Subtoxic Hepatic Vitamin A Concentrations in Captive Rhesus Monkeys (Macaca mulatta)\textsuperscript{1,2}

Kristina L. Penniston and Sherry A. Tanumihardjo\textsuperscript{3}

Integrated Graduate Program in Nutritional Sciences, University of Wisconsin-Madison, Madison, WI 53706

ABSTRACT Although the rhesus monkey (Macaca mulatta) is a widely used experimental animal, its exact vitamin A requirement is unknown. An amount of 430-3600 IU/d [129-1080 retinol equivalents (RE)] is recommended, largely on the basis of depletion studies. Normal hepatic vitamin A appears to be 1 μmol/g liver. Our goal was to determine hepatic vitamin A concentrations of captive monkeys. Liver autopsy samples from rhesus and marmoset (Callithrix jacchus) monkeys were obtained from the Wisconsin Regional Primate Research Center. The rhesus monkeys consumed a diet with 40 μ (12 RE) retinyl acetate/g. Male and female monkeys consumed an estimated 250 and 175 g diet/d, respectively. Marmosets were fed a powder-based diet consisting of 20 μ (6 RE) retinyl acetate/g. The marmosets consumed an estimated 25 g of the diet/d. Liver samples were extracted and analyzed by HPLC. The vitamin A concentration of the rhesus monkey livers was very high at 17.0 ± 6.3 μmol/g liver. The hepatic vitamin A of the marmosets was 1.25 ± 0.58 μmol/g liver. Histologic examination of the livers revealed Ito cell hypertrophy and hyperplasia in the rhesus monkeys compared with the marmosets. Considering that the natural diet of the rhesus monkey (fruits, seeds, roots and insects) is not high in preformed vitamin A, the vitamin A content of the diet appears excessive, supplying four times the NRC recommendation and resulting in high liver stores. J. Nutr. 131: 2904–2909, 2001.

KEY WORDS: • rhesus monkeys • marmoset monkeys • vitamin A intake • liver storage • subtoxicity • vitamin A status

The rhesus monkey (Macaca mulatta) is a widely used experimental animal. Detailed knowledge of its specific nutritional needs is important to ensure that data obtained are not confounded by unintended nutrient deficiency or excess (1). However, the systematic study of the nutrient requirements of animals, particularly of vitamins, has largely passed, predating the extensive use of nonhuman primates for clinical investigation (2). Thus, estimated nutrient requirements for nonhuman primate species are based largely on the experimental production of deficiencies and on nutrient levels that produce adequate health, growth and reproduction (1,3). Additionally, information has been obtained from results of nutrition studies whose primary design was not necessarily to establish nutrient requirements (1). Extensive extrapolation from the nutritional needs of other species, including humans, has also contributed to the estimation of nutrient requirements for nonhuman primates. In formulating the diets for nonhuman primates held in captivity, general practice appears to be to provide some excess of most nutrients to allow for losses during manufacturing and storage (3,4).

The vitamin A requirement for the rhesus monkey has not been adequately determined experimentally (1,2,4–6). Although vitamin A depletion in the rhesus monkey has been studied (7–11), especially regarding ocular and reproductive health, the effects of toxicity other than its teratogenicity and of subtoxicity via chronic ingestion of high physiologic doses have been studied by few investigators. In light of the extensive use of the monkey in research, the recent decrease in dietary vitamin A recommendations for humans (12) and the observation that vitamin A requirements appear to vary with the stages of the life cycle (1), it is of considerable interest to examine the vitamin A status of monkeys used in biomedical research.

Dietary vitamin A is obtained from animal products as preformed retinol (retinyl esters) or as provitamin A carotenoids (e.g., b-carotene, α-carotene, b-lycopene, (14). Retinyl esters are hydrolyzed to retinol in the intestinal lumen. Retinol is absorbed in the intestine, bound to a specific cellular retinol-binding protein (CRBP II)\textsuperscript{4}, and esterified to retinyl esters. The retinyl esters are transported by chylomicrons through the lymph and then as chylomicron remnants to the liver (13). Once in the liver, retinyl esters are hydrolyzed into retinol and either bound to retinol binding protein (RBP) or reesterified for storage, preferentially as palmitate (14). The retinyl esters


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\textsuperscript{3}To whom correspondence should be addressed.

E-mail: sherry@nutrisci.wisc.edu.

\textsuperscript{4}Abbreviations used: CRBP II, cellular retinol-binding protein; RBP, retinol binding protein; RE, retinol equivalents; WRPRC, Wisconsin Regional Primate Research Center.

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TABLE 1

Anthropometric data for rhesus and marmoset monkeys

<table>
<thead>
<tr>
<th>Age</th>
<th>Total body weight kg</th>
<th>Liver weight g/100 g body</th>
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</thead>
<tbody>
<tr>
<td>Rhesus</td>
<td>14.1 ± 10.1</td>
<td>8.3 ± 2.4</td>
</tr>
<tr>
<td>Marmoset</td>
<td>5.0 ± 3.9</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>

P-value | <0.0001 | <0.0001 | <0.025 | <0.0001 |

1 Values are means ± sd, n = 10.

in these stores remain there unless needed to maintain vitamin A balance, in which case they are again hydrolyzed to retinol, bound to RBP and transported in the bloodstream to the tissues. Carotenoids are cleaved in the intestine to retinal, reduced to retinol and then converted to retinyl esters for incorporation into chylomicrons. In humans, the absorption rate of the preformed vitamin A is much higher than that of carotenoids, 70–90% vs. 20–50% (13).

Vitamin A is a necessary nutrient for the rhesus monkey and is efficiently obtained by cleavage of β-carotene (15) or by ingestion of preformed vitamin A. The term vitamin is essential for vision, growth and cellular differentiation (16). As in humans, vitamin A status in monkeys appears to be influenced by many factors, including fat composition of the diet, protein-energy deficiency, physical activity, stress, infection and parasites (1). The concentration and source of micronutrients in the diet also appear to influence vitamin A status, e.g., carotenoids (16), zinc (17) and vitamin E (18). Vitamin A status most reliably corresponds to the size of the liver stores and not to plasma levels because plasma vitamin A concentration does not begin to decrease until liver reserves have been depleted (19), which is usually <0.07 μmol/g in humans (13). Excess vitamin A, that which is not required for immediate use by the tissues, is stored in the liver, mainly in the Ito cells (also known as stellate cells, lipocytes, lipid- and vitamin A-storing cells) as retinyl esters of fatty acids (14,19,20). Hypervitaminosis A is accompanied by appetite loss, nausea and vomiting, weakness, dry itchy skin, alopecia, bone thickening, joint stiffness, enlarged liver and spleen, and hepatocellular damage (15,16). Liver reserves > 1.05 μmol/g (300 μg/g) are considered excessive (21). In human adults, chronic intakes > 30,000 retinol equivalents (RE) (100,000 IU) for ≥6 mo can result in symptoms of hypervitaminosis A but individuals vary, with hypervitaminosis symptoms occurring in some with lower intakes (22,23). Hepatic vitamin A concentration is the most accurate means by which to assess whole-body vitamin A status; however, an exhaustive review of the literature revealed only one study in which the hepatic vitamin A concentration of normal (i.e., control) rhesus monkeys was determined. In their study of vitamin A deficiency and reproduction, O’Toole et al. (7) analyzed the livers of two rhesus monkeys that were not fed a vitamin A–deficient diet and found hepatic vitamin A concentrations of 1.08 and 1.07 μmol/g, respectively.

The rhesus monkey is naturally frugivorous (3), subsisting in the wild mainly on fruits, seeds, roots, leaves, insects and grubs (4,24). Nonhuman primates can dramatically modify their nutritional intake to such environmental influences as seasonal availability and competition from other mammals. Nevertheless, it would appear that the rhesus monkey naturally derives much of its vitamin A from plant matter, and thus, from carotenoids. The fact that the rhesus monkey is not a good carotene absorber (15) but is very efficient at cleaving β-carotene lends weight to this conclusion. Most commercial primate diet manufacturers, however, provide the majority of their vitamin A as the preformed vitamin (e.g., as retinyl acetate) and provide 20–40 IU/g of dry food. Our goal was to determine the vitamin A status of monkeys held in captivity and fed these diets.

MATERIALS AND METHODS

Animals and diet. Fresh frozen liver autopsies samples from rhesus monkeys (n = 10; 6 male and 4 female; 14.1 ± 10.1 g, range 3.5 to 28.2 g) and from marmoset monkeys (n = 10; 3 male and 7 female; 5.0 ± 3.9 g, range 1.2 to 12.6 g) were obtained from the Wisconsin Regional Primate Research Center (WRPRC). Refer to Table 1 for anthropometric data for the monkeys. Research and animal care at the WRPRC are regulated by University committees and national agencies to ensure compliance with the Animal Welfare Act. The WRPRC is fully accredited by the American Association for the Accreditation of Laboratory Animal Care-International. Rhesus monkeys were fed Lab Diet #5038 (Purina Mills, St. Louis, MO) consisting of 40 IU vitamin A (as retinyl acetate) per gram dry food and 2.5 μg/g carotene (Table 2). According to primate center staff, males and females consumed an estimated 250 and 175 g of the diet/d, accounting for a daily preformed vitamin A intake of 10,000 and 7000 IU for males and females, respectively. Marmoset monkeys were fed Mazuri callitrichid high fiber diet #5M16 (Purina Mills) consisting of 20 IU vitamin A/g, also as retinyl acetate (Table 3). According to staff, the marmosets consumed an estimated 25 g of the diet/d, for a daily preformed vitamin A intake of 500 IU.

Determination of retinyl esters. Rhesus and marmoset liver (100 and 200 mg portions, respectively) were ground with mortar and pestle and dried with sodium sulfate. Purified retinyl acetate (0.5 μl) was added to calculate extraction efficiency. The material was extracted exhaustively and brought to volume (50 mL) with dichloromethane/ethylmethane. Aliquots (0.100 mL) were dried down with argon gas and reconstituted with an equal volume of 75:25 methanol/dichloromethane.
TABLE 3

Composition of Mazuri callitrichid high fiber diet #5M16 (Purina Mills)\(^1\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g</td>
</tr>
<tr>
<td>Protein2</td>
<td>22.4</td>
</tr>
<tr>
<td>Fat3</td>
<td>5.7</td>
</tr>
<tr>
<td>Fiber (crude)4</td>
<td>5.1</td>
</tr>
<tr>
<td>Nitrogen-free extract (by difference)(^5)</td>
<td>58.1</td>
</tr>
<tr>
<td>Vitamins(^6)</td>
<td></td>
</tr>
<tr>
<td>Minerals(^7)</td>
<td></td>
</tr>
</tbody>
</table>

1 The nutrient composition is of the dry powder before reconstituting with water.
2 Provided primarily as soybean meal, casein, corn gluten meal, gelatin, dried whole whey, dried whey, dehydrated alfalfa meal and fish meal.
3 Provided primarily as soybean oil and animal fat preserved with butylated hydroxyanisole.
4 Provided primarily as ground beef pulp, ground whole aspen, solka-floc (nonnutritive fiber) and dehydrated alfalfa meal.
5 Provided primarily as glucose, corn flour, wheat middlings, fructose and sucrose.
6 Vitamins, as mg/kg: vitamin K as menadione dimethylpyrimidinol bisulfite, 2.7 (menadione); thiamin hydrochloride, 17; riboflavin, 17; niacin, 98; pantothenic acid, 62; choline chloride, 1500; folic acid, 23; pyridoxine, 17; biotin, 0.45; ascorbic acid, 610; vitamin A as retinyl acetate, 6 (retinol equivalents); vitamin D-3 as cholecalciferol, 0.095; vitamin E as d-a-tocopheryl acetate, 250 (a-tocopherol equivalents); vitamin B-12, 0.085.
7 Minerals, as mg/kg: calcium, 12,300; phosphorus, 6300; phosphorus (nonphytic), 5200; potassium, 6500; magnesium, 1500; sodium, 3200; chlorine, 4200; sulfur, 1400; iron, 185; zinc, 163; manganese, 130; copper, 27; iodine, 2.4; selenium, 0.28; cobalt, 0.69.

RESULTS

The total vitamin A concentration of the rhesus monkey livers, represented by retinyl esters, was 17.0 ± 6.3 μmol/g liver compared with 1.25 ± 0.58 μmol/g liver for the marmosets (P < 0.0001). The predominant retinyl ester in both monkeys was retinyl palmitate, accounting for 52.2 ± 2.9% of the total in the rhesus monkeys and 63.7 ± 6.4% of the total in the marmosets (P < 0.0001). Retinyl oleate, retinyl stearate and retinyl myristate plus retinyl palmitoleate (separation of the myristate and palmitoleate fractions was not achieved) accounted for the majority of the remainder (see Table 4 for full ester profile). Retinol was detectable in only 1 of the 10 rhesus monkeys and represented 0.2% of total hepatic vitamin A in that monkey. Retinol was also detected in only one of the marmosets and represented 1.3% of total hepatic vitamin A in that monkey. The HPLC system limit of detection for retinol was 0.035 μmol/g because the system was not optimized for detection of relatively small amounts of retinol compared with the substantial retinyl esters present.

Figure 2 illustrates the Ito cell hypertrophy and hyperplasia of the rhesus monkey livers compared with the marmoset monkey livers. Although irregular, the livers showed no fibrosis or overt pathology as confirmed by the pathologist.

Hepatic vitamin A concentration was not correlated with age in this limited sample (P > 0.5). A weak yet nonsignificant inverse relationship existed between hepatic vitamin A concentration and liver weight (P > 0.2).

DISCUSSION

The rhesus monkey livers had very high concentrations of retinyl esters, with a mean of 17.0 ± 6.3 μmol/g (range, 7.2–31; median, 17 μmol/g) or ~16 times more than expected.
TABLE 4

Hepatic retinyl ester concentration of rhesus and marmoset livers

<table>
<thead>
<tr>
<th></th>
<th>Rhesus</th>
<th>Marmoset</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/100 g total esters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0 + 16:1</td>
<td>12.0 ± 3.8</td>
<td>10.1 ± 3.2</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>15:0</td>
<td>0.60 ± 1.2</td>
<td>None detected</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>16:0</td>
<td>52.2 ± 2.9</td>
<td>63.7 ± 6.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>17:0</td>
<td>1.5 ± 0.2</td>
<td>0.10 ± 0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>18:0</td>
<td>15.0 ± 7.0</td>
<td>8.7 ± 2.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>18:1</td>
<td>18.6 ± 2.5</td>
<td>17.2 ± 3.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Vitamin A, μmol/g</td>
<td>17.0 ± 6.3</td>
<td>1.25 ± 0.58</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 10.
2 Retinyl esters: 14:0, retinyl myristate; 16:1, retinyl palmitoleate; 16:0, retinyl palmitate; 17:0, retinyl heptadecanole; 18:0, retinyl stearate; 18:1, retinyl oleate.

on the basis of O'Toole's earlier characterization of normal rhesus monkeys (7). The retinyl ester concentration of the marmoset livers was closer to, yet higher than our expectations at 1.25 ± 0.58 μmol/g (range, 0.44–2.4; median, 1.2 μmol/g). The rhesus monkey diet provided a greater amount of vitamin A than the marmoset diet (see Tables 2 and 3). Specifically, the rhesus monkey diet provided 40 IU/g of food. According to primate center staff, male and female rhesus monkeys consumed an estimated 250 and 175 g diet/d for a daily preformed vitamin A intake of 10,000 and 7000 IU (3000 and 2100 RE), respectively, and an average of 1200 IU (360 RE)/(kg body · d) for the males and 840 IU (250 RE)/(kg body · d) for the females. The marmoset diet provided 20 IU/g of dry powder or half the vitamin A of the rhesus diet. The marmosets, whose daily food intake was an estimated 25 g of dry powder, obtained 500 IU (150 RE) of vitamin A/d for a mean of 1600 IU (480 RE)/(kg body · d) for the males and 1500 IU (450 RE)/(kg body · d) for the females. Although the marmosets actually consumed more vitamin A/kg body than did the rhesus monkeys, their hepatic vitamin A concentration was 93% less. This is explained in part by the marmosets having nearly double the liver weight of the rhesus monkeys when expressed as a percentage of total body weight (Table 1). Perhaps the vitamin A needs are higher than those of rhesus monkeys when expressed on a body weight basis. The marmoset diet also contains a higher fiber content, which may affect the bioavailability of the vitamin A from the diet. Based upon the 2001 dietary recommendations for adult humans (12), the upper limit of what is considered safe for human intake has been achieved in these rhesus monkeys, i.e., 3000 RE. Considering that the mean body weight of the rhesus monkeys in this study is 8–9 times less than that of the mean healthy human (8.2 vs. 70 kg), this study supports the gradual decrease of vitamin A in the diet and continued monitoring of rhesus monkey vitamin A status.

Bendich and Langseth (23) noted that the livers of various carnivore species may contain > 3.4 μmol/g vitamin A and those of herbivores (e.g., chickens, cows, lambs) appear to average 0.17 μmol/g. Schweigert (24) reported similar hepatic vitamin A concentrations for carnivores and herbivores of 0.35–10.5 μmol/g and 0.17–1.4 μmol/g, respectively. In the wild, the rhesus monkey is neither a strict herbivore nor a carnivore. Its diet is based largely on fruits, seeds, roots, leaves, insects and grubs. Therefore, the hepatic vitamin A concentration of a rhesus monkey in its natural environment could be expected to be closer to that reported for herbivores. However, the rhesus monkeys we analyzed had even higher levels of hepatic vitamin A than most of the carnivorous arctic mammals whose vitamin A concentration is known [Table 5 (23–27)].

The physiology of vitamin A storage in the liver has been well characterized. Much (50–80%) of the total vitamin A (retinol plus retinyl esters) in mammals is stored in the liver (28), mainly in the stellate cells (also known as Ito cells). Stellate cells are specialized lipid-storing cells that also synthesize collagen. With administration of excess vitamin A, the lipid droplets in the stellate cells increase in size (29). Analysis of the lipid droplets in rats by Moriwaki et al. (30) indicates that the major component is retinyl esters. The volume of the lipid droplet can be correlated with the quantity of vitamin A stored in the stellate cell (29). Animals with high vitamin A intakes will thus have a large quantity of vitamin A in stellate cells. Other components of the lipid droplets are triacylglycerol, unesterified retinol, cholesterol esters, cholesterol, free fatty acids and phospholipids, but these appear to be less than half the total in animals with normal vitamin A stores (30).

As the stellate cells enlarge with greater lipid and retinyl ester concentration, there is less room for organelles within the cell and compression of nearby cells (31). It is unclear whether there are pathological implications of this compression, at least at subtoxic levels of vitamin A. Zou et al. (32) hypothesized that under a nonpathological (i.e., subtoxic) condition the enlargement of stellate cells due to high vitamin A stores may protect the liver from fibrogenetic insults by preventing the development of the intracellular organelles that synthesize collagen. This would explain the absence of fibrotic pathology in the rhesus monkey livers we analyzed. We therefore conclude that although the vitamin A liver stores of the rhesus monkeys were high, placing them in a subtoxic range, their livers showed no fibrosis and no overt pathology. It appears that the natural physiologic defenses against vitamin A toxicity, including the conversion of dietary vitamin A to retinyl esters, the significant storage capacity of the liver and the controlled release of retinol from the liver (33), were sufficient in these monkeys to prevent observable hepatic symptoms of hypervitaminosis A.

Recent research in humans and rats suggests that vitamin A needs may change with age. Specifically, the elderly may have a decreased need for vitamin A due to higher circulating levels. It is unclear whether this is due to increased absorption of vitamin A (34) or decreased clearance of vitamin A from chylomicrons (35,36). Epidemiologic studies have linked excess vitamin A in the elderly with a high incidence of osteoporosis in northern Europe (36). In light of this and other data, the Dietary Reference Intakes for humans for vitamin A were recently lowered to 900 and 700 RE for men and women, respectively (12). Given that monkeys in experimental conditions may live longer than 30 y and that they are used in a variety of research initiatives involving multiple human disease conditions, investigation into their vitamin A needs throughout the life cycle is immediately necessary.

In addition to providing a source for the preformed vitamin A, carotenoids appear to provide other health benefits that are increasingly appreciated. It is widely accepted that β-carotene and other carotenoids are potent antioxidants. Cell culture and human nutrition studies have shown that β-carotene offers protection against some types of cancer and cardiovascular disease and that it stimulates the immune system (13,37).

Studies among nonhuman primates suggest that diets high in carotenoids may be important not only for protection against cancer but for longevity as well (38). To maintain healthy
of carotenoids. The subtoxic-toxic hepatic concentrations of vitamin A in the rhesus monkeys we analyzed shows that the diet contains too much preformed vitamin A, substantially more than is required to meet their physiologic needs. A review of the dietary formulations fed to captive monkeys with regard to vitamin A is immediately warranted with a gradual decrease of vitamin A content and continued monitoring of the vitamin A status of the captive monkeys.

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LITERATURE CITED


