

# Nitrogen Metabolism and Protein Synthesis During Pneumococcal Sepsis in Rats

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Markedly increased synthesis of  $\alpha_2$  and  $\beta$  globulins and  $\alpha_1$ ,  $\alpha_2$ , and  $\beta$  glyco-globulins occurs during pneumococcal sepsis in the rat simultaneously with decreased albumin formation, diminished tritiated leucine incorporation into muscle protein, and enhanced excretion of nitrogen. This augmented synthesis of specific serum proteins does not become evident until fever and bacteremia develop, and it appears to be a fundamental aspect of host response to a proliferating bacterial infection in that it occurs even in rats fed a protein-deficient (6% protein) diet after weaning and before exposure to *Diplococcus pneumoniae*.

Although amino acid catabolism, in general, appears to be increased during infection, tryptophan degradation via the kynurenine pathway, as assessed by measuring diazotizable urinary metabolites, changes little or is, at times, significantly less than in control animals. Coincidentally, functional tryptophan oxygenase activity decreases at 16 hr after exposure. Total tryptophan oxygenase activity, however, is unchanged.

Anabolic as well as catabolic phenomena occur during sepsis. Increased synthesis of serum proteins has been demonstrated in man (23, 26) and rodent (11, 27) during bacterial infection. Induction of hepatic tyrosine transaminase with a resultant decrease in circulating tyrosine has been noted in rats exposed to *Diplococcus pneumoniae* (21). Wasting of body nitrogen is a long-standing and well-documented observation under these circumstances (3, 7, 20). The enhanced excretion of metabolites of tryptophan has been shown to occur in a variety of illnesses (18). Evidence is accumulating that a number of the infection-induced alterations in host metabolism arise from the release of an endogenous mediator(s) (13, 25) and are, therefore, interrelated. We have, therefore, begun a series of studies in an attempt to gain a more complete perspective of the concomitant catabolic and anabolic aspects of generalized host response during the early stages of bacterial infection. The first of these studies deals with an acute, lethal bacterial illness, pneumococcal sepsis, in rats.

## MATERIALS AND METHODS

**Animals.** Fisher Dunning rats (obtained from Microbiological Associates, Walkersville, Md.) weighing 150 to 200 g, were housed in a room which was maintained at 25 to 26 C and lighted from 0600 to 1800 hr. During the week before their use in these studies, all rats were trained to consume, between 0800

and 1600 hr, an agar gel diet (1) which contained 18% casein. To assess nitrogen balance, 12 rats were placed in individual metabolic cages 1 week prior to sample collection. Six control rats and six infected rats were fed in pairs on the day prior to inoculation and on the 2 days after exposure to the microorganism. Urine and fecal collections were made one day before and for 2 days after inoculation with *D. pneumoniae*. To study the effects of dietary protein deprivation on protein synthesis, weanling rats were fed either a 6 or an 18% agar gel diet for 28 days prior to their exposure to the microorganisms.

**Microorganism and the resultant infection.** Virulent *D. pneumoniae* type 1, strain A5, served as the infectious agent. The organisms were grown to early stationary phase in 3.7% brain heart infusion supplemented with 1% bovine serum albumin and were then frozen at -65 C in 15% glycerol. When needed, these stock cultures were diluted to 1 to  $2 \times 10^7$  organisms/ml with sterile saline. Infection was initiated by the subcutaneous administration of 0.1 ml of saline containing 1 to  $2 \times 10^6$  *D. pneumoniae*. Subsequent fever was measured by rectal probe and bacteremia quantitated by plating 0.1 ml of serial 10-fold dilutions of blood onto blood agar and counting the resultant colonies after 24 hr of incubation at 37 C.

Fever became apparent by 14 hr after exposure and was maximal at 24 to 30 hr. Body temperature then dropped as the animals approached agonal stage. Bacteremia was evident by hr 14, became massive at 24 to 28 hr, and persisted thereafter. Death generally occurred between 48 and 72 hr. Rats serving as controls were given 1 to  $2 \times 10^6$  heat-killed (56 C for 20 min) organisms.

**Amino acid incorporation.** An intraperitoneal injection of 10  $\mu\text{Ci}$  of L-[4,5- $^3\text{H}$ ]leucine (55.5  $\mu\text{Ci}/\text{mm}$ ; New England Nuclear, Boston, Mass.) per 100 g of body weight was administered 2 hr before the rats were to be killed. At the indicated times, rats were anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane), and blood was collected from the axillary fold pouch after severing of the brachial artery. The blood was centrifuged at  $1,500 \times g$  for 15 min, and the serum was removed. The rats were killed by cervical dislocation and perfused with cold 0.9% NaCl until the livers were cleared of visible blood. The entire liver and rear leg muscles were removed, weighed, and homogenized in two and four volumes, respectively, of cold 0.9% NaCl.

A 1-ml sample of serum or tissue homogenate was added to 100 mg of sulfosalicylic acid, mixed, and centrifuged at  $1,500 \times g$  for 15 min. The supernatant fluid was removed, and the precipitate was washed twice with 0.2 M perchloric acid (PCA), dissolved in 2 N NaOH, and incubated for 1 hr at 96 C. After the samples were cooled, concentrated HCl was added, and the protein precipitate was recovered by centrifugation at  $1,500 \times g$  for 20 min. This precipitate was washed with 0.2 M PCA and dissolved in 2 N NaOH by incubation at 96 C for 1 hr. A 0.5-ml sample of the dissolved protein was added to 2 ml of Scintisol TD, after which 10 ml of Scintolute (Isolab, Elkhart, Ind.) was added. The samples were counted in a 3-channel Nuclear-Chicago Corp. scintillation counter with external standardization. Another sample of the NaOH extract was assayed for protein content by an automated procedure (10).

**Amino acid incorporation into individual serum electrophoretic fractions.** At 24 hr after the injection of *D. pneumoniae* or heat-killed organisms, the rats were injected intraperitoneally with 100  $\mu\text{Ci}$  of L-[ $^3\text{H}$ ]leucine per 100 g of body weight and killed 2 hr later. Serum was collected from each animal, and 50  $\mu\text{l}$ iters were applied to a cellulose polyacetate strip (Sephaphore III, Gelman Instrument Co., Ann Arbor, Mich.). The strip was electrophoresed in barbital buffer (pH 8.6, ionic strength 0.075), stained for protein with Ponceau S, and cleared and quantitated by using an Analytrol integrating densitometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a B-2 cam and 550-nm interference filters. The strips were run through a 4 *Pi* radioactive chromatograph scanner (Actigraph III, Nuclear-Chicago Corp., Chicago, Ill.). Individual protein fractions on the cellulose polyacetate strips were cut out, placed in liquid scintillation vials, and dissolved in acetone, followed by addition of 1 ml of Scintisol GP and 10 ml of Scintolute. The samples were counted in a liquid scintillation counter with external standardization. The percentage of total serum radioactivity in each fraction was calculated for the two groups of animals. Serum glycoprotein electrophoretic determinations were done by the method of Klainer et al. (9).

**Tryptophan oxygenase (TO) activity.** The rats were decapitated at various times after infection. Their livers were immediately removed and kept in chilled, normal saline until weighed. A portion of each liver was homogenized in a constant volume of 0.14 M KCl

containing 0.02 M phosphate buffer, pH 7.2. The resulting 16 to 25% homogenates were frozen at  $-65$  C for assay later for TO activity. TO was assayed in the presence of (i) 54  $\mu\text{moles}$  of tryptophan alone (functional activity) and (ii) 54  $\mu\text{moles}$  of tryptophan and 0.03  $\mu\text{mole}$  of hematin (total activity) in a final volume of 9 ml. Kynurenine production was assessed by diazotization (15). Activity is defined as the difference in values between samples of the homogenates incubated for 30 and 90 min at 37 C.

**Urine and fecal analysis.** Urine samples (24 hr) were collected in 2 ml of 6 N HCl, diluted to 30 ml, and stored at  $-20$  C until analyzed for various nitrogen constituents. Daily fecal samples were homogenized in 5 ml of water and stored at  $-30$  C. Urinary total nitrogen, urea nitrogen, ammonia,  $\alpha$ -amino nitrogen, creatinine, and fecal nitrogen were all determined by previously described automated techniques (3). A 1-ml sample of urine was analyzed for diazotizable substances by the procedure of Pitot et al. (15).

## RESULTS

Rats fed a diet adequate for growth eliminated more nitrogen when exposed to *D. pneumoniae* than their pair-fed controls which had been injected with heat-killed pneumococci (Fig. 1). The average increase in nitrogen excretion amounted to 37 mg/24 hr on day 1 and 25 mg/24 hr on day 2. These differences were significant at the 0.005 and 0.05 levels, respectively, as judged by the paired *t* test. Analysis of the urine samples for specific nitrogen metabolites showed urea nitrogen, ammonia,  $\alpha$ -amino nitrogen, and creatinine to be excreted in greater quantities by the infected animals than by the controls (Table 1).

The uptake of  $^3\text{H}$ -leucine into serum and liver proteins during the first 24 hr after exposure is detailed in Fig. 2. Little change was noted in total liver protein incorporation, but serum protein contained significantly more radioactivity at 18

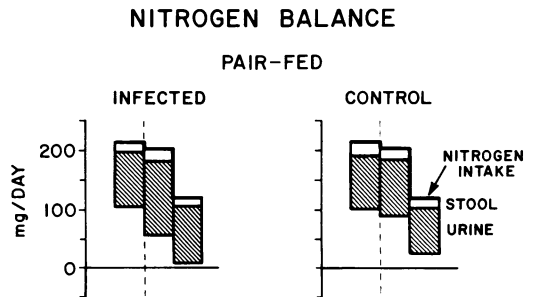


FIG. 1. Nitrogen balance of pair-fed rats. On the day prior to and for two days subsequent to the initiation of infection, 24-hr urine and feces collections were made. The dark line across the top of the bars represents nitrogen intake. There were six animals per group except for day 2, wherein the groups contained only four rats each.

TABLE 1. Effect of infection on excretion of various urinary nitrogen metabolites in pair-fed rats<sup>a</sup>

Days	Body temp (C)		Urea N <sub>2</sub> (mg/day)		Ammonia (mg/day)		α-Amino N <sub>2</sub> (mg/day)		Creatinine (mg/day)	
	I	C	I	C	I	C	I	C	I	C
-1	37.2 ±0.02	37.0 ±0.04	82.7 ±6.1	87.0 ±6.1	8.35 ±0.37	9.50 ±0.44	0.51 ±0.04	0.73 ±0.06	3.9 ±0.2	3.8 ±0.1
1	40.1 <sup>c</sup> ±0.03	36.9 ±0.10	105.5 ±9.9	94.7 ±7.9	12.80 <sup>b</sup> ±0.86	9.90 ±0.79	1.04 <sup>c</sup> ±0.06	0.82 ±0.04	5.7 ±0.3	5.2 ±0.3
2	39.5 <sup>c</sup> ±0.08	36.8 ±0.02	89.0 <sup>b</sup> ±6.4	69.0 ±4.8	11.33 <sup>c</sup> ±0.23	7.98 ±0.77	1.00 <sup>c</sup> ±0.03	0.51 ±0.08	6.3 <sup>c</sup> ±0.6	3.8 ±0.3

<sup>a</sup> The values presented are the mean ± 1 standard error mean of six animals, except for day 2 which had only four rats per group. I, Infected animals; C, control animals.

<sup>b</sup>  $P < 0.05$ .

<sup>c</sup>  $P < 0.01$ .

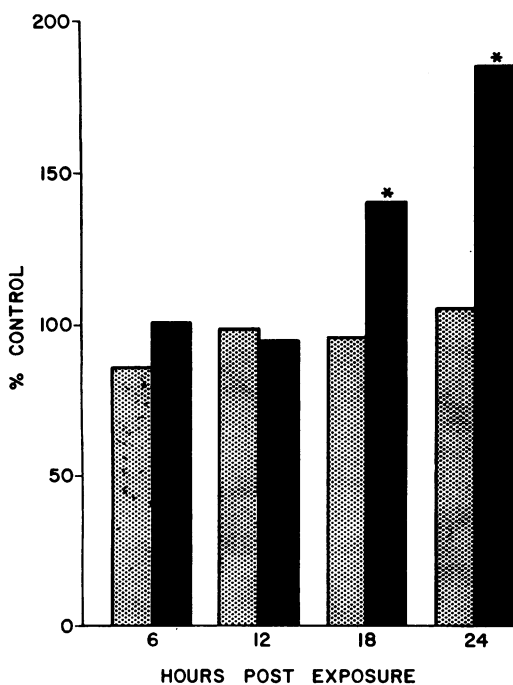


FIG. 2. Incorporation of <sup>3</sup>H-leucine into tissue protein after a 2-hr pulse dose of 10 μCi/100 g of body weight at various times after rats were exposed to 10<sup>8</sup> *D. pneumoniae*. Data shown for infected rats are expressed as a percentage of the paired control values. The stippled bar represents total liver protein; the shaded bar represents serum protein. There were five animals per group. \*,  $P < .005$ .

and 24 hr postexposure, which corresponds to the onset of fever and bacteremia in these animals. A similar increase in synthesis of serum protein was observed in rats 24 hr after inoculation with *D. pneumoniae*, irrespective of whether the animals

were fed a protein-deficient diet (6% protein) or an adequate protein diet (18% protein) for 28 days prior to their exposure to infecting organisms. Coincidentally, highly significant decreases in muscle protein synthesis occurred in both groups (Table 2).

The serum from animals, exposed to 10<sup>6</sup> *D. pneumoniae* 24 hr previously, contained more protein in the α<sub>2</sub> and β fractions and markedly less in the albumin and the γ-globulin fractions in comparison with pair-fed controls (Table 3). Radiochromatograms of the electrophoretic strips, as well as analysis of the individual fractions, revealed that the α<sub>1</sub>, α<sub>2</sub> and β serum protein fractions of the infected animals contained more <sup>3</sup>H-leucine than similar fractions in noninfected pair-fed controls, whereas radioactivity in albumin and γ-globulin fractions was significantly decreased (Fig. 3, Table 3). When the serum protein electrophoretic strips were stained for glycoproteins, the α<sub>2</sub> and β globulin fractions were increased, whereas γ and α<sub>1</sub> fractions were decreased in the infected animals in comparison with pair-fed controls (Table 3). If, however, these data were rendered as milligrams of protein-bound carbohydrates, instead of as percent of distribution, the α<sub>1</sub>, α<sub>2</sub> and β globulin fractions were shown to increase and the γ fraction was unchanged.

Measurement of the diazotizable metabolites of tryptophan indicated that tryptophan degradation via the kynurenine pathway was significantly decreased during the first 24 hr after exposure to *D. pneumoniae* (Table 4). This was true whether the data was expressed as total diazotizable material or as diazo-reactive substances per milligram of creatinine. Consistent with this finding was the decrease noted in functional TO activity at 16 hr postexposure (Table 5). There was, however, no change in total TO activity during this period.

TABLE 2. Effect of diet on protein synthesis during infection

Diet protein (%)	Group	Disintegrations per min/ $\mu$ g of protein $\pm$ 1 standard error mean (8/group)		
		Serum	Liver	Muscle
6	Control	4.2 $\pm$ 0.5	3.7 $\pm$ 0.4	0.44 $\pm$ 0.03
	Infected (Change)	10.3 $\pm$ 0.7 <sup>a</sup> (+147% <sup>a</sup> )	3.8 $\pm$ 0.2 (+3%)	0.15 $\pm$ 0.01 <sup>a</sup> (-65% <sup>a</sup> )
18	Control	4.7 $\pm$ 0.2	3.0 $\pm$ 0.1	0.43 $\pm$ 0.01
	Infected (Change)	9.6 $\pm$ 0.5 <sup>a</sup> (+104% <sup>a</sup> )	3.2 $\pm$ 0.2 (+7% <sup>c</sup> )	0.20 $\pm$ 0.02 <sup>a</sup> (-54% <sup>a</sup> )

<sup>a</sup>  $P < 0.001$ .TABLE 3. Percent distribution of serum fractions at 24 hr<sup>a</sup>

Determination	Albumin	$\alpha_1$	$\alpha$	$\beta$	$\gamma$
Serum proteins					
Control	45.3	12.3	7.2	25.8	9.2
	$\pm 1.4$	$\pm 0.5$	$\pm 0.3$	$\pm 0.9$	$\pm 0.8$
Infected	33.4	13.3	14.9	32.6	5.7
	$\pm 0.7$	$\pm 0.4$	$\pm 0.3$	$\pm 1.2$	$\pm 0.3$
(Change)	-	0	+	+	-
Serum glycoproteins					
Control	2.9	51.9	16.2	17.7	11.3
	$\pm 0.8$	$\pm 0.9$	$\pm 0.5$	$\pm 0.9$	$\pm 0.4$
Infected	1.3	38.4	25.6	28.9	5.9
	$\pm 0.6$	$\pm 2.1$	$\pm 1.6$	$\pm 1.0$	$\pm 1.4$
(Change)	0	-	+	+	-
<sup>3</sup> H-Leucine incorporation					
Control	32.6	13.7	22.3	20.8	10.3
	$\pm 1.3$	$\pm 0.8$	$\pm 0.9$	$\pm 0.8$	$\pm 0.8$
Infected	8.3	19.0	32.4	32.1	8.0
	$\pm 0.6$	$\pm 1.0$	$\pm 1.1$	$\pm 1.5$	$\pm 0.6$
(Change)	-	+	+	+	0

<sup>a</sup> All changes display a  $P$  value of less than 0.005.

## DISCUSSION

Infected rats fed on a diet optimal for growth excreted significantly more nitrogen than their pair-fed controls, 34% more on day 1 postexposure and 25% more on day 2, yet they still remained in nitrogen equilibrium. This is a result of using young, growing rats in these studies for such animals, when fed a diet adequate to produce optimum growth, retain approximately 50% of the dietary nitrogen, thus decreasing the likelihood of a negative nitrogen balance developing during an acute illness (1).

Despite the enhanced excretion of nitrogen, the infected animals demonstrated a significant increase in the uptake of label into serum proteins, which appeared to parallel the accelerated flux of

amino acids into liver (24) and did not become evident until fever and bacteremia developed. Increased <sup>3</sup>H-leucine incorporation into serum protein was observed even in rats fed a protein-deficient diet for 28 days after weaning and prior to inoculation with *D. pneumoniae*. Thus, it would seem that serum protein synthesis by the liver is a fundamental host response to this severe bacterial infection.

The decrement in uptake of radioactivity into muscle protein during febrile illness in rats fed either the 6 or 18% protein diet is consistent with the increased excretion of creatinine noted in this study. It is also consonant with the hypothesis that infection induces a flow of amino acids from skeletal muscle to liver (24).

Examination of the pattern of serum protein synthesis, 24 hr after exposure in rats fed the 18% protein diet, reveals an augmented uptake of <sup>3</sup>H-leucine by the  $\alpha_1$ ,  $\alpha_2$ , and  $\beta$  fractions, which correlates positively with the increases in the

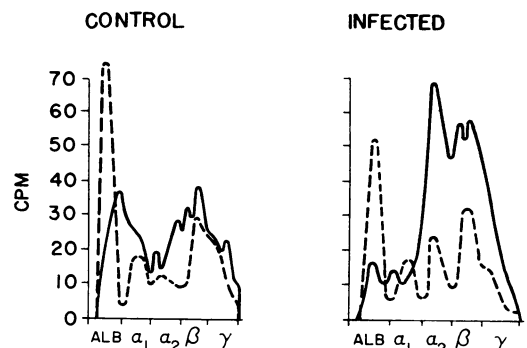


FIG. 3. Typical patterns of <sup>3</sup>H-leucine incorporation into serum proteins. Serum (50  $\mu$ liters) was applied to cellulose acetate strips. These were subjected to electrophoresis, stained with Ponceau S, and subsequently scanned for radioactivity on an Actigraph III chromatograph scanner (Nuclear-Chicago). The dashed line represents the serum protein pattern; the solid line represents leucine incorporation.

TABLE 4. *Diazotizable substances in the urine*<sup>a</sup>

Days	Total diazotizable material expressed as OD <sub>550</sub>		Diazotizable material/mg of creatinine	
	Infected	Control	Infected	Control
-1	48.9 ±3.3	56.7 ±3.9	12.67 ±0.88	14.97 ±1.24
1	56.4 <sup>c</sup> ±2.4	69.0 ±2.1	10.02 <sup>c</sup> ±0.42	13.60 ±0.60
2	33.0 ±3.3	40.5 ±7.8	6.84 <sup>b</sup> ±0.08	12.32 ±2.34

<sup>a</sup> Samples of the urine were acidified and diazotized. The values presented are the mean ± 1 standard error mean of six animals, except for day 2 which had only four rats per group.

<sup>b</sup>  $P < 0.05$ .

<sup>c</sup>  $P < 0.01$ .

TABLE 5. *Hepatic tryptophan oxygenase activity in fed rats at various times after the initiation of infection*<sup>a</sup>

Clock hr	Post-infection hr	Functional activity		Total activity	
		Control	Infected	Control	Infected
1600	8	8.06 ±2.23	7.38 ±2.21	16.59 ±3.12	18.52 ±2.97
2400	16	14.52 ±2.27	6.92 <sup>b</sup> ±1.52	24.15 ±4.28	20.49 ±4.21
0800	24	7.91 ±1.91	7.21 ±1.27	19.31 ±4.50	25.63 ±5.97

<sup>a</sup> Tryptophan oxygenase activity is expressed as  $\mu$ moles of kynurenine formed per hour per 100 g of body weight. The mean ± 1 standard error mean of five animals are shown.

<sup>b</sup>  $P < 0.25$ .

$\alpha_1$ ,  $\alpha_2$ , and  $\beta$  glycoprotein fractions when the latter are expressed as milligrams of protein-bound carbohydrate/100 ml of serum. However, only the  $\alpha_2$  and  $\beta$  fractions are shown to increase when the serum protein patterns are examined. The difference between protein and glycoprotein patterns may arise from the increased synthesis of a carbohydrate-rich  $\alpha_1$  glycoprotein representing but a small fraction of the  $\alpha_1$  serum protein population, or from the faster turnover of the  $\alpha_1$  serum proteins in the infected animal.

Both rat and man respond to infection by excreting greater than normal quantities of body nitrogen, suggesting the breakdown of proteins and the subsequent deamination of the constituent amino acids. However, contrary to what has been documented for a number of infectious illnesses in human beings (18), in this study we noted a lessened degradation of tryptophan via

the kynurenine pathway. This difference cannot be attributed to the oral administration of tryptophan in the human studies for increased excretion of tryptophan catabolites can be demonstrated during sandfly fever, even in the absence of such a tryptophan load (16). Rather, the observed decrease in the degradation of tryptophan during pneumococcal sepsis in the rat may originate in the seeming essentiality of tryptophan in regard to protein synthesis in rodents (5, 22), the marked increase in serum protein synthesis making less tryptophan available for degradation. Pneumococcal sepsis may also cause the diversion of a greater proportion of endogenous tryptophan toward serotonin formation. Such a diversion appears to occur when endointoxicated mice are given a bolus of tryptophan (12).

Rapoport et al. observed a transitory increase in hepatic TO activity in mice during pneumococcal sepsis and suggested that tryptophan metabolism via the kynurenine pathway was transiently enhanced during the first 24 hr after exposure (18). Because the present study indicates decreased tryptophan during the first 24 hr of illness, TO activity was measured at 8, 16, and 24 hr postexposure to see if this enzyme's activity was also diminished. An estimate of functional activity, the amount of enzymatic protein complexed to reduced hematin and, hence, requiring no further activation or cofactor, can be gained by assaying the enzyme in the presence of tryptophan alone. The addition of hematin gives a measure of total TO activity (6). Consistent with the decrement in urinary excretion of diazotizable substances, there was a significant reduction in functional TO activity at 16 hr postinitiation of the infection. There was, however, no change in total TO activity during this period. It should be stressed that the decrease in functional TO activity may be merely fortuitous in that, in rodents, tryptophan degradation via the kynurenine pathway appears to be controlled by substrate availability (8, 17) rather than by alterations in TO activity.

The alterations in host nitrogen metabolism noted above may, in part, be attributed to the febrile state. Certainly, increased excretion of urea nitrogen and creatinine were observed to occur in man during experimental hyperthermia (2). The augmented synthesis of serum proteins may also require the presence of functioning adrenal glands, because other proteins formed in increased amounts by the liver during infectious illness appear to do so (18, 21). But, evidence is accumulating that the anabolic and, perhaps also, the catabolic aspects of nitrogen metabolism observed during sepsis are due to the elaboration of a substance (or substances) from leukocytes. This

mediator induces depressions in serum zinc and iron (13), causes a movement of these elements into the liver (14), induces a flux of amino acids from muscle to liver (25), and elicits the synthesis of acute-phase globulins (4). Thus, the decrement in muscle protein synthesis may merely reflect the loss of amino acids from this tissue. The increased nitrogen excretion may stem from the degradation of amino acids moving to the liver, in excess of the synthetic requirements, or from the conversion of the amino acids into other intermediary metabolites. Also, whether the heightened availability of amino acids to the liver could, of itself, induce the rapid and dramatic shift in the pattern of serum protein synthesis must be considered.

Although the above study deals only with an acute, terminal illness in rats, it may have implications with regard to chronic systemic infections in man, particularly in malnourished individuals. Continued sublethal infection in such persons would tend to exaggerate the malnutrition by causing the liver to synthesize increased amounts of serum proteins at the expense of muscle mass.

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