Vitamin A Deficiency Increases Protein Catabolism and Induces Urea Cycle Enzymes in Rats

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Abstract
Chronic vitamin A deficiency induces a substantial delay in the rates of weight and height gain in both humans and experimental animals. This effect has been associated with an impaired nutrient metabolism and loss of body protein. Therefore, we analyzed the effect of vitamin A deficiency on endogenous proteolysis and nitrogen metabolism and its reversibility with all-trans retinoic acid (RA). Male weanling rats, housed in pairs, were pair-fed a vitamin A-deficient (VAD) or control diet until they were 60 d old. A group of deficient rats were further treated with daily intraperitoneal injections of all-trans RA for 10 d. Final body and tissue (i.e. liver and heart) weights were significantly lower and tissue:body weight ratios were similar in VAD rats and in controls. Conversely, the epididymal white fat:body weight ratio and the plasma concentrations of alanine aminotransferase and adiponectin were significantly higher in VAD rats, which also had hepatic macrovesicular lipid accumulations. Plasma and gastrocnemius muscle 3-methylhistidine, urine nitrogen, and plasma and concentrations of alanine aminotransferase and adiponectin were significantly higher in VAD rats, which also had hepatic macrovesicular lipid accumulations. Plasma and gastrocnemius muscle 3-methylhistidine, urine nitrogen, and plasma and urine urea concentrations were all significantly higher in the VAD group. The expression of the genes encoding urea cycle enzymes and their activities increased in VAD livers. These changes were partially reverted by all-trans RA. We propose that fuel partitioning in vitamin A deficiency may shift from fatty acids to protein catabolism as an energy source. Our results emphasize the importance of vitamin A on the energy balance control system and they provide an explanation for the role of vitamin A in protein turnover, development, and growth. J. Nutr. 140: 792–798, 2010.

Introduction
Vitamin A (all-trans retinol) is an essential nutrient involved in many physiological functions, including vision, immunity, cell differentiation, embryological development and growth, and it also acts as a physiological antioxidant (1). Retinol is the precursor in the body for 2 especially important metabolites, retinaldehyde and retinoic acid (RA), particularly all-trans and 9-cis RA. RA exerts its broad range of biologic effects mainly by controlling gene expression. RA binds to and activates heterodimeric nuclear receptors, which comprise 2 subfamilies, RA receptors and retinoid X receptors. Moreover, retinoid X receptors form heterodimers with other members of the nuclear receptor family, including PPAR, and vitamin D and thyroid hormone receptors (2).

Vitamin A deficiency is still a nutritional problem in the world, with important implications for global health policies. This deficiency has a wide range of undesirable effects, among which are visual problems, increased susceptibility to severe infection, and disturbances in growth (3). Since its discovery, several studies have linked vitamin A deficiency with delayed rates of weight and height gain in both humans and experimental animals (3–7). However, the underlying mechanism involved in the relationship between vitamin A status and growth has not been convincingly stated. Former studies (8) have reported that vitamin A deficiency causes a loss of appetite with a subsequent reduction in food intake and that gastrointestinal disturbances associated with this deficiency could lead to a decreased absorption of nutrients (8). These features might influence growth rate after chronically withdrawing vitamin A from the diet. However, thorough in vivo studies of body composition and nutrient absorption in rats using the paired feeding method have concluded that these factors could not justify the weight loss and growth failure observed with vitamin A deficiency. The effect of vitamin A deficiency on body weight appears to be mostly due to alterations in nutrient utilization and metabolism after absorption; moreover, difference in weight was significantly due to protein loss in deficient rats (8).
Recently, in vivo studies have established a link between retinoid status and the expression of those genes encoding several enzymes of intermediary metabolism. The results suggest that vitamin A deficiency may induce a general catabolic state that could relate with resistance to growth hormone (GH) effects (10). However, despite the interest in understanding the metabolic effects of vitamin A deficiency, particularly those concerning delays in tissue growth rate and development, the effect of this deficiency on protein metabolism has not yet been convincingly stated.

Proteolysis along with protein synthesis is a major process that contributes to body protein turnover and tissue development, and a slight decrease in synthesis or a slight increase in degradation rates, if sustained, can result in a marked loss of mass in the organism as a whole. Experimental and clinical studies have pointed out that the most important feature in the muscle protein loss in several catabolic states, including sepsis, cancer, burn injury, metabolic acidosis, diabetes, or hypothyroidism, is enhanced muscle protein degradation, especially of myofibrillar proteins (11). Under such conditions, muscle protein catabolism increases to provide the organism with amino acids for energy production, gluconeogenesis, or new protein synthesis (11), and in vivo studies have shown that increased amino acid utilization responds mostly to a decreased availability of nonprotein substrates as an energy supply (12). In this context, it is worth mentioning that retinoid status modulates fatty acid utilization in different tissues. In fact, chronic vitamin A deficiency decreases the expression of the genes involved in mitochondrial fatty acid oxidation in liver, whereas treatment with RA increases the lipid oxidation capacity in liver, skeletal muscle, and white adipose tissue (13–16). The adaptive response in vitamin A deficient (VAD) rats to a lower capacity of lipid oxidation may result in an increased utilization of proteins as an alternative energy source, leading to progressive protein loss and greater nitrogen excretion as urea, a nontoxic metabolite synthesized by the liver from amino acid catabolism.

In this work, we studied the effect of chronic vitamin A deficiency on protein catabolism and its reversibility by all-trans RA treatment. We evaluated growth, myofibrillar protein catabolism by determining 3-methylhistidine concentration in plasma and muscle, total nitrogen urinary excretion, urea production, and hepatic urea cycle enzyme activities and their mRNA. Moreover, because the liver is involved in most aspects of vitamin A homeostasis, expresses the RA-regulated transcription factors at high levels, plays a key role in lipid balance, and is a singular tissue in that it has a complete metabolic capability of detoxifying ammonia to urea, we also evaluated the histological ultrastructure of livers from control and VAD rats.

Materials and Methods

Animals and diets. All procedures using rats were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Ethics Committee for Animal Research and Welfare at the University of Valencia. VAD rats were prepared as previously described (17). Briefly, pregnant rats (Charles River) were housed in individual cages at 22°C using a 12-h light-dark cycle. On the day after delivery, dams with their litters were randomly divided into 2 groups. The first was fed a complete purified diet (AIN-93, ICN Biomedicals) following the recommendations of the AIN (18) (control group). The second was fed the same diet but devoid of vitamin A (VAD group). Feeding dams the vitamin A-free diet during the 21-d lactation shortened the interval of vitamin A depletion in pups (19). After lactation, male pups were weaned to take their dams’ diet (control or VAD diet) until they were 60 d old. To study the effect of all-trans RA, a group of 60-d-old VAD rats were treated further with 10 daily intraperitoneal injections of 100 μg of all-trans RA in 100 μL of sunflower seed oil (VAD RA-treated group) or with an equal amount of vehicle (vehicle-treated group) (20). This oil contains less vitamin traces than others (A. Catharine Ross, The Pennsylvania State University, personal communication). For growth evaluation, some rats of both the control and VAD groups were not killed and continued with their corresponding diets for 10 additional days until they were 70 d old. Food intake and body weights were determined periodically over the experimental period in all the rats. Rats were housed in pairs and pair-fed isocaloric, isonitrogenous diets, either control or VAD, with ad libitum intake for the VAD group. All rats used for the N metabolism studies were deprived for 18–24 h before the experimental assays.

Sampling procedure. Experiments were performed between 10:00 and 12:00 h. Rats were anesthetized with pentothal (50 mg/kg body weight, intraperitoneally) before being killed. Blood was collected from the aorta in heparinized syringes. Then the liver and gastrocnemius muscle were taken and processed as previously described (21). Heart, kidney, lung, and epididymal adipose tissue were then excised and weighed. For the ultrastructural studies, whole anesthetized rats were perfused with 0.5% glutaraldehyde and 4% formaldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) (20). For urine collection, rats were placed in individual metabolic cages at the end of the experimental period for 24 h, as described previously (22).

Retinoids determination. Plasma retinol and liver retinoids were extracted, dissolved in methanol:ethanol (1:1, v:v) (20), and measured using an isocratic HPLC method (23).

Ultrastuctural studies. Small fragments of rat liver were postfixed in 1.5% glutaraldehyde and 1% formaldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 60 min at 4°C, dehydrated in graded concentrations of ethanol, and embedded in Epon 812 (6 blocks per sample) (24). Ultrathin sections were examined in a Philips CM 100 transmission electron microscope.

Liver protein. Liver total protein was measured by the method of Lowry et al. (25).

Plasma biochemistry. Plasma glucose, triglycerides, cholesterol, and alanine aminotransferase activity were measured in 18- to 24-h food-deprived rats using enzymatic methods following the manufacturer’s instructions (Spinreact). Adiponectin was measured with a competitive ELISA from Linco Research according to the manufacturer’s manual.

Plasma and urine nitrogenous compounds. Creatinine, urea, and total N were measured in plasma and in 24-h urine samples, as described in Barber et al. (22).

Plasma and gastrocnemius muscle 3-methylhistidine. Methylhistidine from the plasma and muscle extracts was determined by the isocratic HPLC method of Nagasawa et al. (26). Muscle methylhistidine was extracted as described (27).

Urea cycle enzyme assays. Liver urea cycle enzyme activities, carbamoyl-phosphate synthetase I, ornithine carbamoyltransferase, argininosuccinate synthetase, argininosuccinate lyase, and arginase I were measured as described (28). One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of product/h at 37°C.

Real-time PCR. RNA extraction and real-time PCR were carried out as described previously (20). One microgram of total RNA was treated with DNase I (Roche) and was reverse transcribed to cDNA using Ready-To-Go You Prime First-Strand beads (Amersham Pharmacia Biotech). Primers for PCR were designed by the Primer3 program and synthesized by Sigma. Three were: carbamoyl-phosphate synthetase I, sense: 5′-TGGTGAACAAATGCGCCAGG-3′, antisense: 5′-GATACTGGAGCACCGCACAAC-3′; ornithine carbamoyltransferase, sense: 5′-
CCAGAGCTCAAGACGCTGAAGG-3, antisense: 5'-TAAGGATTTCCCTTGGAAATAGC-3; arginosuccinate synthetase, sense: 5'-TCTACAAGAACATGCTGG-3; arginosuccinate lyase, sense: 5'-CCGAGCTCAGAGTCGAG-3; arginase I, sense: 5'-AGTTTTGATGTTGATGGACTGGA-3, antisense: 5'-CAGGATGGTGGTGATGGACATTA-3; argininosuccinate lyase, sense: 5'-CAGTTCACCACCCATCAGATAG-3, antisense: 5'-ATTCAGTGAGCAGAGTATGAGCAG-3; argininosuccinate synthetase, sense: 5'-TGAGCATCGAGTGACCGAGAA-3, antisense: 5'-TCAAGAGTTGGGTTCACTTCC-3; b2-microglobulin, sense: 5'-CAGTTCACCACCCATCAGATAG-3, antisense: 5'-ATTCAGTGAGCAGAGTATGAGCAG-3. The PCR was done using the SYBR Green PCR Master Mix and the AB 7900HT Fast Real-Time PCR system (Applied Biosystems). Standard curves were plotted with quantified cDNA templates for each gene and the absolute number of copies for mRNA in samples was determined by the cycle threshold method. Samples were tested in triplicate. The amounts of mRNA were normalized to the mRNA of 2-microglobulin. Product purity was confirmed by agarose gel electrophoresis and by melt-curve analysis.

Statistics. Data are presented as means ± SEM. Control and VAD groups were compared using Student’s t tests. When the effect of treatment with RA was assessed, the data were analyzed by 1-way ANOVA followed by Tukey’s multiple comparison test. Differences were considered significant at P < 0.05. All analyses were conducted using GraphPad Prism software.

Results

Retinoid concentrations in plasma and liver. The rats fed a vitamin A-free diet from birth (through the dam’s milk) until reaching the age of 60 d had plasma retinol concentrations that were 5% of the control value (Table 1). The VAD rats treated with all-trans RA had almost negligible plasma retinol and liver retinyl palmitate concentrations (Table 1). The results obtained with the vehicle-treated group did not significantly differ from the VAD group, and, for simplicity, they are not shown.

Body and tissue weights. VAD rats grew more slowly than those fed the control diet and their body weight was lower at the end of the study (Fig. 1A). We detected growth retardation in VAD rats by the age of 56 d (wk 8) despite equivalent food consumption. Differences in body weight were significant by wk 9, by which time VAD rats had reached a weight plateau.

At the end of the experiments (d 60), VAD rats were smaller, but their liver weight was also significantly lower than control rats (Table 2); accordingly, the liver weight as a percentage of the total body weight (liver:body weight ratio) was similar in both groups (2.8% of body weight). The liver protein content was also similar in both the control and VAD rats (Table 2). The absolute and relative heart weights of both groups followed the same pattern as that in the liver (heart weight: 0.89 ± 0.04 g in control rats, 0.75 ± 0.02 g in VAD rats, P < 0.05; heart:body weight ratio: 0.35% in both the control and VAD groups); lung and kidney weights did not significantly differ between groups and, for simplicity, they are not shown. Interestingly, we found a greater accumulation of white fat, as the epididymal fat pad:body weight ratio was significantly higher in VAD rats than in control rats (Fig. 1B) despite the similar food intake of both groups.

Liver ultrastructural studies. Because the liver plays a key role in lipid homeostasis, we examined representative sections of the livers from both the control and VAD rats (Fig. 1C). Electron microscopic studies showed numerous lipid vacuoles in the hepatocytes of VAD rats, which were very scarce in control rats. In a previous study (29), we also noticed vacuole formation in VAD livers. Such vacuolization is often considered an index of steatosis, which has also been described by other authors in humans and rats with decreased hepatic vitamin A content (15,30).

Plasma biochemistry. The lower body and tissue weights and the greater fat accumulation in VAD rats (Table 2; Fig. 1B,C), despite similar food intake, suggested a shift in total energy

### Table 1

<table>
<thead>
<tr>
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<th>Control</th>
<th>VAD</th>
<th>VAD + RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma all-trans-retinol, µmol/L</td>
<td>1.69 ± 0.01 (8)</td>
<td>0.09 ± 0.01 (8)</td>
<td>0.05 ± 0.01 (4)</td>
</tr>
<tr>
<td>Liver all-trans-retinyl palmitate, nmol/g</td>
<td>121 ± 21 (8)</td>
<td>9 ± 4 (6)</td>
<td>6 ± 2 (4)</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (n). Means in a row without a common letter differ, P < 0.05.
Alanine aminotransferase, total cholesterol, triglycerides, mmol/L.

Control group, except for ornithine carbamoyltransferase, which activities in the VAD group were significantly higher than in the control group. Liver urea cycle enzyme activities.

TIONS (Fig. 2). Liver creatinine excretion were similar in the 2 groups. Consequently, the concentration of creatinine in plasma and the 24-h urine excretion, the major N compound found in the urine of rats. The difference in urinary N excretion can account for an increase in urea function, which agrees with a greater removal of urea, the end-product of amino acid catabolism. These data indicate that the metabolic response to vitamin A deficiency implies a catabolic phase that is preceded by the increased production of 3-methylhistidine, suggesting that protein catabolism may be an important factor in the growth retardation induced by vitamin A deficiency.

Other authors have proposed that vitamin A deficiency may induce a catabolic state associated with GH resistance and the decreased expression of insulin-like growth factor 1 (IGF-1) (10,33). Although the effect of RA on pituitary GH synthesis in vivo has been controversial (34), several studies have also established a close linkage between the secretion of GH, the principal hormone stimulus for IGF-1 and vitamin A status in increased proteolysis in vitamin A deficiency.

### TABLE 2 Body and liver weights, liver protein concentration, and plasma biochemistry of rats fed control or VAD diets for 60 d

<table>
<thead>
<tr>
<th>Control</th>
<th>VAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight, g</td>
<td>273.1 ± 6.8 (10)</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>8.2 ± 0.3 (10)</td>
</tr>
<tr>
<td>Liver proteins, mg/g</td>
<td>215 ± 12 (8)</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>7.1 ± 0.3 (10)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.70 ± 0.05 (5)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>1.73 ± 0.15 (5)</td>
</tr>
<tr>
<td>Alanine aminotransferase, U/L</td>
<td>12.7 ± 1.8 (8)</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (n). Means in a row without a common letter differ, P < 0.05.
2 One unit of alanine aminotransferase activity is defined as the amount of enzyme that catalyzes the transformation of 1 μmol of substrate/minute at room temperature.

### TABLE 3 Concentrations of major N compounds in plasma and urine of rats fed control or VAD diets for 60 d

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Control</th>
<th>VAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea, mmol/L</td>
<td>6.7 ± 0.5 (10)</td>
<td>9.6 ± 0.7 (10)*</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>141.4 ± 8.8 (10)</td>
<td>132.6 ± 8.8 (16)</td>
</tr>
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<table>
<thead>
<tr>
<th>Urine</th>
<th>Control</th>
<th>VAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total N, mg/24 h</td>
<td>196.0 ± 11.2 (4)</td>
<td>330.3 ± 40.2 (4)*</td>
</tr>
<tr>
<td>Urea, mg/24 h</td>
<td>5.4 ± 0.7 (7)</td>
<td>8.4 ± 0.5 (7)*</td>
</tr>
<tr>
<td>Creatinine, mg/24 h</td>
<td>0.010 ± 0.002 (8)</td>
<td>0.010 ± 0.002 (8)</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (n). Means in a row without a common letter differ, P < 0.05.
vivo and in vitro (35–37). GH levels are associated with growth, body weight and size, positive nitrogen balance, muscular protein synthesis, gluconeogenesis, lipolysis, and decreased deposition of fat, and these effects depend on IGF-1 secretion, which is delivered mostly by the liver (38). As mentioned above, the expression of hepatic IGF-1 has been reported to be depressed with vitamin A deficiency, and this may explain at least in part the impaired growth and fat accumulation in the VAD group. Moreover, IGF-1 has additional specific actions, such as inhibition of muscular proteolysis and stimulation of glucose uptake by different tissues (39). In fact, it has been reported that IGF-1 blocks muscle proteolysis, an effect that could be an important factor in growth stimulation by IGF-1 (39). Thus, the endocrine status induced by vitamin A deficiency (10,33) and the role ascribed to IGF-1 agree with the significantly greater muscle proteolysis that we found in retinol-depleted rats and suggest a possible mechanism for protein loss and delayed growth in retinol deficiency.

Our work shows that vitamin A deficiency induces a coordinated change of the urea cycle enzymes despite being located in different cellular compartments, which reflects quite closely that of the change in the urinary N output. In agreement with this finding, recent studies using Affymetrix oligonucleotide microarrays have observed that retinol deficiency induces the expression of the urea cycle enzymes in the depleted liver (10). Concerted changes in N excretion and in all urea cycle enzymes have been described under different physiological and pathological conditions (22,40,41). Specifically, the increased amino acid availability induced by high-protein diets leads to an adaptive increase in amino acid utilization, N and urea excretion, and urea cycle enzyme activities (40,41). However, we found higher N excretion and induction of the urea cycle enzyme in rats that had the same N intake as controls, thus supporting the idea of a greater availability of amino acids in the deficient group as a result of the enhanced catabolism of the endogenous proteins.

After RA treatment, protein catabolism decreased to the levels in control rats. Conversely, the urea cycle enzyme activities remained elevated, which is in agreement with their long half-lives (40). Although not to control levels, RA diminished significantly (P < 0.02) and tended to diminish (P = 0.1) the mRNA for argininosuccinate synthetase and carbamoyl-phosphate synthetase, respectively. Some authors have found an upregulation of the mitochondrial urea cycle enzymes induced by mild oxidative stress (42) and we have shown in a recent report that RA treatment increases the oxidative stress generated by VAD (17,20). Whether this oxidative status is responsible for the elevated urea cycle enzyme mRNA remains to be clarified.

Additionally, fat accumulation and a histologically apparent fatty liver with hepatocytes revealing large lipid droplets were evident in the VAD group. Consistent with these data, several studies have shown that retinoid signaling plays an important role in adipocytes and energy balance. At the molecular level, a
cross-talk is well established between retinoid signaling and PPAR, which are the nuclear receptors that play a central role in the regulation of energy and lipid metabolism (43).

RA triggers an upregulation, and vitamin A deficiency a downregulation, of the PPARα gene in liver and white adipose tissue, which may be a key event in the regulation of fatty acid oxidative metabolism by retinoid status in different tissues (15,44). In this sense, the functional loss of RA in transgenic mice expressing the RA receptor α-dominant negative form in hepatocytes developed both massive hepatic steatohepatitis, as a result of impaired mitochondrial fatty acid oxidation, and liver tumor (13). RA treatment also induced a dose-dependent increase in the skeletal muscle mRNA expression levels of PPARγ in vivo, which was associated with not only an increased lipid oxidative capacity in this tissue but also decreased muscle lipid content (16). Moreover, retinaldehyde and/or RA antagonized PPAR-γ activity in vivo producing a loss of body adipose tissue with changes including a reduction of adipocyte size, triacylglycerol content, and lipogenic capacity (44,45). Transgenic mice that did not express retinaldehyde dehydrogenase, which consequently have elevated retinaldehyde concentrations, had decreases in subcutaneous and visceral adipose tissue, liver lipid deposition, and plasma adiponectin (45). In agreement with these data, our results show that loss of retinoid signaling in vitamin A deficiency induces the opposite effect, i.e. greater relative epididymal-adipose tissue and liver lipid deposition and higher concentrations of alanine aminotransferase and adiponectin in plasma. Plasma elevations of alanine aminotransferase have also been described in several models of hepatic steatosis, including those induced by an altered retinoid status (13,46).

The increased expression and release of the PPARγ target gene adipocytokine adiponectin (also known as apM1, adipQ, Gbp28, and Acrp30) and enhanced triglyceride accumulation are specific and distinct indicators of adipogenesis (45). Therefore, the fact that vitamin A deficiency induces an increase in fat depots with enlarged hepatic lipid droplets and a rise in adiponectin plasma concentrations suggests that vitamin A deficiency induces adipogenesis.

In conclusion, our work shows that chronic dietary vitamin A deficiency, without changing the intake of dietary protein, increases protein catabolism and raises the gene transcription rate and the corresponding activities of hepatic urea cycle enzymes. The elevation of both the muscle protein catabolic response and urinary nitrogen excretion provides insight into the origin of protein losses following vitamin A deficiency. This elevated protein catabolism was accompanied by higher fat mass and lower body and tissue weight in VAD rats than in control rats. Thus, in the absence of vitamin A, fuel partitioning may shift from fatty acids to protein catabolism as an alternative energy source, leading to progressive protein loss. RA partially reversed the changes observed in VAD rats. The integration of all these results could contribute to an understanding of the complex interaction between vitamin A status and the transcriptional regulation of metabolism.

The accelerated protein breakdown, increased N elimination, and abnormal deposition of triacylglycerols in tissues may contribute to disease development in chronic vitamin A deficiency. Moreover, we have previously reported that vitamin A deficiency produces hepatic oxidative stress (17), which may play an important role in the initiation and/or progression of hepatic lesion (13), and also that it results in profound alterations in the basement membrane of different tissues (20,24). All these could be contributing factors to tissue malfunction in vitamin A deficiency.

Acknowledgments
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Literature Cited

Increased proteolysis in vitamin A deficiency


