INFECTION AND IMMUNITY, July 1974, p. 60-65

Vol. 10, No. 1
Printed in U.S.A.

Amino Acid Flux and Protein Synthesis After Exposure of Rats to Either *Diplococcus pneumoniae* or *Salmonella typhimurium*

ROBERT W. WANNEMACHER, JR., MICHAEL C. POWANDA, AND RICHARD E. DINTERMAN
U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

Received for publication 7 December 1973

At 26 h after inoculation of rats with *Diplococcus pneumoniae*, the serum concentrations of 10 and 20 individual amino acids were lower than corresponding values observed in pair-fed controls. In contrast, only 2 of 20 serum amino acids were similarly decreased in rats inoculated with *Salmonella typhimurium*. Despite these serum differences, a greater accumulation of labeled non-metabolizable amino acids occurred in the livers of rats infected with *S. typhimurium*. These data suggested a greater increase in the flux of amino acids from muscle to liver in the rats infected with *S. typhimurium* as compared to those infected with *D. pneumoniae*. A similar increase in serum protein synthesis was observed in rats infected with *D. pneumoniae* or *S. typhimurium*. However, with the latter infection, a larger percentage of the amino acids appeared to be utilized as a source of energy in addition to their role as precursors of proteins.

It has been suggested that the infection-related alterations in plasma amino acids are the result of an elevated rate of flux of amino acids from muscle to liver and a subsequent increase in a utilization for synthesis of serum protein (13, 15). It has been demonstrated further that many of the infection-related alterations in amino acid and protein metabolism are mediated by an endogenous substance or substances released by phagocytic cells (14; R. W. Wannemacher, Jr., In *Parenteral Nutrition—Rationale and Clinical Experience*, in press). Despite this apparent common mediator, there appear to be differences in host metabolism as a function of the genus of invading microorganisms. For example, the pattern of amino acid changes in serum of volunteers infected with *Salmonella typhi* (12) differs from that found in volunteers exposed to sandfly fever virus (13). Serum amino acid concentration is a result of many factors including their transport and utilization in various tissues. Accordingly, we have resorted to studying rats infected with either *Diplococcus pneumoniae* or *Salmonella typhimurium* in which non-metabolizable amino acid analogues could be used to measure amino acid transport phenomena in tissues and radiolabeled metabolizable amino acids could simultaneously be used to gain some measure of amino acid utilization for protein synthesis.

Investigation of the transport of amino acids is complicated by the fact that external cell membranes contain a number of transport sites which have preference for different families of amino acids (10). The alanine-preferring "A" transport system which carries alanine, glycine, and other neutral amino acids with small or polar side chains can best be studied by utilizing the model amino acid α-aminoisobutyric acid (AIB), which is subject to intracellular transport and concentration but is neither incorporated into proteins nor metabolized (4). Cycloleucine is a non-metabolizable amino acid analogue which is an indicator for the transport of neutral amino acids with branched chains or with aromatic rings at the so-called "L" site (1). Recently, another model amino acid, 2-aminobicylo-[2,2,1]-heptane-2-carboxylic (BHC), has been described, which is highly specific for the Na^+-independent transport of neutral amino acids with large apolar side chains. Furthermore, BHC is neither metabolized nor rapidly excreted in rats (5, 6). By use of these several analogues, it seemed possible to determine whether the difference in serum amino acids during infections was due to alterations in their rates of transport.

When measuring alterations in transport of amino acids into cells, it is important to determine whether this represents changes in the intracellular concentration of that analogue or difference in extracellular volumes in tissues of

infected rats. To achieve these measurements, it was necessary to determine intra- and extracellular volumes in various tissues.

MATERIALS AND METHODS

Animals and treatment. Fisher Dunning rats (Microbiological Associates, Walkersville, Md.), weighing 150 to 200 g, were housed in a room maintained at 25 to 26 C and lighted from 6 a.m. to 6 p.m. The rats were fed a diet of agar gel containing 18% casein (2). A rat-passed culture of *D. pneumonia* type I, strain A was maintained as previously described (15). The infectious process was initiated by subcutaneous inoculation of 0.1 ml of a saline-diluted culture containing 10^6 to 2×10^6 *D. pneumoniae*. All rats were inoculated at 8 a.m. and killed 26 h later. Infections were confirmed by elevation in body temperature and pneumococcal bacteremia. A group of six rats was studied at each time period, and an equal number of control pair-fed rats inoculated with saline was studied at equivalent time periods.

S. typhimurium MIT strain (16) was rat-passed and grown on nutrient agar slants. Before usage the organisms were grown to the early stationary phase (22 h) in tryptose broth (Difco Laboratory, Detroit, Mich.). The infectious process was initiated by intraperitoneal injection of 0.1 ml of culture containing 10^8 to 2×10^8 *S. typhimurium*; infections were confirmed by elevation in body temperature and recovery of organisms from blood, liver, and colon.

At 8 a.m. on the day before the inoculation with either infectious organism, all rats were injected subcutaneously with 1 μCi (per 100 g of body weight) of either: (i) [^{14}C]1-aminocyclopentane-1-carboxylic acid and [^{14}C]cycloleucine (6.68 mCi/mol) and [^{14}C]AIB (10.31 mCi/mmol), or (ii) DL-[^{14}C]β-2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BHC; 4.28 mCi/mmol) from New England Nuclear Corp., Boston, Mass. At 2 h before the killing, each rat was injected intraperitoneally with 10 μCi of one of the ^{3}H-labeled L-amino acids per 100 g of body weight (L-[3-^{3}H]alanine, 2.93 Ci/mmol; L-[2-^{3}H]glycine, 6.8 Ci/mmol; L-[4,5-^{3}H]leucine, 38.5 Ci/mmol) from New England Nuclear Corp., Boston, Mass. The radiochemical purity of each labeled amino acid was determined by chromatography prior to its use. All rats were killed at 10 a.m. on the day after inoculation with the microorganisms or saline.

Twenth-six hours postinfection all rats were anesthetized with 1-bromo-2-chloro-1,1,1-trifluorethane (halothane); blood was collected from the axillary fold pouch after severing the brachial artery. The rats were then killed by cervical dislocation and were perfused via the aorta with cold 0.25 M sucrose until the livers were cleared of visible blood. The entire liver and rear leg muscles were removed, weighed, and homogenized in cold, distilled water (2 to 4 volumes, respectively).

Analytical procedures. A 0.1-ml sample of blood was used to quantitate bacteremia on a blood agar plate for *D. pneumoniae* and on nutrient agar plates for *S. typhimurium;* the remainder of the blood sample was centrifuged at 1,500 $\times$ *g* for 15 min, and serum was removed. A 1-ml sample of serum or tissue homogenate was added to 25 mg of sulfosalicyclic acid, mixed, and centrifuged at 1,500 $\times$ *g* for 15 min. Supernatant fluid was removed and analyzed for ^{14}C- and ^{3}H-labeled and individual free amino acids by previously described procedures (15). The precipitate was analyzed to determine protein-bound ^{3}H content as described previously (11).

A 2-ml sample of either liver or muscle homogenate was placed in a boiling-water bath for 30 min, cooled, and centrifuged at 1,500 $\times$ *g* for 15 min, and the supernatant fluid was removed. The chloride concentrations in supernatant fluids from liver and muscle and in the serum were utilized for determination of extra- and intracellular fluid volumes by the procedure of Cheek et al. (3).

The data were analyzed for statistical significance. Group means were calculated; values for control and infected groups were compared by the *t* test.

RESULTS

In the rats inoculated with *S. typhimurium*, fever became apparent by 8 h after inoculation and was maximum by 26 h. Bacterial growth was evident in serum, liver, and colon by 8 h, became massive by 26 h, and persisted thereafter. By 24 h, food consumption was reduced to approximately 40% of preinoculation intake, and death occurred within 72 to 96 h. The course of infection in rats inoculated with the selected dose of *D. pneumoniae* was similar to that reported previously (15), with elevated body temperatures and bacteremia by 14 h and death within 60 to 78 h.

At 26 h postinoculation with *D. pneumoniae*, the serum concentrations of alanine, asparagine, glutamine, glycine, isoleucine, leucine, proline, serine, threonine, tyrosine, and total amino acid were significantly below the values observed in pair-fed controls (Table 1). In contrast, only serum lysine and tyrosine concentrations were significantly decreased in rats inoculated with *S. typhimurium*. During both experimentally induced infections, serum phenylalanine concentrations were significantly increased, which resulted in marked elevations of the phenylalanine/tyrosine ratio from 0.82 $\pm$ 0.03 in pair-fed controls to 1.56 $\pm$ 0.04 and 1.45 $\pm$ 0.03 in rats inoculated with *S. typhimurium* or *D. pneumoniae*, respectively.

By 26 h after inoculation with either *D. pneumoniae* or *S. typhimurium*, the liver and muscle concentrations of intracellular water were significantly increased, whereas concentrations of extracellular water were decreased in these tissues when compared to pair-fed controls (Fig. 1). Therefore, all subsequent calculations of concentrations of tissue amino acids

TABLE 1. *Serum individual free amino acids 26 h postinoculation of rats injected with either Diplococcus pneumoniae or Salmonella typhimurium and pair-fed controls*

Amino acid	Serum concn[a] (μmol/liter)		
	Control	*D. pneumoniae*	*S. typhimurium*
Alanine	430 ± 26	326 ± 20[b]	601 ± 32[c]
Arginine	55 ± 4	65 ± 7	54 ± 6
Asparagine	71 ± 5	50 ± 4[b]	81 ± 4
Aspartic acid	71 ± 2	66 ± 4	85 ± 9
Glutamine	498 ± 38	297 ± 25[b]	600 ± 23
Glutamic acid	185 ± 12	175 ± 11	212 ± 31
Glycine	250 ± 8	202 ± 7[b]	261 ± 10
Histidine	55 ± 5	57 ± 4	51 ± 3
Isoleucine	139 ± 5	103 ± 5[b]	150 ± 6
Leucine	196 ± 10	158 ± 5[b]	239 ± 18
Lysine	243 ± 13	203 ± 13	186 ± 12[b]
Methionine	43 ± 5	38 ± 2	42 ± 9
Ornithine	66 ± 7	90 ± 22	94 ± 19
Phenylalanine	64 ± 3	84 ± 4[c]	95 ± 1[c]
Proline	155 ± 7	126 ± 5[b]	174 ± 14
Serine	256 ± 9	209 ± 4[b]	257 ± 5
Threonine	246 ± 8	142 ± 8[b]	289 ± 13
Tyrosine	78 ± 3	54 ± 3[b]	64 ± 3[b]
Valine	140 ± 7	124 ± 6	151 ± 6

[a] The values presented are the mean ± standard error of the mean of six rats.

[b] Decrease with a significance of $P < 0.01$ when compared to pair-fed control.

[c] Increased with a significance of $P < 0.01$ when compared to pair-fed control.

were calculated on a basis of intracellular water content of liver or muscle.

The data in Table 2 illustrate the intracellular concentrations of AIB and cycloleucine in liver, serum, and muscle of rats inoculated 26 h earlier with either *D. pneumoniae* or *S. typhimurium* and their respective pair-fed controls. In control rats equilibrated with either AIB or cycloleucine, a small but definite gradient was established between serum and either liver or muscle. When rats were infected with *D. pneumoniae*, their liver concentrations of cycloleucine or AIB were twice that of control animals. In contrast, a five- to sevenfold increase in hepatic AIB or cycloleucine content was observed in animals exposed to *S. typhimurium*. Both infections induced significant decreases in the muscle concentration of these markers. Similar increases in hepatic concentration of [^{14}C]BHC were observed in rats exposed to either *D. pneumoniae* or *S. typhimurium*. To better illustrate the concentration gradients that were developed in liver and muscle, data were expressed as ratios of tissue to serum content of cycloleucine or AIB. Since AIB is cleared more rapidly by rat kidneys than is cycloleucine, the ratios in control rats were much larger. During either infection, the liver-to-serum ratio was markedly increased for both model amino acids. However, those rats infected with *S. typhimurium* showed greater increases in this ratio than rats with *D. pneumoniae* infection. Concomitant with the increase in liver gradient there was a decrease in the muscle-to-serum ratio of the infected rats, with the changes again being more marked in the rats exposed to *S. typhimurium*.

The incorporation of tritiated leucine, glycine, and alanine was significantly increased in the serum proteins of rats infected with *D. pneumoniae* or *S. typhimurium* when compared to pair-fed controls (Fig. 2). In contrast to the much greater uptake of cycloleucine and AIB that was noted in the rats inoculated with *S. typhimurium* as compared to *D. pneumoniae* (Table 1), the incorporation of leucine, glycine, and alanine into serum proteins was approximately the same in both infections.

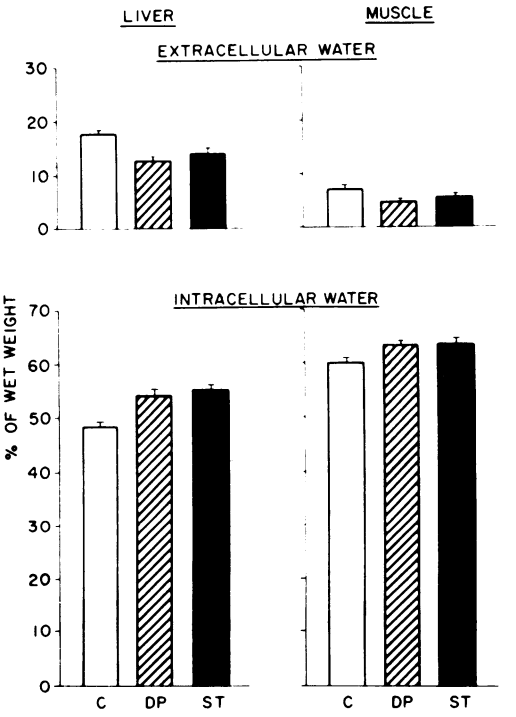

FIG. 1. *Concentration of intracellular and extracellular water in liver and skeletal muscle from rats inoculated 26 h earlier with either D. pneumoniae (DP) or S. typhimurium (ST), and pair-fed controls (C). Each value is the mean ± standard error of the mean of six rats.*

TABLE 2. *Distribution of [^{14}C]cycloleucine, AIB, or BHC in serum, liver, and muscle after exposure to either D. pneumoniae or S. typhimurium*

Treatment	Serum[a] (disintegrations/min/μliter of water)	Liver[a]		Muscle[a]	
		Disintegrations/min/μliter of water	Liver/serum	Disintegrations/min/μliter of water	Muscle/serum
Cycloleucine					
Control	26 ± 1	40 ± 5	1.6 ± 0.2	37 ± 2	1.44 ± 0.01
D. pneumoniae	24 ± 1	75 ± 6[b]	3.2 ± 0.3[b]	30 ± 2[b]	1.30 ± 0.12
S. typhimurium	21 ± 1[b]	250 ± 20[b]	12.0 ± 1.1[b]	26 ± 3[b]	1.24 ± 0.20
AIB					
Control	5.1 ± 0.4	41 ± 7	9.0 ± 1.0	23 ± 1	4.9 ± 0.2
D. pneumoniae	5.0 ± 0.3	110 ± 15[b]	22.0 ± 3.0[b]	18 ± 1	3.0 ± 0.4[b]
S. typhimurium	5.2 ± 0.4	225 ± 14[b]	46.0 ± 3.5[b]	12 ± 1[b]	2.5 ± 0.3[b]
BHC					
Control		1.2 ± 0.1			
D. pneumoniae		1.8 ± 0.1[b]			
S. typhimurium		2.7 ± 0.3[b]			

[a] The values presented are the mean ± standard error of the mean of six rats.
[b] The difference between control and infected rats had a $P < 0.01$.

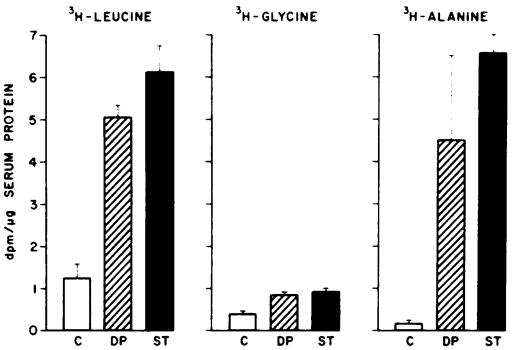

FIG. 2. *Specific activity of serum proteins after a pulse dose of either [^3H]leucine, [^3H]glycine, or [^3H]alanine in rats exposed 26 h earlier to D. pneumoniae (DP), S. typhimurium (ST), or pair-fed controls (CT). Each value is the mean ± standard error of the mean from six rats.*

DISCUSSION

In rats exposed to *D. pneumoniae*, the decrease in many of the serum amino acids is associated with a flux of amino acids from muscle to liver (15). This flux of amino acids to liver in infected rats begins before onset of fever or other overt clinical signs of the infectious disease. The concept of an infection-related stimulation of a flow of amino acids from muscle to liver was substantiated by the observations that a non-metabolizable amino acid analogue, cycloleucine, decreased in muscle while accumulating in the liver of rats exposed to *D. pneumoniae*. In contrast, however, the concentrations of most of the metabolizable free amino acids within the liver were similar to, or less than, values found in pair-fed controls. These observations, plus the observed increase in serum protein synthesis, lead to the conclusion that many of the amino acids which were rapidly entering the liver of the pneumococcus-infected rat were, in turn, being rapidly utilized for the increased rates of protein synthesis. This concept of an increased flux of amino acids to liver in infected rats could be used to explain the observed decreases in serum amino acid concentrations of volunteers exposed to *S. typhi* (9, 12), *Pasturella tularensis* (7), live attenuated Venezuelan equine encephalomyelitis vaccine virus (8), or sandfly fever virus (13).

In the present study, only serum lysine and tyrosine were depressed in rats infected with *S. typhimurium*, whereas a majority of the serum amino acids were decreased in rats exposed to *D. pneumoniae*. The data on the pneumococcal infection were essentially similar to those reported in an earlier study (15); however, the pattern of serum amino acid change is a function of the severity and duration of the infection. These parameters are related to the route and dose of inoculum and may vary slightly from one study to another. When interpreting changes in serum amino acids, it must be realized that the concentration of each amino acid in the serum represents, at any given time, the algebraic product relating rates of efflux and

influx into serum of that amino acid from various compartments of the body as well as from dietary gains or excretory losses.

Despite slight alterations in the concentration of serum amino acids, two to three times more non-metabolizable amino acids (cycloleucine, AIB, or BHC) accumulated in the liver of the animals exposed to *S. typhimurium* as compared to those inoculated with *D. pneumoniae*. Thus, an even greater flux of amino acids from muscle to liver was stimulated in the rats infected with the *S. typhimurium*. But the influx of the amino acids to serum from muscle was equal to or slightly greater than the efflux of amino acids from serum to liver, resulting in little change in the concentration of many of the serum amino acids of the *S. typhimurium*-infected rats as compared to their pair-fed controls. Because of increases in the intracellular water content of liver and muscle of rats infected with either microorganism, all calculations on the distribution of metabolizable amino acids in tissues of the infected rats were based on intracellular water concentration and related to distribution in extracellular fluid. As noted previously (15), significant infection-related increases in the hepatic concentrations of the model amino acids were also apparent when the data were calculated as concentrations per milligram of liver. The current data, however, provide conclusive evidence that alterations in tissue concentrations of the amino acid analogues are the result of change in the intracellular content and not differences in the extracellular volume.

Since *D. pneumoniae* and *S. typhimurium* can be isolated from perfused livers of the infected rats, the possibility exists that the elevated concentration of the non-metabolizable amino acid in liver is due to the uptake of these metabolites by the microorganism in liver. Quantitation of the bacteria in liver of rats infected with either *D. pneumoniae* or *S. typhimurium* is quite variable, but the data do indicate that the total bacterial mass is very small relative to the total mass of hepatic cells in liver (R. W. Wannemacher, Jr., personal communication). In addition, a number of hormones or hormone-like mediators can stimulate hepatic uptake of the model amino acids (R. W. Wannemacher, Jr.). Recently, it has been reported that sterile serum from rats infected with *D. pneumoniae* or from men with *S. typhi* will stimulate hepatic uptake of cycloleucine when injected into normal rats (12, 22). Therefore, it has been concluded that the microorganism in liver of infected rats are not responsible per se for the prominent increase in hepatic uptake of the model amino acids.

As noted earlier, the model amino acids selected for use in these studies allowed different key transport sites to be studied. Since bacterial infections stimulated significant accumulations of all model amino acids in the liver, it may be concluded that the infectious process stimulated the hepatic transport of the families of amino acids which are transferred at various L and A sites on the liver membrane, which are both energy dependent and independent. Although factors affecting amino acid losses from cells are not necessarily identical to those influencing cellular uptake (10), it may be suggested that the infection-related process influenced significant increases in the efflux of both model amino acids from skeletal muscle.

As in an earlier study on the distribution of tritiated leucine in rats during pneumococcal sepsis (15), the serum concentrations following a pulse dose of the labeled metabolizable amino acids were increased in the infected rats. Previously, this increase in synthesis of serum protein was shown to be associated exclusively with the α_1-, α_2-, and β-globulin fractions (11). Because of the very rapid course of these lethal model infections and the early collection of serum, 26 h postinoculation, it is doubtful that immunoglobulin synthesis contributed significantly to the increased protein synthesis observed. Early increases in the hepatic synthesis of serum proteins were noted in rats infected with either *S. typhimurium* or *D. pneumoniae*. Despite the fact that a two- to threefold greater flux of amino acids to liver was noted in the rats infected with *S. typhimurium* as compared to *D. pneumoniae*, little differences were observed in the rates of serum protein synthesis when either tritiated leucine, glycine, or alanine were used as precursors. In addition to being utilized for the synthesis of proteins, most of the amino acids can enter other pathways which may involve their metabolism as a source of energy, in the production of glucose, or as precursors of other essential metabolites (10). Therefore, it is possible that in rats infected with the *S. typhimurium* a greater percentage of the amino acids of the liver were entering nonprotein metabolic pathways to meet the metabolic needs of the host. These possibilities are under current investigation.

It could be argued that the increased specific activity of the serum proteins of the infected rats was the result of a decrease in the precursor free amino acid pool size of liver. However, the increased flux of amino acids, minor change in

the concentration of hepatic free amino acids (15), and unaltered or decreased specific activity of liver, serum albumin, and muscle proteins (11, 15) would tend to disprove this argument. Therefore, it can be concluded that the infectious process has a specific effect which results in an increase in synthesis of serum "acute-phase" globulins.

LITERATURE CITED

1. Akedo, H., and H. N. Christensen. 1962. Transfer of amino acids across the intestine: a new model amino acid. J. Biol. Chem. **237**:113-117.
2. Allison, J. B., R. W. Wannemacher, Jr., W. L. Banks, Jr., and W. H. Wunner. 1964. The magnitude and significance of the protein reserves in rats fed at various levels of nitrogen. J. Nutr. **84**:383-388.
3. Cheek, D. B., C. D. West, and C. C. Golden. 1957. The distribution of sodium and chloride and the extracellular fluid volume in rat. J. Clin. Invest. **36**:340-351.
4. Christensen, H. N. 1968. Relevance of transport across the plasma membrane to the interpretation of the plasma amino acid pattern, p. 40-52. *In* J. H. Leathem (ed.), Protein nutrition and free amino acid patterns. Rutgers Univ. Press, New Brunswick, N.J.
5. Christensen, H. N., and M. Cullen. 1969. Behavior in rats of a transport-specific bicyclic amino acid. Hypoglycemic action. J. Biol. Chem. **244**:1521-1526.
6. Christensen, H. N., M. E. Handlogten, I. L. Howard, S. Tager, and R. Zand. 1969. A bicyclic amino acid to improve discrimination among transport systems. J. Biol. Chem. **244**:1510-1520.
7. Feigin, R. D., and H. G. Dangerfield. 1967. Whole blood amino acid changes following respiratory-acquired *Pasteurella tularensis* infection in man. J. Infect. Dis. **117**:346-351.
8. Feigin, R. D., R. F. Jaeger, R. W. McKinney, and A. C. Alevizatos. 1967. Live, attentuated Venezuelan equine encephalomyelitis virus vaccine. II. Whole-blood amino acid and fluorescent-antibody studies following immunization. Amer. J. Trop. Med. **16**:769-777.
9. Feigin, R. D., A. S. Klainer, W. R. Beisel, and R. B. Hornick. 1968. Whole-blood amino acids in experimentally-induced typhoid fever in man. N. Engl. J. Med. **278**:293-298.
10. Munro, H. N. 1970. Free amino acid pools and their role in regulation, p. 299-380. *In* H. N. Munro (ed.), Mammalian protein metabolism, vol. 4. Academic Press Inc., New York.
11. Powanda, M. C., R. W. Wannemacher, Jr., and G. L. Cockerell. 1972. Nitrogen metabolism and protein synthesis during pneumococcal sepsis in rats. Infect. Immunity **6**:266-271.
12. Wannemacher, R. W., Jr., H. L. DuPont, R. S. Pekarek, M. C. Powanda, A. Schwartz, R. B. Hornick, and W. R. Beisel. 1972. An endogenous mediator of depression of amino acids and trace metals in serum during typhoid fever. J. Infect. Dis **126**:77-86.
13. Wannemacher, R. W., Jr., R. S. Pekarek, P. J. Bartelloni, R. T. Vollmer, and W. R. Beisel. 1972. Changes in individual plasma amino acids following experimentally-induced sandfly fever virus infection. Metabolism **21**:67-76.
14. Wannemacher, R. W., Jr., R. S. Pekarek, and W. R. Beisel. 1972. Mediator of hepatic amino acid flux in infected rats. Proc. Soc. Biol. Med. **139**:128-132.
15. Wannemacher, R. W., Jr., M. C. Powanda, R. S. Pekarek, and W. R. Beisel. 1971. Tissue amino acid flux after exposure of rats to *Diplococcus pneumoniae*. Infect. Immunity **4**:556-562.
16. Young, V. R., S. C. Chen, and P. M. Newberne. 1968. Effect of infection on skeletal muscle ribosome in rats fed adequate or low protein. J. Nutr. **94**:361-368.