou & Taylor-Roberts, 1969; Jeevanandam *et al.* 1986). During measurement of protein metabolism the patients were given hourly sip feedings with amounts of protein and energy that were identical to the initial and final dietary intakes. Plasma hormones and substrates believed to regulate protein synthesis and degradation were also measured during sip feeding. In addition, in order to examine the response to a standardized single test meal, plasma substrates and hormones were measured at the beginning and at the end of the 4-week study. In the present study the content of energy and protein in the meal was kept constant.

Finally, in order to elucidate the mechanism of the increased protein requirement, a comparison of protein metabolism was made between the patients who had an increased requirement and the patients who had a requirement within the recommendation for healthy subjects.

METHODS

The methods employed in the refeeding protocol were essentially as described previously (Nielsen *et al.* 1995) and will be described in brief only.

Patients

Malnourished patients were recruited for the study when they were clinically stable, at least 1 week after admission. They all had biopsy-confirmed alcoholic cirrhosis, except one patient who had biopsy-confirmed cryptogenic cirrhosis. Patients with hepatic encephalopathy, positive results for hepatitis B_sAg, overt diarrhoea, renal disease, fever, or malignant diseases were not included. One patient with insulin-dependent diabetes mellitus was included in the 2-week protocol. The patients were considered malnourished if one or both of the following criteria were met: body weight (BW) $< 80\%$ of reference weight, or lean body mass (LBM) $< 80\%$ of reference value. LBM was calculated from three 24 h urinary creatinine excretions (Nielsen *et al.* 1995). The clinical status of the patients was classified according to the Child-Pugh scores A, B or C, employing measures of plasma bilirubin, plasma albumin, prothrombin time and the presence or absence of ascites and hepatic encephalopathy (Pugh *et al.* 1973). Patients were included in the study when the ability to co-operate had been verified during an initial 3 d screening procedure (Nielsen *et al.* 1995). The patients stayed in the department throughout the study under metabolic ward conditions. To the best of our knowledge the patients abstained from alcohol during the study period but this was not controlled by measurement of alcohol in blood or urine. A large number of the patients were studied while being evaluated for liver transplantation, and any suspicion of continued alcohol intake would have precluded transplantation.

Refeeding protocol

The patients in the 4-week protocol were the last eight of the fifteen patients described earlier (Nielsen *et al*. 1995). After a 3 d baseline period in which protein and energy intake was kept at the pre-study level of the 3 d screening period, protein and energy intake was increased by 15–20 % in subsequent periods of 4–6 d. The energy composition of diets with respect to protein, carbohydrate and fat was kept fixed at the composition recorded during the pre-study period. The diets were customary mixed daily diets, prepared in the kitchen associated with the department. Two of the eight patients did not continue to increase dietary intake and the data from these patients are only included in the extended analysis of initial results (Table 7).

Patients included in the 2-week protocol were treated similarly, except that intake of energy and protein was increased by approximately 20 % after each 3 d period, in order to reach a doubling of dietary intake after 2 weeks. This seemed feasible since no patients developed encephalopathy during the preceding more cautious 4-week protocol.

Standardized test meal

At the beginning and at the end of the 4-week protocol, four of the eight patients were given a standard liquid meal containing (per kg body weight) 50 kJ and 0·4 g protein, after an overnight fast. Blood samples were taken for analysis of substrates and hormones before the meal and 1 and 3 h after the meal.

Analytical methods

Nitrogen balance and energy intake. In the 4-week protocol initial and final N balance was calculated from weighed dietary intake, with the N content given in food tables, and from urinary N excretion to which was added an amount equal to 17 % of mean dietary N intake (2·5 g N) to correct for skin and faecal loss (Bingham & Cummings, 1985). N excretion was measured in 24 h urine collections during the whole study by a microKjeldahl technique (Keltec Systems; Tecator AB, Hoganaes, Sweden). Protein balance was then calculated as:

$$\text{protein balance} = \text{protein intake} - (6·25 \times (24 \text{ h urinary N} + 2·5)). \qquad (1)$$

This procedure gave reliable results according to a validation study in the same patients (Nielsen *et al*. 1995). In the 2-week protocol, N content of food (duplicate-portion technique) and faecal N content were measured in an initial and final 3 d period just before the [^{15}N]glycine study (Nielsen *et al*. 1995). Skin N loss was taken to be 4 % of protein intake (Bingham & Cummings, 1985). Protein balance was then calculated as:

$$\text{protein balance} = \text{protein intake} - (6·25 \times (24 \text{ h urinary N} + \text{faecal N})$$
$$+ 0·04 \times \text{protein intake}). \qquad (2)$$

A correction for loss of urine was performed in the 4-week protocol by giving 4-aminobenzoic acid tablets (Bingham & Cummings, 1983). The correction turned out to be of minor quantitative importance in these patients, selected because of ability to cooperate (Nielsen *et al*. 1995), and it was omitted from the 2-week protocol.

As described by Nielsen *et al*. (1995), the protein requirement for N balance, and protein utilization, were calculated for each patient by linear regression analysis of N balance *v*. protein intake for each period of protein intake, i.e. at least six balance periods

for each patient. The requirement for protein equals the intercept at balance $= 0$ and utilization is the the slope of the regression line.

Energy intake was calculated from weighed dietary intake by means of food tables. A validation study showed that measurement in duplicate portions of food on average gave 13 % lower energy content (Nielsen *et al.* 1995) but this was ignored in the present study where exact energy balance was not of crucial importance.

Protein metabolism. On day 3 of the baseline period a primed continuous intravenous infusion of [^{15}N]glycine (98 %, Sigma Chemical Company, St Louis, MO, USA) was initiated at 11.00 hours. A bolus of 0.52 mg ^{15}N/kg BW was given over 10 min followed by a continous infusion of 0.42 mg ^{15}N/kg BW per h for 29 h (Jeevanandam *et al.* 1986). From the next morning the same amount of energy and protein was given in equally-divided doses at hourly intervals from 07.00 to 20.00 hours, in the form of a mixture of casein-based (Nutridrink; Nutricia, Zoetermeer, The Netherlands) and soyabean-protein-based (Top-Up; Ferrosan, Copenhagen, Denmark) liquid supplements (casein–soyabean protein; 2 : 1, w/w). Urine samples for analysis of isotope enrichment were taken at 2 h intervals from 12.00 to 16.00 hours, i.e. after 25–29 h isotope infusion when isotopic steady-state was established. The amount of [^{15}N]glycine administered during the continuous infusion was about 6 % of the amount of glycine given in dietary protein. The tracer study was performed twice in each subject and the final [^{15}N]glycine investigation also took place after 3 d of fixed, increased, dietary intake.

A pre-infusion urine sample was used to determine the ^{15}N background. NH$_3$ in urine and NH$_3$ derived from urinary urea was isolated as described previously (Nielsen *et al.* 1994). The samples were analysed by using a RoboPrep$^{®}$ elemental analyser connected with a TracerMass$^{®}$ isotope-ratio mass spectrometer (Europa Scientific, Crewe, Ches.). NH$_3$ and urea fluxes were calculated separately from the atom percentage enrichment (APE) at plateau ^{15}N enrichment (Picou & Taylor-Roberts, 1969). Average flux was calculated as the geometric average of NH$_3$ and urea fluxes since this calculation does not favour any of the two extreme models discussed by Fern *et al.* (1985). Protein synthesis and degradation was calculated according to the equation:

$$Q_{geo} = I + D = E + S, \qquad (3)$$

in which Q_{geo} is flux, I is intake, D is degradation, E is excretion of urinary N recalculated to protein by multiplication by 6.25, and S is synthesis. Blood samples were taken at 14.00 hours, i.e. when the second of the three urine samples was collected.

Substrates and hormones

Routine laboratory tests were analysed by standard methods. As a quantitative test of liver function, galactose elimination capacity was determined according to Tygstrup (1966). Plasma insulin and glucagon were determined as described previously (Nielsen *et al.* 1995). IGF-I in serum was determined after acid–ethanol extraction and cryoprecipitation as described by Bang *et al.* (1991) and Juul *et al.* (1995), and IGF-binding protein (GFBP)-3 was determined according to Blum *et al.* (1990). Due to large inter-assay variations each hormone was measured in all samples from one protocol on the same day. Plasma amino acid composition was measured by the Pico-Tag$^{®}$ method, Waters Chromatography Division, Milford, MA., USA. In Table 5 the concentration of total amino acids in plasma was calculated by summation of individual amino acids. In Tables 6 and 7, total amino acids in plasma was measured by the dinitrofluorobenzene method (Goodwin, 1970).

Statistics

Results are expressed as means with their standard errors. Statistical significance was evaluated by Student's *t* test for paired or unpaired observations, as appropriate.

Ethics

The study was approved by the local Ethics Committee and conducted in accordance with the principles for human experimentation as defined in the Declaration of Helsinki II.

RESULTS

The initial clinical and nutritional indices for the eleven patients who completed the two protocols are shown combined in Table 1. Eight of the ten patients with alcoholic liver cirrhosis admitted to alcohol abuse. Their mean intake had been 93 (SE 12; range 60–180) g/d for a mean of 12 (SE 2; range 5–20) years. Refeeding was begun at a mean of 18 (SE 3; range 7–44) d after admission. The patients were refed for a mean of 31 (SE 3; range 20–

Table 1. *Clinical and nutritional status of eleven patients with liver cirrhosis before refeeding*

(Mean values with their standard errors)

	n	Mean	SE
Male	8		
Female	3		
Child–Pugh group*: A	1		
B	6		
C	4		
Age (years)		45	2
Height (m)		174	2
Wt†: kg		57	3
% of reference		83	3
Lean body mass: 72 h creatinine (kg)		29	1
% reference		57	8
Triceps skinfold: cm		0·68	0·07
% reference		51	6
Mid-arm-muscle-area: cm^2		38	3
% reference		65	3
Albumin (μmol/l)‡		462	18
Prothrombin index‡		0·55	0·05
Alanine aminotransferase (*EC* 2.6.1.2; IU/l)‡		34	7
Alkaline phosphatase (*EC* 3.1.3.1; IU/l)‡		294	46
Bilirubin (μmol/l)‡		71	38
Galactose elimination capacity: mmol/min		1·17	0·09
% reference		48	4
IgG (μmol/l)‡		134	14
IgA (μmol/l)‡		59	8
IgM (μmol/l)‡		3·6	1

Ig, immunoglobulin.

* Child–Pugh group classification is a clinical score for severity of liver disease (Pugh *et al.* 1973).

† All patients had ascites.

‡ Reference values given by the Department of Clinical Chemistry: albumin 550–830 μmol/l, prothrombin index 0·70–1·30, alanine aminotransferase 10–40 IU/l, alkaline phosphatase 80–275 IU/l, bilirubin 4–17 μmol/l, IgG 36–89 μmol/l, IgA 3·8–21·0 μmol/l, IgM 0·3–3·7 μmol/l.

Table 2. *Protein intake and nitrogen balance of eleven patients with liver cirrhosis during refeeding (g protein/kg per d)* *

(Mean values with their standard errors)

	2-week protocol (n 5)		4-week protocol (n 6)	
	Mean	SE	Mean	SE
Protein intake: Initial	1·04	0·16	0·95	0·07
Final	2·12	0·38	1·78	0·16
N balance: Initial	−0·08[a]	0·24	−0·02[c]	0·08
Final	0·90[b]	0·36	0·51[d]	0·11
Utilization† (g/g)	0·95	0·17	1·08	0·15

[a,b,c,d] Mean values with different superscript letters within the same column were significantly different: [a,b] $P = 0.027$; [c,d] $P = 0.001$.

*For details of patients and procedures, see Table 1 and pp. 198–200.

†Calculated by linear regression analysis of N balance v. protein intake.

41) d in the 4-week protocol and 14·2 (SE 0·52; range 13–16) d in the 2-week protocol. All patients had ascites and BW increased by a mean of 4 % during refeeding (data not shown).

Table 2 shows that, in both protocols, protein intake doubled and N balance improved. As calculated by linear regression analysis of N balance v. protein intake, protein utilization was close to 1 in both protocols. Estimated protein requirement was 0·98 (SE 0·13) g/kg per d in the 2-week protocol and 0·81 (SE 0·10) g/kg per d in the 4-week protocol. The r^2 of regression analysis was 0·86 (SE 0·05) in the 2-week protocol and 0·87 (SE 0·04) in the 4 week protocol. The patients were initially in energy balance (intake 7·9 (SE 0·7) MJ/d v. energy expenditure 7·5 (SE 0·3) MJ/d), as calculated by the factorial method described previously (Nielsen *et al.* 1995). During refeeding, energy intake was increased in proportion to protein intake.

Table 3 shows the background ^{15}N atom percentage, and the APE as averages of three samples taken 25, 27 and 29 h after initiating isotope infusion. The analytical precision (CV) was 0·8 ‰ when applying 10 samples of 100 μg N sample (consisting of glycine enriched by 0·05 APE by mixing with [^{15}N]glycine) to the mass spectrometer. To evaluate whether an isotope steady-state was actually achieved, the CV for APE of NH$_3$ and urea was calculated for each set of three measurements (i.e the three 2 h sampling periods). The average CV for the total of twenty-two sets of urinary NH$_3$ was 5·2 (SE 0·7) %, and for urea 3·4 (SE 0·4) %. At the second investigation the background atom percentage of NH$_3$ and urea was negligibly higher than the first background measurements.

Table 4 shows that increased dietary intake stimulated flux and protein synthesis to the same extent in both protocols. Protein degradation decreased after 2 weeks refeeding but was unchanged after 4 weeks. Protein balance in Table 4 (degradation subtracted from synthesis) is higher than that of Table 2 since it is customary in the calculation of protein synthesis to subtract only urinary N from protein flux, rather than the sum of N excretion in urine, faeces and skin. N in faeces and skin may to an unknown extent be proteins synthesized in the body. For comparison, results are shown from a study in healthy individuals by Pacy *et al.* (1994).

Two of the eight patients originally included in the 4-week protocol had returned to their initial protein intake and N balance at the end of the study (after 28 and 31 d respectively), after an intermediate period of increased intake. Despite this, these two patients were also subjected to the second [^{15}N]glycine investigation. On average, flux, synthesis and degradation had decreased by 20–25 % at the second investigation.

Table 3. *Individual values of background isotope enrichment and atom percentage excess (APE) during steady-state isotope infusion in eleven patients with liver cirrhosis**

Patient		First study		Second study	
		Background	APE	Background	APE
4-week protocol					
JH	NH_3	0·366	0·076	0·371	0·086
	Urea	0·367	0·065	0·374	0·045
LQ	NH_3	0·371	0·070	0·369	0·083
	Urea	0·371	0·071	0·370	0·089
PM	NH_3	0·371	0·115	0·371	0·084
	Urea	0·370	0·084	0·371	0 088
JL	NH_3	0·368	0·120	0·376	0·112
	Urea	0·371	0·080	0·374	0·072
BTO	NH_3	0·372	0·115	0·372	0·070
	Urea	0·368	0·100	0·371	0·061
LO	NH_3	0·368	0·115	0·379	0·065
	Urea	0·368	0·071	0·375	0·052
2-week protocol					
OM	NH_3	0·366	0·129	0·373	0·086
	Urea	0·368	0·095	0·371	0·072
IGN	NH_3	0·367	0·052	0·373	0·040
	Urea	0·367	0·034	0·368	0·045
TW	NH_3	0·370	0·075	0·371	0·070
	Urea	0·370	0·065	0·372	0·058
CFP	NH_3	0·369	0·088	0·374	0·049
	Urea	0·367	0·055	0·370	0·042
CN	NH_3	0·368	0·130	0·383	0·086
	Urea	0·367	0·059	0·376	0·066

*For details of patients and procedures, see Table 1 and pp. 198–200.

Table 4. *Protein metabolism in eleven patients with liver cirrhosis during hourly feeding before and after refeeding (g/kg per d)**

(Mean values with their standard errors for no. of patients shown)

	n	Before		2 weeks refeeding		4 weeks refeeding		Statistical significance of the difference‡:
		Mean	SE	Mean	SE	Mean	SE	P
Flux	5	3·95	0·55	4·80	0·53	–	–	0·016
Synthesis	5	3·14	0·48	3·88	0·44	–	–	0·044
Degradation	5	2·91	0·51	2·69	0·51	–	–	0·040
Flux	6	2·86	0·22	–	–	3·83	0·28	0·022
Synthesis	6	2·15	0·24	–	–	2·81	0·35	0·033
Degradation	6	1·92	0·28	–	–	2·06	0·37	
Protein intake†								
(g/kg per d)...		0·75		1·5				
Flux†		4·37		4·70				
Synthesis†		4·22		5·00				
Degradation†		3·62		3·20				

*For details of patients and procedures; see Table 1 and pp. 198–200.
†Data recalculated from Pacy *et al.* (1994). After 2 weeks of the protein intakes specified, healthy subjects were investigated by [^{13}C]leucine infusion during hourly feeding for 12 h (for further details, see p. 207).
‡Mean values were significantly different from those before refeeding by paired *t* test.

Table 5 shows changes in plasma substrates and hormones in the 2-week protocol, as measured at 14.00 hours during hourly feeding and [^{15}N]glycine infusion. Lysine, tyrosine, branched-chain amino acids (BCAA), proline, glucagon and T_3 showed significant increases.

In the 4-week protocol only total plasma amino acids, IGF-I and IGFBP-3 were measured at 14.00 hours during the [^{15}N]glycine infusion. Also in this protocol, total plasma amino acids increased (by 55%; $P = 0.008$) and neither IGF-I nor IGFBP-3 increased significantly.

Table 5. *Plasma substrate and hormone levels of five patients with liver cirrhosis during hourly feeding before and after 2 weeks of refeeding*†*

(Mean values with their standard errors)

	Initial value		Final value		Statistical significance of difference (paired *t* test): *P*
	Mean	SE	Mean	SE	
Glucose (mmol/l)	10·00	3·27	13·51	5·77	
Lactate (mmol/l)	1·71	0·21	1·83	0·19	
Free fatty acids (mmol/l)	0·32	0·09	0·28	0·08	
β-hydroxybutyrate (μmol/l)	53	17	39	4	
Total amino acids (mmol/l)	3·85	0·24	4·82	0·21	0·049
Lys (μmol/l)	180	11	262	26	0·026
Thr (μmol/l)	144	27	186	21	
His (μmol/l)	95	11	95	16	
Trp (μmol/l)	74	8	102	12	
Phe (μmol/l)	115	15	172	26	
Tyr (μmol/l)	145	23	259	33	0·011
Met (μmol/l)	78	31	72	16	
Cys (μmol/l)	260	35	274	29	
Leu (μmol/l)	85	3	133	11	0·011
Ile (μmol/l)	46	6	79	7	0·024
Val (μmol/l)	135	12	234	26	0·004
Ala (μmol/l)	392	61	528	31	
Glu (μmol/l)	55	7	89	17	
Gln (μmol/l)	766	100	775	54	
Asn (μmol/l)	223	19	295	17	
Pro (μmol/l)	248	19	355	8	0·027
Arg (μmol/l)	160	17	190	19	
Orn (μmol/l)	94	9	120	16	
Cit (μmol/l)	56	19	68	24	
Gly (μmol/l)	286	42	281	38	
Ser (μmol/l)	173	19	200	9	
Tau (μmol/l)	44	12	48	14	
Insulin (mU/l)	61	21	121	52	
Glucagon (ng/l)	312	107	453	178	0·008
Growth hormone (μU/l)	8·66	2·76	9·53	3·42	
IGF-I (μg/l)	35	8	45	13	
IGFBP-3 (μg/l)	857	292	1095	342	
Free T_3 (pmol/l)	4·66	0·64	5·94	0·97	0·018
Free T_4 (pmol/l)	14·8	1·8	16·4	2·3	
TSH (mU/l)	1·59	0·37	1·27	0·20	

IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; T_3, triiodothyronine; T_4, thyroxine; TSH, thyroid-stimulating hormone.

*For details of patients and procedures, see Table 1 and pp. 198–200.

†Reference values cannot be given for this fed state condition. However, some hormones are believed not to fluctuate with diurnal dietary intake and 95% reference values for these hormones can be given tentatively. IGF-I 67–264 μg/l, IGFBP-3 1957–5065 μg/l, free T_3 5·0–10·0 pmol/l, free T_4 9·0–28·0 pmol/l, TSH 0·40–3·80 mU/l.

Table 6 shows the effect of a standardized meal-test (50 kJ/kg and 0·4 g protein/kg) after an overnight fast in four patients in the 4-week protocol, at the beginning and at the end of the refeeding period. The results given for the 'meal' condition are means of the 1 h and 3 h post-meal measurements. At the first meal test, free fatty acids decreased significantly and insulin increased significantly. When repeated at the end of the refeeding period the response of all substrates and hormones were similar to that of the first test-meal, except that the increase in insulin did not reach statistical significance ($P = 0·052$) and the increase in total amino acids was statistically significant, but quantitatively similar. No significant changes were observed in fasting levels of substrates and hormones before the second meal test. The composition of plasma amino acids was only measured in three of the four patients included in the meal test study. After the first meal, plasma leucine increased by 83 (SE 12)% ($P = 0·02$) and total BCAA increased by 60 (SE 11)% ($P = 0·03$). The increases in other amino acids did not reach statistical significance. These changes were the same after the second meal.

In order to identify the disturbance in protein metabolism underlying the increased protein requirement, the thirteen patients who participated in the present study were divided into two groups (Table 7). The patients were divided according to whether or not their protein requirement for N balance was within the value recommended for healthy individuals on a mixed diet (0·8 g/kg per d). The mean requirement in the 'high-requirement' group was almost twice as high compared with the 'normal-requirement' group (Table 7). The utilization of protein (i.e. the slope of linear regression of N balance v. protein intake) was identical in the two subgroups. The other data in Table 7 are from the initial investigation of protein metabolism. Protein intake coincidently was similar in the two subgroups. Urinary N excretion recalculated to protein was higher in the 'high-requirement' group. Protein synthesis was similar in the two groups. Protein degradation was higher in the 'high-requirement' group and although this difference was not

Table 6. *The effect of a single standard meal on plasma substrate and hormone levels in four patients with liver cirrhosis before and after 4 weeks of refeeding**

(Mean values with their standard errors)

		Initial test		Statistical significance of difference†: P	Final test		Statistical significance of difference†: P	Reference values‡	
		Mean	SE		Mean	SE		Mean	SE
Glucose (mmol/l)	Fasting	5·64	0·39		5·83	0·17		4·74	0·11
	Meal	8·49	1·52		7·77	1·55			
Lactate (mmol/l)	Fasting	1·40	0·30		1·20	0·10		–	–
	Meal	1·55	0·13		1·36	0·06			
Free fatty acids (mmol/l)	Fasting	0·65	0·09		0·70	0·11		–	–
	Meal	0·14	0·03	0·032	0·16	0·0-3	0·014		
β-hydroxybutyrate (μmol/l)	Fasting	97	10		93	11		–	–
	Meal	80	5		79	4			
Total amino acids (mmol/l)	Fasting	2·95	0·20		3·45	0·31		3·40	0·11
	Meal	3·38	0·17		3·78	0·36	0·036		
Insulin (mU/l)	Fasting	10	4		15	3		8	1
	Meal	51	9	0·021	52	12			
Glucagon (ng/l)	Fasting	79	20		99	30		76	9
	Meal	82	13		99	18			

*For details of patients and procedures, see Table 1 and pp. 198–200.
†The difference between mean meal and fasting values was analysed by paired *t* test.
‡Fasting values for healthy individuals from this laboratory (Hamberg & Vilstrup, 1994).

Table 7. *Protein metabolism of patients with liver cirrhosis who had low or high protein requirements**

(Mean values with their standard errors)

Requirement (g/kg per d)...	< 0·8		> 0·8		
n ...	5		8		Statistical significance of difference: P
	Mean	SE	Mean	SE	
Requirement (g/kg per d)	0·63	0·05	1·12	0·12	
Utilization (g/g)	1·04	0·15	0·91	0·12	
Intake (g/kg per d)	1·06	0·18	1·12	0·12	
Excretion (g/kg per d)†	0·52	0·07	0·93	0·08	0·005
Flux (g/kg per d)	3·24	0·36	3·71	0·41	
Synthesis (g/kg per d)	2·72	0·33	2·77	0·38	
Degradation (g/kg per d)	2·19	0·34	2·58	0·38	
Degradation:synthesis	0·79	0·04	0·94	0·04	0·03
Plasma amino acids (mM)	2·82	0·29	3·45	0·22	

*For details of patients and procedures, see Table 1 and pp. 198–200.
†Calculated from urinary N excretion × 6·25.

statistically significant, it was numerically similar to the difference in excretion, in accordance with equation 3. When expressed relative to protein synthesis, protein degradation was significantly higher in the 'high-requirement' group. The 'high-requirement' group had a higher (but not significant) mean value of total amino acids ($P = 0·11$).

DISCUSSION

According to Table 1 the patients were moderately malnourished and had a moderate degree of liver disease. All patients were ambulatory and ten patients had ascites. In these respects the patients were similar to those studied earlier (Nielsen *et al.* 1995). None of the patients developed encephalopathy during refeeding. Table 2 shows that oral protein (and energy) intake was doubled within the 2-week protocol. The more rapid increase in dietary intake in the 2-week protocol did not decrease protein utilization.

Results obtained with the [^{15}N]glycine method have been found previously to be similar to those obtained with labelled leucine (Golden & Waterlow, 1977; Pacy *et al.* 1994). However, if the experimental conditions affect intermediary metabolism of glycine by other routes than protein synthesis and degradation, results can be interpreted erratically (Golden & Jackson, 1981). In patients with liver cirrhosis, plasma [^{15}N]glycine elimination rate and urinary ^{15}N appearance did not differ between patients and healthy controls after an oral dose of [^{15}N]glycine (Kohno *et al.* 1990). A number of physiological and pathological conditions (dietary amino acid composition, glucose infusion, fasting, refeeding, hypophysectomy, treatment with growth hormone or corticotropin) do not affect intermediary glycine-N metabolism substantially, as discussed recently (Nielsen *et al.* 1994). The initial values in the present study for flux and rates of synthesis and degradation were, within 20% identical to those reported in healthy individuals at a similar protein intake, investigated by the same isotope method (Jeevanandam *et al.* 1986). In addition, the ratio between flux calculated from urinary NH$_3$ and urinary urea was 0·75 (SE 0·04) in our initial study (data not shown) which is very close to the value reported by Jeevanandam *et al.* (1986). These observations indicate that patients with cirrhosis do not

pose specific problems with the [^{15}N]glycine method. After refeeding, the ratio between flux calculated from urinary NH_3 and urinary urea was changed slightly but not significantly (0·84 (SE 0·03)), and flux calculated from NH_3 or urea both increased significantly after refeeding (data not shown), indicating that refeeding did not perturb the system substantially.

Previously, protein metabolism has been investigated in patients with liver cirrhosis in the diurnal fasting–fed state transition, after a single meal. Milikan *et al.* (1985) found a decrease in protein degradation while protein synthesis was unaffected. Swart *et al.* (1988) reported a significant improvement in N balance but the increase in protein synthesis and the decrease in protein degradation failed to reach statistical significance. Tessari *et al.* (1994) found a decrease in protein degradation but protein synthesis was not measured. Only one previous study (Bonkovsky *et al.* 1991) dealt with the effect of prolonged (3 weeks, intravenous) refeeding, but they only studied protein metabolism in the fasting state and found no effect of refeeding on protein synthesis or degradation.

As discussed by Millward (1990), regulatory mechanisms involved in growth probably differ from regulatory mechanisms involved in the diurnal fasting–fed state transition. The same distinction could apply to repletion after weight loss *v.* the diurnal fasting–fed state transition. In healthy volunteers, Pacy *et al.* (1994) found that in the fasting–fed state transition, protein synthesis increased and protein degradation decreased, and this was associated with a large increase in plasma insulin. The 2-week adaptation to various protein intakes (from insufficient to excessive levels) showed that an increased daily protein intake also led to increased protein synthesis and decreased protein degradation, as measured in the fed state. However, this was not associated with changes in plasma levels of insulin (or free T_3 or IGF-I) but with an increase in plasma leucine. Part of their data is reproduced in Table 4. To facilitate a comparison with the protein intake in our 2-week protocol (Table 3), we have reproduced their results after 2-weeks feeding of either 0·75 or 1·5 g/kg per d.

Our results agree with those of Pacy *et al.* (1994) in that increased protein synthesis, rather than decreased protein degradation, accounted quantitatively for most of the increase in N retention. In addition, our meal test study also showed a large increase in insulin, while prolonged refeeding only led to non-significant increases in insulin but significant increases in plasma amino acids, especially leucine and other BCAA.

The increased plasma level of amino acids may contribute to regulation of protein metabolism, as indicated by experiments with human subjects, in which amino acid infusion increased protein synthesis. This occurred to the same extent whether or not plasma insulin was kept at a basal fasting level or at a high level (Tessari *et al.* 1987; Castellino *et al.* 1987). With respect to individual amino acids, a possible regulatory role for leucine was suggested by studies in isolated rat muscle (Fulks *et al.* 1975) and in rats *in vivo* (Garlick & Grant, 1988). Alanine and proline were suggested to increase hepatic protein synthesis in rats *in vivo* (Perez-Sala *et al.* 1987). However, the mechanism whereby amino acids may regulate protein synthesis is uncertain. Experiments in fasted mice suggest that dietary protein enhances protein synthesis via stimulation of both initiation and elongation activity (Yoshizawa *et al.* 1995).

In contrast to the results of Pacy *et al.* (1994), we found an increase in T_3 from initial borderline low T_3 levels (Table 5). This could be due to increased intake of both protein and energy in our patients who initially were in a state of chronic malnutrition, known to be associated with low T_3 levels. In the study of Pacy *et al.* (1994), energy intake was kept constant. An increase in T_3 may facilitate protein synthesis under some conditions, but a direct effect of T_3 administration on protein synthesis could not be shown during refeeding of starved rats (Millward *et al.* 1988). Growth hormone and IGF-I did not increase

significantly in our study, suggesting that these hormones were not responsible for the increase in protein synthesis. It is, however, difficult to give a definite interpretation of these results since, for example diurnal fluctuation of growth hormone and several binding proteins for IGF-I other than IGFBP-3 may affect the biological activity of these hormones. The observation that the response to a single meal was unchanged after 4 weeks refeeding (Table 6) is compatible with the view that refeeding did not substantially alter the hormonal milieu of the patients. Our results suggest that repletion after weight loss, with respect to whole-body protein metabolism, is due primarily to increased protein synthesis and that this increase is associated primarily with elevated levels of plasma amino acids. This agrees with conclusions from the study of healthy individuals (Pacy et al. 1994).

Concurrent with the high protein utilization which is considered to be similar to that of underweight, otherwise healthy, subjects (Nielsen et al. 1995), patients with liver cirrhosis in general require an increased amount of protein to reach N balance. In the present study eight of thirteen patients had an increased requirement (Table 7). Taken together with the high utilization this means that in patients with a high protein requirement there is a parallel shift to the right of the N retention v. protein intake curve (Nielsen et al. 1995) and, at any given intake, N retention will be less than that of underweight, otherwise healthy, subjects. In the preceding study (Nielsen et al. 1995), faecal energy and N content was similar to that of healthy individuals and the same applied to faecal N excretion in the patients of the present study (data not shown). In the absence of malabsorption the decreased N retention must be due to increased N excretion associated with decreased protein synthesis and/or increased protein degradation. According to Table 7 the increased protein requirement, and increased N excretion, was associated with increased protein degradation, rather than decreased protein synthesis. The results in Table 7 further suggest that the primary disturbance is increased protein degradation, rather than increased N excretion, since a primarily increased N excretion, as induced by infusion of glucagon, decreases protein synthesis but it does not increase protein degradation (Pacy et al. 1990). In addition, glucagon infusion decreases plasma amino acid levels (Couet et al. 1990) but the 'high-requirement' group in Table 7 tended to have an elevated total plasma amino acid concentration, consistent with the hypothesis that increased degradation is the primary disturbance.

Increased protein degradation has previously been reported after a single meal in patients with liver cirrhosis, compared with healthy control subjects (Swart et al. 1988; Tessari et al. 1994). However, these studies failed to explain the increased protein requirement. In the first study (Swart et al. 1988), protein synthesis was also increased and N balance was more positive in the patients as compared with normal-weight healthy controls, as would be expected when feeding underweight individuals. In the other study (Tessari et al. 1994), N balance was not measured and the metabolic fate of amino acids produced by protein degradation (i.e. protein synthesis v. N excretion) was not investigated. Protein degradation, also, has been found to be increased after an overnight fast, but not in all studies (McCullough & Glamour, 1993). In the present study, protein degradation was observed to be increased during feeding in patients who had an increased protein requirement, as estimated from subsequent 24 h balance periods of several weeks duration. This indicates that the mechanism underlying the increased protein degradation, and increased protein requirement, is not confined to the nocturnal fasting period, as suggested by Swart et al. (1988).

It was shown previously that there is no correlation between protein requirement and resting energy expenditure (Nielsen et al. 1995). One possible explanation for the increased rate of protein degradation could be the low plasma levels of IGF-I (Table 5), since IGF-I is

known to inhibit protein degradation (Elahi *et al.* 1993; Giordana *et al.* 1995) and to improve N balance in individuals with low IGF-I levels, such as semi-starved healthy individuals (Clemmons *et al.* 1992) and normal-weight elderly women given a weight-maintaining diet (Thompson *et al.* 1995). In the present study, initial plasma concentrations of IGF-I and IGFBP-3 were 22 and 24 % respectively of age- and sex-matched mean values (Juul *et al.* 1995 and unpublished results relating to data for 316 healthy individuals at age 50 years). The persistence of low levels in our patients during refeeding indicates that the low levels are due to impaired liver function since plasma IGF-I is normalized within 8 d of refeeding healthy individuals (Clemmons & Underwood, 1991). The lack of increase in IGF-I during refeeding could require a compensatory higher protein intake, before the balance between protein synthesis and degradation converts to net anabolism.

With the aim of identifying a clinical correlate to the increased protein requirement, we subdivided all twenty patients in whom protein requirement had been estimated in the present and the preceding studies (Nielsen *et al.* 1995). Patients with an increased protein requirement (i.e. > 0·8 g/kg per d; *n* 11 (of the twenty patients)) tended to have higher levels of plasma bilirubin and alanine aminotransferase (*EC* 2.6.1.2), a decreased galactose elimination capacity and a more severe degree of liver disease (higher Child–Pugh score (Pugh *et al.* 1973)), but none of these differences were significantly different. Further, nutritional status (LBM from three × 24 h creatinine excretions, mid-arm-muscle area or triceps skinfold), amount and duration of alcohol intake, or time lapse from admission to starting the refeeding protocol, or any other variable included in Table 1, did not differ between the two groups (data not shown). Linear regression analysis and multiple regression analysis were also unable to identify clinical correlates to the increased protein requirement. More specifically, it was considered that protein wasting in the form of myopathy is common in chronic alcoholics, also in those without liver disease (Martin *et al.* 1985; Preedy *et al.* 1994), and that lack of alcohol abstinence in patients with cirrhosis is associated with increased protein turnover, compared with abstinent individuals (Hirsch *et al.* 1995). However, according to our analysis, neither alcohol intake, duration of alcohol abuse or duration of abstinence before refeeding were related to increased protein requirement.

In summary, the present and the preceding studies (Nielsen *et al.* 1993, 1995) have shown, by application of the 'N retention *v*. N intake' methodology, that increased requirement, rather than decreased utilization, is the abnormality of N retention in patients with liver cirrhosis. Furthermore, our results suggest that these two components of the retention *v*. intake curve are distinct entities since utilization was the same in the two subgroups divided according to protein requirement (Table 7). It also appears, at least in our patients, that the two components are controlled in distinctly separate ways: utilization by protein synthesis and requirement by protein degradation.

Even when clinically stable, the spontaneous intake of these patients is very close to the requirement for balance (Nielsen *et al.* 1993) and, therefore, they are prone to rapid protein wasting in periods with decreased appetite (due to ascites, infection, bleeding etc.). In the absence of clinical correlates that can identify patients with an increased protein requirement, we suggest (from the data in Table 7) that these patients are identified by measurement of urinary N excretion at an intake of 1 g protein/kg per d. Patients in positive balance at this intake can be considered not to have an increased requirement. Other patients require larger amounts of protein to achieve N balance. A therapeutic approach with partial substitution of ordinary protein with BCAA failed to reduce the protein requirement for N balance (Swart *et al.* 1989), compatible with the view that BCAA may be especially important for protein utilization which is already highly efficient.

Instead, dietary substances or hormonal factors (such as IGF-I) that can reduce protein degradation specifically may be useful in these patients.

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