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Effect of excessive vitamin A intake on muscle protein turnover in the rat

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3-Methylhistidine excretion *in vivo* and *in vitro* was monitored in hypervitaminotic and pair-fed control rats. Feeding with excess of retinyl palmitate (40000i.u./day per 100g body wt.) significantly increased urinary 3-methylhistidine and creatinine output during a 4-day treatment interval. 3-Methylhistidine release from perfused rat hindquarters was also elevated after 5 days of vitamin treatment. To determine whether the adrenals were involved in mediating the above response, a study was conducted on adrenalectomized and sham-operated rats. Excessive vitamin A intake stimulated 3-methylhistidine excretion *in vivo* and *in vitro* in both adrenalectomized and sham-operated animals, thus suggesting that the vitamin A-induced acceleration in myofibrillar protein breakdown was not mediated by the adrenals. In both groups of rats, vitamin A treatment had no effect on the rate of protein synthesis, on the basis of incorporation *in vitro* of [^{3}H]phenylalanine into muscle protein. Additional studies revealed that the addition of excess retinol to the perfusion medium (10i.u./ml) had no significant effect on the rates of 3-methylhistidine release or [^{3}H]phenylalanine incorporation *in vitro*. Finally, high doses of cortisol (7mg/day per 100g body wt.) administered to intact rats for 5 days significantly increased rates of 3-methylhistidine excretion, both *in vivo* and *in vitro*.

One of the important early manifestations of acute vitamin A toxicity is a marked stimulation of hepatic gluconeogenesis (Singh *et al.*, 1969; Dileepan *et al.*, 1977; Rao *et al.*, 1977). It has been postulated that the effects of hypervitaminosis A on gluconeogenesis are mediated by the adrenals. Supporting this hypothesis is an increase in blood corticoids and decreased cholesterol and ascorbic acid content of the adrenal cortex in hypervitaminotic-A rats, suggesting the increased synthesis and secretion of adrenocorticoids (Singh *et al.*, 1972). Additionally, bilateral adrenalectomy abolished the effects of large doses of retinol on hepatic gluconeogenesis (Singh *et al.*, 1976).

Excessive vitamin A intake produced a negative nitrogen balance and an increase in blood urea in rats (Nerurkar & Sahasrabudhe, 1956; Strebel *et al.*, 1969). Since amino acids are excellent substrates for gluconeogenesis (Exton, 1972) and since skeletal muscle comprises the largest labile amino acid pool in the body (Young, 1970), it seems likely that the negative nitrogen balance observed may be due to alterations in muscle protein turnover.

Asatoor & Armstrong (1967) have suggested that 3-methylhistidine release from muscle and subsequent excretion from the body might be of use in determining rates of myofibrillar protein degradation in various species. Actin and myosin contain 3-methylhistidine, which is formed by the post-translational methylation of peptide-bound histidine by *S*-adenosylmethionine (Young *et al.*, 1972; Haverberg *et al.*, 1974). 3-Methylhistidine is neither re-utilized for protein synthesis nor metabolized in the rat (Cowgill & Freeburg, 1957); therefore, its rate of excretion offers a direct, non-invasive method of determining the degradation rate of myofibrillar protein.

To ascertain whether acute vitamin A toxicity alters myofibrillar protein degradation, a study was performed to monitor 3-methylhistidine excretion in normal rats and rats given excess vitamin A. It was demonstrated that giving excessive amounts of vitamin A stimulated 3-methylhistidine excretion both *in vivo* and *in vitro*. Additional experiments using adrenalectomized and sham-operated rats were conducted to assess the involvement of the adrenals in mediating the above response. The effect of excessive glucocorticoid administration on myofibrillar protein breakdown is also reported.

Methods and materials

Reagents

Bovine serum albumin (Cohn fraction V), L-

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(+)-lactic acid, sodium pyruvate, retinol, retinyl palmitate, cortisol acetate and amino acids were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin used in the perfusion medium was dialysed against deionized water 48h to remove low-molecular-weight compounds. L-Phenyl-[2,3-^{3}H]alanine was purchased from New England Nuclear Corp., Boston, MA, U.S.A. Chemicals and enzymes used in the glucose, phenylalanine and creatinine determinations were products of Sigma Chemical Co. All other chemicals used were of reagent grade.

Animals and treatments

Male Sprague–Dawley rats (Simonsen Farms, Gilroy, CA, U.S.A.) weighing 160–250g were housed in individual stainless-steel metabolic cages. The environment of the animal quarters was maintained at 22–24°C (26°C for adrenalectomized and sham-operated animals) with a day–night cycle of 12h light, 12h dark. In all experiments, animals were fed on a purified diet containing (w/w) 25% casein, 32% glucose, 32% corn starch, 5% Mazola oil, 4% Rogers–Harper salt mixture, 1% vitamin mixture and 0.3% methionine. Bilateral adrenalectomies and sham operations were performed on the same day and animals were allowed to recover 7 days before the start of experimentation. Adrenalectomized rats were provided with 0.9% NaCl. Success of the adrenalectomy was ascertained after the animal was killed.

A total of five separate experiments were conducted during the course of this study. Acute vitamin A toxicity was induced in intact-unoperated rats in Expts. I and II and adrenalectomized and sham-operated rats in Expt. IV. With the aid of a stomach tube, vitamin A, as retinyl palmitate (40000i.u./100g body wt.; 1i.u. = 0.53μg of retinyl palmitate) was given once daily for 5 consecutive days. In Expt. III, subcutaneous injections of cortisol acetate (7mg/100g body wt.) were given to intact rats. Weight- and age-matched controls were either given the same amount of corn oil (vehicle solution) or injected with the same volume of 0.9% NaCl in lieu of vitamin A and steroid-hormone treatments, respectively. In addition, controls were pair-fed, matching food being given to them to the food eaten by either the hyper-vitaminotic or glucocorticoid-treated animals during the treatment interval. In Expt. IV, sham-operated rats were pair-fed, by using as a guide the food eaten by adrenalectomized animals, for 6 days before each group was divided into (1) control and (2) vitamin A-treated subgroups. Vitamin A treatment then commenced and the sham–control, sham–vitamin A-treated and adrenalectomized–control groups were pair-fed, by using as guide the food eaten by the adrenalectomized–vitamin A-treated animals. In Expt. V, food was offered *ad libitum* until the day of perfusion. Crystalline retinol dissolved in ethanol was added to the perfusion medium (10i.u./ml, 1i.u. = 0.3μg of retinol) at zero time of each perfusion. The ethanolic retinol solution was stored at −20°C, gassed with nitrogen and protected from light to prevent oxidation.

Urinary 3-methylhistidine excretion

Complete 24h urine collections were obtained from each rat 4 days before and 4 days after the initiation of vitamin A or glucocorticoid treatment. Thymol was added as a preservative. A sample was taken for creatinine analysis (Slot, 1965). Another sample of urine was hydrolysed in 6M-HCl for 24h at 110°C in a sealed ampoule (Young *et al.*, 1972). The hydrolysate was dried by evaporation and reconstituted in 0.2M-lithium citrate dilution buffer (pH2.2) before application to a column of a Beckman 121M amino acid analyser. The analytical procedure for all 3-methylhistidine determinations involved a starting buffer of 0.2M-lithium citrate, pH3.70. After 12min, the buffer was changed to 1M-lithium citrate, pH3.70, and 3-methylhistidine was eluted 60min later. Penicillamine, which was used as an internal standard, was eluted with a third buffer, 1.6M-lithium citrate, pH6.5. Regeneration was performed with 0.3M-LiOH. The total run time was 2h.

Hindquarters-perfusion technique

Rats were anaesthetized with sodium pentobarbital and surgically prepared for hindquarters perfusion by a modification of the technique of Ruderman *et al.* (1971). The first 50ml of perfusate that passed through the tissues was discarded. The muscle preparation was then transferred to a perfusion chamber, where an initial perfusate volume of 150ml began to recirculate. Timing of the perfusion began at this point. The perfusion apparatus was similar to that described by Goodman *et al.* (1978) and was housed in a wooden cabinet maintained at 37°C.

The perfusion medium was composed of Krebs–Henseleit (1932) bicarbonate buffer containing 6% (w/v) bovine serum albumin, 15mM-glucose, 1.5mM-lactate, 0.15mM-pyruvate and amino acids at the following concentrations (μM): alanine (530), arginine (230), asparagine (70), aspartate (45), cystine (160), glutamate (75), glutamine (100), glycine (500), histidine (100), isoleucine (106), leucine (160), lysine (430), methionine (70), phenylalanine (80), proline (290), serine (550), threonine (400), tryptophan (80), tyrosine (100) and valine (200). In experiments in which protein synthesis was measured, the phenylalanine concentration was increased to 800μM. During the course of perfusion, the pH of the perfusate was maintained at 7.40 by

continuous gassing with O_2/CO_2 (19:1) in a silastic-tubing oxygenator and by dropwise addition of 1 M-NaOH as needed. Perfusate was pumped at a flow rate of 35 ml/min, which usually produced an arterial pressure of 9.0–9.3 kPa (60–70 mmHg). Tissue viability of each hindquarters preparation was assessed by monitoring the rate of glucose uptake between 30 and 120 min of perfusion. The rate of glucose uptake was constant during this period for all perfusions reported in the present paper.

Determination of the rate of 3-methylhistidine release from perfused rat hindquarters

The rate of 3-methylhistidine release from perfused rat hindquarters was measured between 30 and 120 min of perfusion. During this perfusion interval, the rate of 3-methylhistidine release is constant (R. J. Hansen & D. Morin, unpublished work) and free intracellular 3-methylhistidine concentrations are unchanged (Wassner & Li, 1981). We assume that changes in 3-methylhistidine concentration of the perfusate were solely the result of myofibrillar protein degradation. The changes observed were not the result of 3-methylhistidine release from skin, since 3-methylhistidine release was not significantly different in skinned and unskinned preparations (R. J. Hansen & D. Morin, unpublished work; Wassner & Li, 1981). In all perfusion experiments reported in this study, 10 ml samples of perfusate were taken at 30 and 120 min of perfusion, mixed with 6% (w/v) sulphosalicyclic acid containing 10 nmol of penicillamine, and centrifuged at 12000 *g* for 15 min. The supernatant was desalted on a cation-exchange column (Dowex AG 50-X8) and 3-methylhistidine was eluted with 25 ml of 7% (v/v) NH_3. The eluate was freeze-dried to dryness and reconstituted in 1 ml of 0.2 M-lithium citrate dilution buffer, pH 2.2, before being applied to the amino acid analyser. The rate of 3-methylhistidine release (nmol/h per g of dried muscle) was calculated by subtracting the total amount of 3-methylhistidine present in the perfusate at 30 min from the total amount at 120 min and expressing this difference per g of dry muscle. In order to determine fractional degradation rate of myofibrillar protein (K_d) *in vitro*, the 3-methylhistidine release rate was divided by the 3-methylhistidine pool size (3150 nmol/g of dried muscle; R. J. Hansen & D. Morin, unpublished work).

Measurement of rates of protein synthesis in vitro

To assess rates of protein synthesis in perfused muscle, [^{3}H]phenylalanine was added to perfusate (0.5 μCi/ml) containing 0.8 mM-phenylalanine (10 times normal plasma value). Incorporation of radioactivity was measured in the myofibrillar and sarcoplasmic protein fractions of gastrocnemius muscle. Rates of protein synthesis were determined between 30 and 120 min of perfusion, the same interval in which the rate of myofibrillar protein degradation (based on 3-methylhistidine release) was measured. The perfusate phenylalanine concentration used in these studies does not alter rates of protein turnover (Flaim *et al.*, 1978) and ensures that the specific radioactivities of the extracellular and intracellular pools of free phenylalanine are the same (Jefferson *et al.*, 1977). Furthermore, phenylalanine is neither synthesized nor degraded by perfused rat hindquarters. To determine the average specific radioactivity of perfusate phenylalanine, samples of perfusate were taken at 60 and 90 min and were deproteinized in 10% (w/v) trichloroacetic acid. After centrifugation (3000 *g* for 30 min), the supernatant was assayed for phenylalanine content (Andrews *et al.*, 1973) and for radioactivity by liquid-scintillation counting. At the end of perfusion, a whole gastrocnemius muscle was rapidly removed from the preparation, blotted, weighed and frozen in liquid nitrogen. The frozen muscle was homogenized in 10 ml of 0.01 M-K_2HPO_4, pH 7.4 (0–4°C), with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.) and centrifuged at 12000 *g* for 10 min at 0–4°C. As defined by Millward (1970), the supernatant contained the sarcoplasmic proteins and the pellet contained the myofibrillar proteins. The pellet was resuspended in 5 ml of the same buffer and centrifuged as above, repeated twice. All supernatants were combined, and the sarcoplasmic proteins were precipitated by addition of an equal volume of cold 10% (w/v) trichloroacetic acid, followed by centrifugation at 3000 *g* for 30 min, repeated twice. The washed myofibrillar pellets and the precipitated sarcoplasmic proteins were dissolved in 3 ml of 0.3 M-NaOH at 60°C for 30 min and centrifuged at 30000 *g* for 15 min. The volume of each fraction was recorded and samples were take for protein determination (Gornall *et al.*, 1949). Then 0.2 ml of each fraction was mixed with 1 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL, U.S.A.), and 15 ml of scintillation cocktail [5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene dissolved in 1 litre of toluene] was added. Samples were counted for radioactivity in a Packard Tri-Carb 2660 liquid-scintillation counter. The rate of protein synthesis, expressed as nmol of phenylalanine incorporated/h per mg of myofibrillar or sarcoplasmic protein, was calculated by dividing the d.p.m. incorporated/mg of protein by the average specific radioactivity of perfusate phenylalanine (d.p.m./nmol) between 30 and 120 min of perfusion.

Statistical procedures

Data in Figs. 1, 2 and 3, involving repeated measurements of the same response variable (either

3-methylhistidine or creatinine excretion), were statistically evaluated by using either one-way or two-way analysis of variance. In order to determine whether vitamin A toxicity, cortisol treatment or adrenalectomy significantly altered urinary 3-methylhistidine and creatinine excretion rates, the output of each of these compounds on days 2 and 4 of the experimental trial was averaged. The resulting value was designated as a covariate. The excretion values on days 6 and 8 were selected as the repeated measurements. Analysis of variance was carried out on a computer with the BMDP-P2V program (Jennrich & Sampson, 1979) for repeated-measures models. In Table 3, conventional one-way analysis of variance was performed followed by the Duncan's Multiple Range Test (Steel & Torrie, 1960). Statistical comparisons in Tables 1 and 2 were done by Student's *t* test.

Results

Effect of vitamin A toxicity and glucocorticoid administration on growth rate

Each of Expts. I–IV consisted of an 8-day experimental trial, which was divided into two separate 4-day intervals. Days 0–4 constituted a basal interval in which none of the groups received vitamin or hormone treatment. Days 4–8 formed a treatment interval in which excessive amounts of either retinyl palmitate or cortisol acetate were administered to certain groups of rats. In Table 1, the average growth rates during the basal and treatment intervals of Expts. I–IV are presented. Both excessive vitamin A intake and glucocorticoid administration markedly decreased the average growth rate of rats during the treatment interval to less than 40% of the growth rate during the basal period. Pair-feeding of age- and weight-matched controls also caused a significant decline in growth rate during the treatment interval; however, the extent of the suppression in growth rate was significantly greater in the hypervitaminotic and hormone-treated animals than in the pair-fed controls. Food intake during the treatment interval was decreased by an average of 16, 17 and 12% in Expts. I, II and III, respectively. Finally, one should note the lower growth rate of adrenalectomized and pair-fed sham-operated rats during the basal interval of Expt. IV when compared with intact animals used in other experiments. The subsequent initiation of acute vitamin A toxicity in adrenalectomized rats caused a further decline in food intake and growth rate.

Effect of excessive vitamin A intake and glucocorticoid administration on myofibrillar protein degradation

Excess vitamin A. The effect of acute vitamin A toxicity on myofibrillar protein degradation in intact rats was studied in Expt. I. An 8-day trial was conducted, and rates of urinary 3-methylhistidine and creatinine excretion were determined on selected days before and after the start of vitamin A treatment. As shown in Fig. 1, urinary 3-methylhistidine excretion/100g body wt. was stimulated significantly by vitamin A toxicity. 3-Methylhistid-

Table 1. *Effect of vitamin A toxicity and glucocorticoid administration on body weight and growth rate*

Daily body weights were recorded during an 8-day experimental trial. Between days 0 and 4, the average basal growth rate (g/day) was monitored in all groups of animals. Beginning on day 4 and continuing until day 8, animals were either given 40000i.u. of retinyl plamitate/day per 100g body wt. (Expts. I, II and IV) or injected with 7mg of cortisol acetate/day per 100g body wt. (Expt. III). Average growth rates during the latter treatment intervals were also measured. In Expts. I, II, III and IV, weight- and age-matched controls were pair-fed as described in the Methods and materials section. Values are means ± S.E.M. for six rats. Significant differences in growth rate between the basal interval (days 0–4) and treatment interval (days 4–8) within the same group are indicated by [a] ($P < 0.001$) and [b] ($P < 0.025$). When comparing the growth rates of vitamin- or hormone-treated animals with their controls on days 4–8, significant differences ($P < 0.025$) are indicated by [c].

Expt. no.	Initial body wt. (g)	Growth rate (g/day) Days 0–4	Days 4–8
I Control	183 ± 3	6.7 ± 0.6	3.6 ± 0.2[a]
Vitamin A-treated	182 ± 2	6.9 ± 0.3	2.3 ± 0.6[ac]
II Control	164 ± 2	6.9 ± 0.3	3.7 ± 0.3[a]
Vitamin A-treated	161 ± 3	6.5 ± 0.4	2.1 ± 0.5[ac]
III Control	185 ± 2	8.4 ± 0.4	7.1 ± 0.4[b]
Cortisol-treated	190 ± 2	8.8 ± 0.3	2.8 ± 0.7[ac]
IV Sham–Control	170 ± 2	4.0 ± 0.5	2.3 ± 0.4[b]
Sham–vitamin A-treated	172 ± 2	3.6 ± 0.4	1.2 ± 0.2[bc]
Adrenalectomized–control	172 ± 1	3.9 ± 0.4	1.8 ± 0.7[b]
Adrenalectomized–vitamin A-treated	168 ± 1	4.6 ± 0.3	1.3 ± 0.4[b]

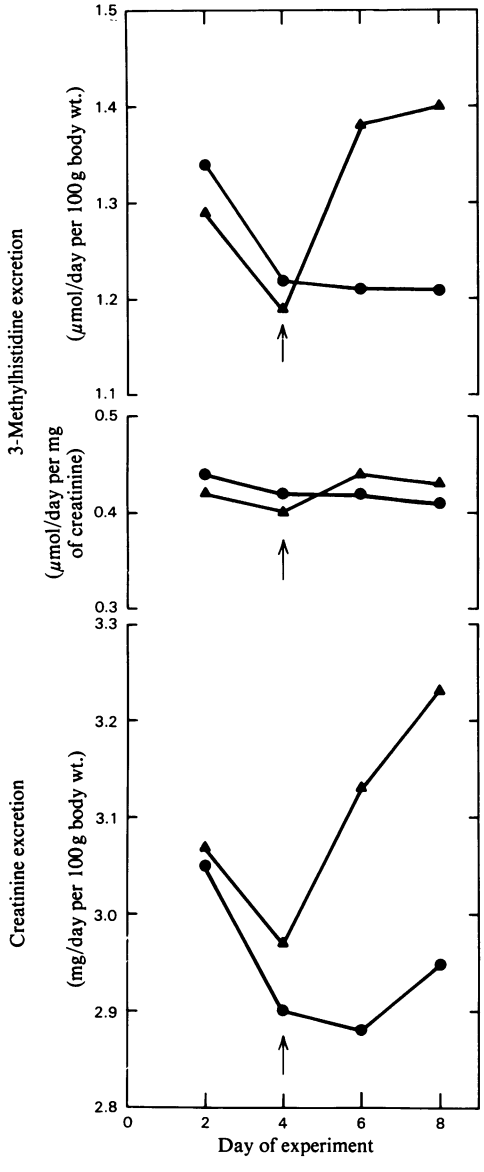

Fig. 1. *Changes in urinary 3-methylhistidine and creatinine excretion owing to excessive vitamin A intake (Expt. I)*

Details of the experimental design, administration of retinyl palmitate and treatment of controls are described in the Methods and materials section. Complete 24h urine samples from individual rats were analysed for 3-methylhistidine and creatinine on selected days during the basal interval (days 2 and 4) and vitamin A-treatment interval (days 6 and 8). The initiation of vitamin A treatment after the collection of urine on day 4 is indicated by (↑). Each point is the mean for six rats in the control (●) and vitamin A-treated (▲) groups. Statistical analysis of the data is described in the Methods and materials section. When the hypervitaminotic group was compared with the controls, significant differences were as follows: 3-methylhistidine/day per 100g body wt., $P<0.001$; 3-methylhistidine/day per mg of creatinine, $P<0.04$; creatinine/day per 100g body wt., $P<0.01$.

ine output of the vitamin A-treated group rose from an average initial rate of 1.25μmol/100g body wt. to 1.40μmol/100g, whereas the excretion rate of controls declined during the 8-day period. Excessive vitamin A intake also caused a significant elevation in urinary creatinine output. The magnitude of the elevation in 3-methylhistidine output caused by vitamin A was decreased when excretion of this amino acid was expressed as a function of creatinine excretion.

After completion of the urine-collection trial and administration of the final vitamin A treatment, hindquarters perfusions were performed on rats used in Expt. I. The degradation rate of myofibrillar protein was determined as described in the Methods and materials section. As shown in Table 2 (Expt. I), the rate of 3-methylhistidine release in the hypervitaminotic group was 40% higher than in the controls, supporting the results from the experiments *in vivo*. Giving excessive amounts of retinyl palmitate produced a similar elevation in 3-methylhistidine release in a second vitamin A-toxicity experiment (Expt. II) in which urinary 3-methylhistidine output was not measured. In both Expts. I and II, vitamin A treatment had no significant effect on glucose uptake by perfused muscle.

Excess glucocorticoid. In Expt. III the effects of glucocorticoid administration on myofibrillar protein degradation in intact rats were studied. As in Expt. I, the experimental design consisted of an 8-day trial followed by a hindquarters-perfusion study. As shown in Fig. 2, cortisol administration significantly increased urinary 3-methylhistidine excretion/100g body wt. and 3-methylhistidine excretion/mg of creatinine, the latter measurement to a smaller extent than the former, since hormone treatment also increased urinary creatinine output. In the hormone-treated group, 3-methylhistidine output rose from 1.19μmol/100g body wt. to 1.39μmol/100g, whereas the excretion rate of controls declined during the 8-day period. In Table 2 (Expt. III), the rate of release of 3-methylhistidine from the cortisol-treated group was 60% higher than the controls, again supporting the observations *in vivo*. Glucose uptake by perfused muscle was not affected by hormone treatment.

Effects of excessive vitamin A intake on rates of muscle protein synthesis and myofibrillar protein degradation in adrenalectomized and sham-operated rats

The effect of acute vitamin A toxicity on muscle protein turnover in adrenalectomized and sham-

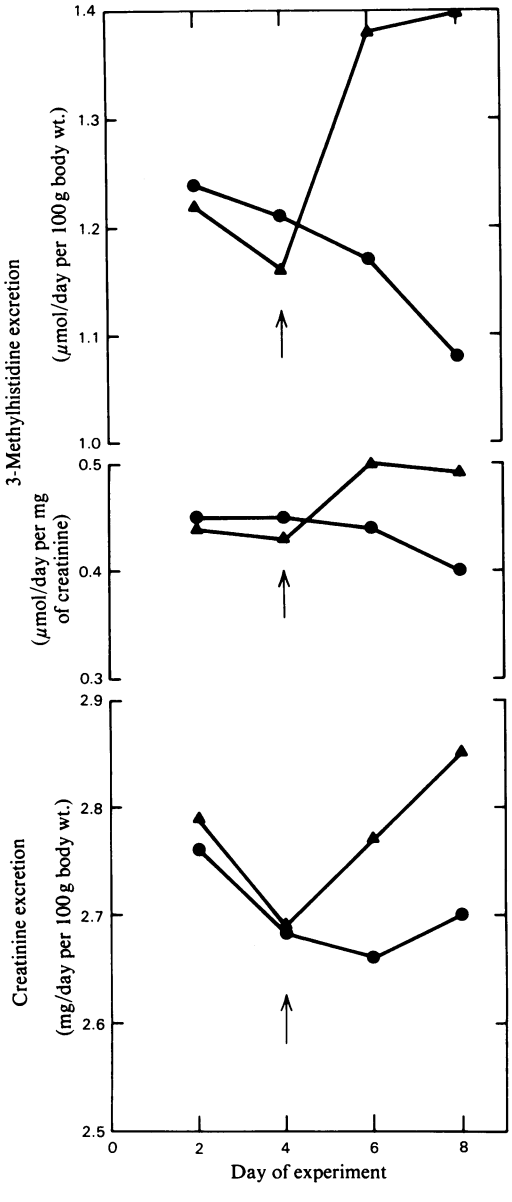

Fig. 2. *Changes in urinary 3-methylhistidine and creatinine excretion owing to cortisol administration (Expt. III)*
Details of the experimental design, administration of cortisol acetate and treatment of controls are described in Table 1. Other details are as in Fig. 1. The initiation of steroid-hormone treatment after the collection of urine on day 4 is indicated by (↑). Symbols designate control (●) and cortisol-treated (▲) groups. When the cortisol-treated group was compared with controls, significant differences were as follows: 3-methylhistidine/day per 100g body wt., $P<0.001$; 3-methylhistidine/day per mg of creatinine, $P<0.001$; creatinine/day per 100g body wt., $P<0.05$.

operated rats was studied in Expt. IV. A urine-collection trial was conducted to measure 3-methylhistidine and creatinine excretion rates before and after the start of vitamin A treatment. As shown in Fig. 3, urinary 3-methylhistidine output of the vitamin A-treated–sham-operated and vitamin A-treated–adrenalectomized groups rose from average initial rates of 1.12 and 1.30 μmol/100g body wt. to 1.34 and 1.61 μmol/100g, respectively. The 3-methylhistidine excretion rate of controls remained constant during the 8-day period. We conclude that the vitamin A-induced elevation in 3-methylhistidine excretion/100g body wt. was similar in magnitude in both adrenalectomized and sham-operated rats, since no significant interaction between the type of surgery and dietary treatment was obtained. However, 3-methylhistidine excretion/100g body wt. was significantly increased in untreated adrenalectomized rats compared with sham-operated animals.

Feeding excess of vitamin A also significantly stimulated creatinine output in both adrenalectomized and sham-operated rats. This finding either nullified or greatly decreased the vitamin A-induced increase in 3-methylhistidine output of sham-operated and adrenalectomized rats, respectively, when excretion of this amino acid was expressed as a function of creatinine excretion. Further analysis of the data revealed that creatinine output was significantly higher in adrenalectomized rats than in sham-operated animals. The latter result accounted for the smaller difference in 3-methylhistidine output between adrenalectomized and sham-operated rats when excretion of this compound was expressed per mg of creatinine rather than per 100g body wt.

After completion of the urine-collection trial, hindquarters perfusions were performed on animals used in Expt. IV. When compared with their respective controls, 3-methylhistidine release rates of the adrenalectomized and sham-operated groups treated with vitamin A were elevated, to a significant degree only in the latter group (Table 3). There was no difference in 3-methylhistidine release *in vitro* between the adrenalectomized and sham-operated control groups. Neither vitamin A toxicity nor adrenalectomy altered the rate of [^{3}H]phenylalanine incorporation into either the myofibrillar or sarcoplasmic protein fractions of perfused gastrocnemius muscle between 30 and 120min of perfusion. Glucose uptake by perfused muscle was similar in each experimental group.

Effect of addition of retinol to the perfusion medium on rates of protein synthesis and myofibrillar protein degradation in perfused skeletal muscle of normal rats

Hindquarters perfusions were performed in order to determine whether addition of retinol (10i.u./ml)

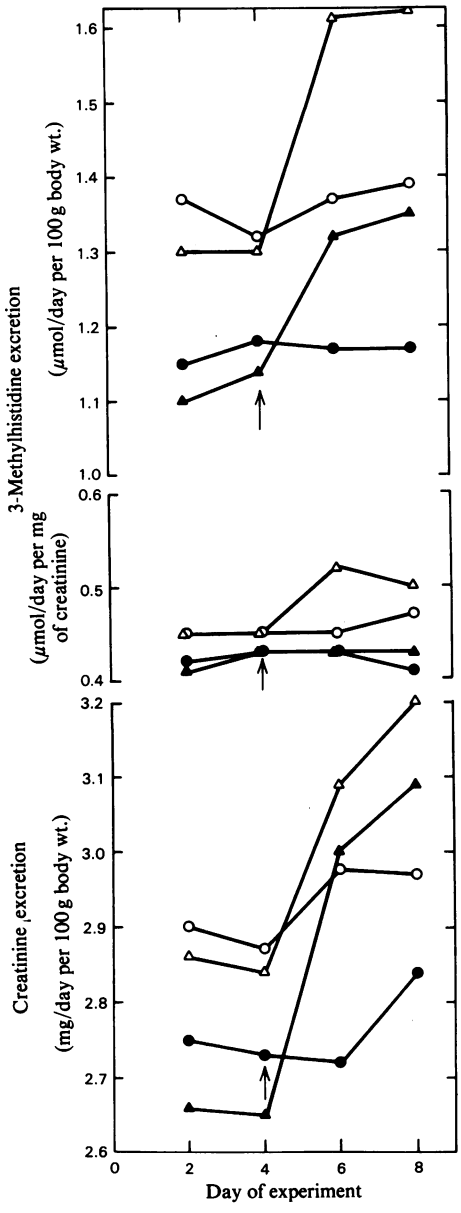

Fig. 3. *Changes in urinary 3-methylhistidine and creatinine excretion of adrenalectomized and sham-operated rats owing to excessive vitamin A intake (Expt. IV)*
Details of the experimental design, administration of retinyl palmitate and pair-feeding procedure are described in the Methods and materials section. Other details are as in Fig. 1. The initiation of vitamin A treatment after the collection of urine on day 4 is indicated by (↑), Symbols designate sham–control (●), sham–vitamin A-treated (▲), adrenalectomized–control (○) and adrenalectomized–vitamin A-treated (△) groups. When hypervitaminotic animals are compared with controls in both adrenalectomized (ADX) and sham-operated groups significances of differences were as follows: 3-methylhistidine/day per 100g body wt., $P<0.001$ for both sham and ADX groups; 3-methylhistidine/day per mg of creatine, $P<0.001$ for ADX group, non-significant for sham group; creatinine/day per 100g body wt., $P<0.001$ for both sham and ADX groups. When adrenalectomized rats are compared with sham-operated animals, 3-methylhistidine excretion/100g body wt. and creatinine excretion/100g body wt. were significantly different, $P<0.001$. There was no interaction between the surgery and dietary treatment in the data for 3-methylhistidine excretion/100g body wt. and creatinine excretion/100g body wt.

to the perfusion medium altered muscle protein turnover. Rates of 3-methylhistidine release from perfused muscle and [^{3}H]phenylalanine incorporation into myofibrillar and sarcoplasmic protein of gastrocnemius muscle between 30 and 120min of perfusion were not significantly changed by retinol addition at the beginning of the perfusion (results not shown). Glucose uptake by perfused muscle was also not affected by retinol addition.

Discussion

When excessive amounts of vitamin A were administered to rats in Expts. I and II, increased release and excretion of 3-methylhistidine was observed, indicating accelerated myofibrillar protein degradation. Since it has been postulated that vitamin A-induced changes in carbohydrate and lipid metabolism are mediated by the adrenals (Singh *et al.*, 1969, 1976), we decided to apply this hypothesis to myofibrillar protein degradation. It was first demonstrated that excess cortisol administration increases 3-methylhistidine excretion *in vivo* and *in vitro* (Expt. III, Fig. 2 and Table 2). These results are consistent with the above hypothesis, but do not prove it. In a more conclusive study, it was shown that excess vitamin A treatment increased urinary 3-methylhistidine excretion to a similar extent in sham-operated and adrenalectomized rats (Fig. 3). Release of 3-methylhistidine from perfused hindquarters of sham-operated and adrenalectomized rats pre-treated with excess vitamin A was also increased, but only to a significant extent in the former group (Expt. IV, Table 3). Thus it is apparent that the effects of vitamin A toxicity are not mediated by the adrenocorticoids.

Further support for the latter conclusion can be derived from the protein-synthesis data in Expt. IV. Rannels & Jefferson (1980) reported that excess cortisone acetate treatment for 3–5 days decreased muscle protein synthesis by 50–60% in the perfused rat hemicorpus. If the effects of excess vitamin A treatment on muscle protein turnover were mediated by adrenocorticoids, one would expect to see a decrease in protein synthesis in muscles of rats so

Table 2. *Effect of excessive vitamin A intake and glucocorticoid administration on the rate of 3-methylhistidine release from perfused hindquarters of intact rats*

Rats in Expts. I, II and III were either given 40000i.u. of retinyl palmitate/day per 100g body wt. or injected with 7mg of cortisol acetate/day per 100g body wt. for 4 days before hindquarters perfusions were performed. In Expts. I and III, perfusions were conducted after the completion of the 8-day urine-collection trial (Figs. 1 and 2). The controls of each experiment were pair-fed as described in the Methods and materials section. Details of the perfusion procedure and calculation of 3-methylhistidine release rate are described in the Methods and materials section. Values are means ± S.E.M. for six animals. Statistical significance for differences from the control group ($P < 0.01$) is indicated by [a].

Expt. no.	3-Methylhistidine release (nmol/h per g of dried muscle)	K_d (%/day)	Glucose uptake (μmol/h per g of dried muscle)
I Control	4.4 ± 0.4	3.4	75.6 ± 9.5
Vitamin A-treated	6.1 ± 0.4[a]	4.6	63.4 ± 9.1
II Control	4.5 ± 0.4	3.4	76.5 ± 5.6
Vitamin A-treated	6.8 ± 0.6[a]	5.2	91.0 ± 7.2
III Control	3.9 ± 0.3	3.0	80.3 ± 8.5
Cortisol	6.2 ± 0.5[a]	4.7	92.0 ± 9.0

Table 3. *Rates of muscle protein synthesis and myofibrillar protein degradation in perfused skeletal muscle of sham-operated and adrenalectomized rats given excessive amounts of vitamin A*

After the completion of the urine-collection trial (Fig. 3) and administration of the fifth and final vitamin A treatment on day 8, animals were surgically prepared for hindquarters perfusion. Further details of the experimental design are given in Table 1. Determinations of the rate of 3-methylhistidine release, myofibrillar protein degradation rate (K_d), and rate of protein synthesis in the myofibrillar and sarcoplasmic protein fractions of gastrocnemius muscle are described in the Methods and materials section. Values are means for six rats. Significant difference ($P < 0.05$) versus the sham-control group is denoted by [a].

	Myofibrillar protein degradation		Phenylalanine incorporation (nmol/h per mg of protein)		
Expt. IV	3-Methylhistidine release (nmol/h per g of dried muscle)	k_d (%/day)	Myofibrillar fraction	Sarcoplasmic fraction	Glucose uptake (μmol/h per g of dried muscle)
Sham–control	4.4	3.4	0.53	0.67	89.5
Sham–vitamin A-treated	5.9[a]	4.5	0.50	0.64	95.0
Adrenalectomized–control	4.9	3.8	0.50	0.61	93.6
Adrenalectomized–vitamin A-treated	5.5	4.2	0.49	0.61	98.4

treated, yet no change in protein synthesis was observed in our experiments with excess vitamin A (Table 3).

Probably the best alternative hypothesis describing the mechanism of increased muscle protein degradation during acute vitamin A toxicity is based on the direct action of excess vitamin A on lysosomal membranes. Excess vitamin A has been shown to stimulate the release of acid hydrolases from lysosomes of liver (Dingle, 1961; Weissmann & Thomas, 1963) and muscle (Berg & Bird, 1970). This release reflects an increased lysosomal membrane fragility. More recently, Schwartz & Bird (1977) have demonstrated that two lysosomal enzymes, cathepsins B and D, can degrade purified myofibrillar protein *in vitro*. It can be postulated that the increased release of the above acid proteinases mediates the elevation in myofibrillar protein degradation during vitamin A toxicity. However, the results of Expt. V do not clearly support this hypothesis, since addition of excess of retinol to the perfusion medium had no significant effect on 3-methylhistidine excretion *in vitro*. The above results may be interpreted in two ways: (1) the effect of vitamin A toxicity on muscle protein degradation is not lysosome-mediated, or (2) a latent period exists between the initiation of lysosomal proteinase release and alterations in myofibrillar protein degradation, so that significant changes in the 3-methylhistidine release rate cannot be observed within the 2h perfusion period.

Excessive vitamin A and cortisol administration

resulted in increased urinary creatinine excretion (Figs. 1, 2 and 3). It would appear that excretion of creatinine relative to muscle mass is increased, owing to some disturbance in creatinine metabolism; therefore the use of creatinine excretion as an index of changes in skeletal-muscle mass may not be accurate during excessive vitamin or hormone treatment. Expressing 3-methylhistidine excretion per unit body weight, rather than per amount of creatinine excreted, is probably more reliable, although it may lead to false interpretations if changes in muscle mass do not parallel changes in body weight.

Our results in Expt. III (Fig. 2, Table 2), showing that urinary 3-methylhistidine excretion increased in glucocorticoid-treated rats, are in agreement with those of Nishizawa *et al.* (1978) and Tomas *et al.* (1979). Our results are in apparent disagreement with the studies of Millward (1976) *in vivo*, and of Shoji & Pennington (1977) and Rannels & Jefferson (1980) *in vitro*, showing by the use of isotopic techniques that total muscle protein degradation is not altered by glucocorticoid treatment. On the basis of studies comparing actomyosin degradation (3-methylhistidine release) with total muscle protein degradation (dilution of [^{14}C]phenylalanine specific radioactivity) in the perfused rat hemicorpus, Li *et al.* (1981) have suggested that the turnover of muscle proteins with long half-lives (e.g. actomyosin) is altered to a greater degree during catabolic conditions than is that of those with short half-lives. Hence the above discrepancy may at least be partially attributed to the specificity of the method used to measure muscle protein degradation. We only monitored actomyosin degradation in the present study, whereas the other groups measured muscle total protein degradation.

In conclusion, our finding of accelerated muscle protein degradation caused by hypervitaminosis A is consistent with the results of others showing that urinary nitrogen excretion (Nerurkar & Sahasrabudhe, 1956) and blood urea concentrations (Strebel *et al.*, 1969) are increased during this condition. Excessive vitamin A intake also causes a marked stimulation in hepatic gluconeogenesis (Singh *et al.*, 1969). The latter effect may be mediated in part by an increased supply of circulating amino acids derived from muscle proteolysis. Exton & Park (1967) have demonstrated in perfused rat livers that the availability of substrates, such as amino acids, is important in regulating hepatic gluconeogenesis. Adrenocortical hormones are also involved in controlling gluconeogenesis by regulating liver enzyme activity and hepatic uptake of gluconeogenic precursors (Thompson & Lippman, 1974). Singh *et al.* (1976) reported that the vitamin A-induced stimulation in gluconeogenesis is not observed in adrenalectomized rats, thus suggesting that this effect was mediated by the adrenals. In contrast, the results of the present study indicate that the vitamin A-induced changes in myofibrillar protein degradation are not dependent upon the adrenals.

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