

# Skeletal Muscle Protein and Amino Acid Metabolism in Experimental Chronic Uremia in the Rat

## ACCELERATED ALANINE AND GLUTAMINE FORMATION AND RELEASE

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**ABSTRACT** The kinetics and factors regulating alanine and glutamine formation and release were investigated in skeletal muscle preparations from control and experimentally uremic rats. These preparations maintained phosphocreatine and ATP levels *in vitro* which closely approximated levels found *in vivo*. Alanine and glutamine release from uremic muscle were increased 45.8 and 36.0%, respectively, but tissue levels were unaltered. The increased release of alanine by uremic muscle was not accounted for by decreased rates of medium alanine reutilization via oxidation to CO<sub>2</sub> or incorporation into muscle protein. The maximal capacity of added amino acids such as aspartate, cysteine, leucine, and valine to stimulate net alanine and glutamine formation was the same in uremic and control muscle. Epitrochlearis preparations were partially labeled *in vivo* with [*guanido*-<sup>14</sup>C]-arginine. On incubation, preparations from uremic animals showed a 54.6% increase in the rate of loss of <sup>14</sup>C-label in acid precipitable protein. Correspondingly, these same uremic preparations showed a 62.7% increase in <sup>14</sup>C-label appearance in the acid-soluble fraction of muscle and in the incubation media. Insulin decreased alanine and glutamine release to an extent threefold greater in uremic than in control preparations, and increased muscle glucose uptake approximately threefold in all preparations. Although basal rates of [4,5-<sup>3</sup>H]leucine incorporation into protein were decreased 25% in uremic muscles as compared with control muscles, insulin stimulated [<sup>3</sup>H]leucine incorporation nearly equally in both preparations.

These data demonstrate increased alanine and glutamine production and release from skeletal muscle of chronically uremic rats. This increase appears to derive in part from an enhancement of net protein degradation which could be caused by an acceleration in the breakdown of one or more groups of muscle proteins, or by an inhibition of protein synthesis, or by both processes. The increased alanine and glutamine formation and release in uremia appears not to result from an insensitivity to insulin action. The implications of these findings for an understanding of the abnormal carbohydrate metabolism of uremia are discussed.

### INTRODUCTION

Diverse alterations of blood amino acid levels have been described in uremic patients maintained on hemodialysis (1-12). Amino acids such as glutamate, serine, and the branched-chain amino acids appeared to be increased 30-50% in anephric patients, compared to normal volunteers (12-15), whereas other amino acids such as glutamine, citrulline, glycine, and the methyl histidines were substantially increased in uremic subjects (12-16). Although blood levels of other amino acids such as aspartate, threonine, histidine, and particularly alanine were found to be unchanged, plasma levels of alanine, aspartate, and threonine were increased in anephric subjects (12-16). Recently we observed a patient with chronic uremia and episodes of spontaneous hypoglycemia in whom hypoalaninemia and decreased rates of gluconeogenesis from alanine were found to account, at least in part, for the pathogenesis of the hypoglycemia (17). Accordingly, we have investigated the kinetics of alanine formation and release in isolated intact epitrochlearis skeletal muscle preparations obtained from rats made experimentally uremic by unilateral nephrectomy and segmental infarction of the contra-

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lateral kidney. Portions of this study have been presented previously (18) and have appeared in abstract form (19, 20).

## METHODS

Adenylate kinase (EC 2.7.4.3), alanine aminotransferase (EC 2.6.1.2), creatine phosphokinase (EC 2.7.3.2), glucose-6-phosphate, dehydrogenase (EC 1.1.1.49), glutamate dehydrogenase (EC 1.4.1.3), glutaminase (EC 3.5.1.2), hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), NAD<sup>+</sup>, NADH, NADP<sup>+</sup>, ATP, ADP, glucose, pyruvate, and amino acids were obtained from the Sigma Chemical Co. (St. Louis, Mo.). [*guanido*-<sup>14</sup>C]arginine (sp act = 55.3 mCi/mmol) was from Amersham/Searle Corp. (Arlington Heights, Ill.), [*U*-<sup>14</sup>C]alanine and [*4,5*-<sup>3</sup>H]leucine were purchased from New England Nuclear (Boston, Mass.). Crystalline glucagon-free insulin was a gift of Dr. J. Galloway (Eli Lilly and Co., Indianapolis, Ind.).

For studies of muscle metabolism *in vitro*, Sprague-Dawley rats weighing from 125 to 150 g were sacrificed by a blow to the high cervical spine. Epitrochlearis skeletal muscle preparations of the upper extremities were visualized and rapidly removed intact as described previously (21). Excess connective tissue was trimmed, and the muscle was rinsed briefly in isotonic sodium chloride, blotted, and placed in 0.5 ml of a modified Krebs-Henseleit buffer (pH 7.4), containing one-half the calcium concentration as originally formulated, and with increased buffering capacity produced by the addition of 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid. Other additions to the medium such as amino acids, glucose, and insulin were as indicated. The incubation flasks were gassed with 95% O<sub>2</sub> - 5% CO<sub>2</sub>, stoppered, and incubations were carried out in a shaking water bath at 37°C for between 30 and 360 min. At the end of the incubation, the muscles were removed rapidly, briefly rinsed in cold buffer, blotted, and then frozen in liquid nitrogen. All muscle samples and incubation media were stored at -80°C. Frozen muscle preparations were weighed while being maintained at -20°C and then rapidly homogenized in ice-cold 3 M perchloric acid, and subsequently centrifuged at 4,500 *g* for 15 min. The resulting supernatant was neutralized with a solution containing 3 M potassium hydroxide, 0.4 M imidazole, and 0.7 M potassium chloride, and the potassium perchlorate precipitate was removed by further centrifugation (22). For studies of isotopically labeled amino acid incorporation into protein *in vitro*, experiments were carried out in incubation flasks using continuous gassing with 95% O<sub>2</sub> - 5% CO<sub>2</sub> for 1- to 6-h periods. The incubation medium was further modified by the addition of a mixture of 16 essential and nonessential amino acids (most at a 2.5-mM final concentration), and insulin at the indicated concentrations. 10 μCi/ml of [*4,5*-<sup>3</sup>H]leucine or [*U*-<sup>14</sup>C]alanine was also added. After the incubation, labeled amino acid incorporation into protein was determined by homogenization of the frozen incubated muscle in hot 12% trichloroacetic acid with two subsequent washings of the acid insoluble pellet with 5% trichloroacetic acid, ether extraction, air drying, and solubilization of the pelleted precipitate with NCS solubilizer (Amersham Corp., Arlington Heights, Ill.). The latter was counted in a liquid scintillation spectrometer using ACS scintillant (Amersham Corp.). Studies of added [*U*-<sup>14</sup>C]alanine oxidation by intact epitrochlearis preparations were carried out in sealed incubation flasks with injection stoppers and a bucket suspended above the media. Alanine was the only added amino acid (final concentration was 0.4 mM), and [*U*-<sup>14</sup>C]alanine was in-

cluded at a 1-μCi/ml final concentration. At the conclusion of the 1- to 4-h incubation period, the experiment was stopped by acidification of the incubation medium with 2 N H<sub>2</sub>SO<sub>4</sub>, and [<sup>14</sup>C]CO<sub>2</sub> was trapped by subsequent absorption of the evolved [<sup>14</sup>C]CO<sub>2</sub> within the suspended bucket containing added triethanolamine base. The latter was then washed and transferred to liquid scintillation vials for counting using ScintiVerse (Fisher Scientific Co., Pittsburgh, Pa.) scintillant. Alanine-specific activities were determined from aliquots of the incubation medium before and after incubation.

For studies of protein labeling *in vitro*, epitrochlearis muscles were obtained from rats previously labeled *in vivo* using an initial intraperitoneal injection of 50 μCi [*guanido*-<sup>14</sup>C]alanine (Sp act = 55 mCi/mmol) followed 6 h later by a second intraperitoneal injection of 20 μmol of unlabeled arginine. After 24 h, the animals were sacrificed, and epitrochlearis preparations were obtained and incubated for between 2 and 4 h in 0.5 ml of Krebs-Henseleit buffer. After incubation, muscles were rinsed, blotted, and immediately frozen in liquid nitrogen. The tissues were subsequently homogenized in 0.9 ml of hot 12% trichloroacetic acid and centrifuged for 20 min at 2,500 *g*. The post-trichloroacetic acid supernate and the incubation medium were directly sampled for liquid scintillation counting. The precipitate in itself was washed twice with additional TCA and subsequently with diethyl ether with final air drying. This precipitate was dissolved in 0.2 ml of NCS solubilizer and transferred to liquid scintillation vials and counted using ACS scintillant. Determinations of protein labeling *in vivo* using [*guanido*-<sup>14</sup>C]arginine were carried out in a similar manner except that skeletal muscles were visualized after the animals had been anesthetized with pentobarbital. These muscles were quickly freeze-clamped using Wollenberger tongs (Fisher Scientific Co.) previously chilled in liquid nitrogen. Isolation of trichloroacetic acid precipitable protein was followed as outlined above. Chronically uremic rats were produced surgically by unilateral nephrectomy and segmental infarction of the contralateral kidney after ligation of two of the renal arterial branches (23, 24). Supplemental feedings of casein, dextrose, and water were given. Control rats were pair-fed. Serum urea nitrogen was 59.1±6.8 and 18.3±2.7 mg/ml (mean±SE) in the uremic and control groups, respectively.

Semimicro- and microfluorometric enzymatic techniques were used to determine levels of intermediates and substrates in perchloric acid extracts of skeletal muscle and in the incubation media. Glucose (22), lactate (25), ATP (22), ADP (22), phosphocreatine (22), alanine (26), aspartate, glutamate, and glutamine (25) were all assayed using coupled enzymatic assays linked to the appearance or disappearance of NADH or NADPH. Cyclic AMP was determined in trichloroacetic acid extracts of muscle by a sensitive, double-antibody radioimmunoassay (27). Statistical assessments were made using Student's *t* test (28).

## RESULTS

To determine the usefulness of the epitrochlearis preparation of rat skeletal muscle for *in vitro* studies of amino acid metabolism in experimental uremia, the effect of incubation was determined on adenine nucleotide and phosphocreatine levels in preparations obtained from chronically uremic and control rats. As shown in Table I, levels of ATP, ADP, and phosphocreatine in epitrochlearis preparations, which were freeze-clamped *in situ*, were not significantly

TABLE I  
Adenine Nucleotide and Phosphocreatine Levels in Muscle from Normal and Chronically Uremic Rats

Treatment	Preparation	ATP	ADP	Phosphocreatine	ATP/ADP
<i>μmol/g muscle, wet wt</i>					
Freeze-clamped, <i>in situ</i>	Control	4.87±0.25	0.59±0.07	15.6±1.09	8.16±0.27
	Uremic	4.82±0.21	0.58±0.08	14.7±0.71	8.18±0.36
Incubation, <i>in vitro</i>	Control	4.72±0.31	0.66±0.12	13.7±0.60	7.21±0.68
	Uremic	4.66±0.36	0.61±0.05	12.8±1.29	7.32±0.73

Epitrochlearis preparations from chronically uremic and control rats were obtained and incubated in Krebs-Henseleit buffer (pH 7.4) for 1 h as described in Methods. For comparison purposes, epitrochlearis preparations from the contralateral extremity of the same rat were freeze-clamped *in situ* using Wollenberger tongs previously cooled in liquid nitrogen. Using fluorometric microtechniques, levels of phosphocreatine, ATP and ADP were determined in the neutralized perchloric acid extracts of each muscle preparation. Values shown are the means (±SEM) for at least six experiments.

different in the uremic animals as compared with the control animals, nor was there any difference in the ratio of ATP to ADP. Epitrochlearis preparations from the contralateral extremity of each animal were incubated for 1 h. Levels of either ATP or ADP in incubated uremic preparations were not significantly altered compared with preparations freeze-clamped *in situ*. Taken as a whole, these data indicate that both preparations remained viable *in vitro*, and that there was no significant difference in muscle preparations from chronically uremic rats as compared with control rats.

An increased rate of release of most of the amino acids studied was found on incubation of skeletal muscle preparations from chronically uremic rats (Table II). With uremic muscle, alanine release was increased 46.8% ( $P < 0.01$ ), aspartate release was in-

creased 200% ( $P < 0.05$ ), glutamate release was increased 61.5% ( $P < 0.02$ ), glutamine release was increased 36.0% ( $P < 0.02$ ), and total amino acid release was increased 42.0% ( $P < 0.02$ ). Although rates of muscle alanine and glutamine release were increased in uremia, tissue levels of these two amino acids were not altered in uremic animals as compared with control animals (Table II). Epitrochlearis preparations from either uremic or control rats also showed no significant decrease in tissue amino acid levels in muscles that were freeze-clamped *in situ* (Table III). Taken together, the data of Tables II and III indicate an increased net formation of alanine and glutamine in skeletal muscle from chronically uremic rats.

TABLE II  
Release of Alanine, Aspartate, Glutamate and Glutamine from Skeletal Muscle of Control and Chronically Uremic Rats

Animal treatment	Alanine release	Aspartate release	Glutamate release	Glutamine release	Total release
<i>nmol/min per g muscle, wet wt</i>					
Control	20.3±1.2	0.7±0.2	10.4±0.7	30.3±0.26	61.4±5.1
Chronic uremia	29.8±2.2	2.1±0.9	16.8±1.2	41.2±3.1	87.2±6.1

Epitrochlearis skeletal muscle preparations were obtained from control and chronically uremic rats. These preparations were placed in Krebs-Henseleit buffer and incubated for 1 h. At the end of the incubation, muscles were removed, rinsed, blotted, frozen in liquid nitrogen, and extracted with perchloric acid. Alanine, aspartate, glutamate, and glutamine were determined enzymatically. Values shown for amino acids released are the means (±SEM) for at least 14 experiments.

TABLE III  
Effect of Incubation on Levels of Alanine and Glutamine in Skeletal Muscle Preparations from Control and Chronically Uremic Rats

Animal treatment	Treatment	Alanine level	Glutamine level
<i>nmol/mg, wet weight</i>			
Control	Freeze-clamped, <i>in situ</i>	1.57±0.10	3.51±0.24
	Incubated	1.51±0.13	3.40±0.27
Chronic uremia	Freeze-clamped, <i>in situ</i>	1.62±0.11	3.39±0.29
	Incubated	1.58±0.09	3.28±0.21

Epitrochlearis preparations from chronically uremic and control rats were dissected and either freeze-clamped *in situ* or incubated in modified Krebs-Henseleit buffer. After incubation, muscles were rapidly removed from the media, rinsed, blotted, and frozen in liquid nitrogen. Alanine and glutamine were determined in the neutralized perchloric acid extracts of these muscles. Values given are the means (±SEM) for at least six experiments.

The metabolic basis for the increased release of alanine in skeletal muscle preparations of chronically uremic rats was next investigated. The effect of experimental uremia on pathways for reutilization of released alanine was studied in a separate series of experiments with [U-<sup>14</sup>C]alanine added to the incubation media (Table IV). 100 mU/ml insulin was also added to the incubation media to insure that [<sup>14</sup>C]alanine transport was not the major limiting step for [<sup>14</sup>C]CO<sub>2</sub> generation from extracellular alanine. The oxidation of [U-<sup>14</sup>C]alanine in the media to [<sup>14</sup>C]CO<sub>2</sub> by muscle preparations from control rats was relatively low and this rate was not substantially altered in muscles from chronically uremic rats. Similarly, the rate of uptake and incorporation from the media of [U-<sup>14</sup>C]alanine into trichloroacetic acid precipitable protein by muscles from control rats was very small compared with the rate of net alanine release by control preparations. In preparations obtained from uremic rats, alanine incorporation into protein was unchanged compared with rates in control preparations at the concentration of insulin used.

The synthesis of alanine and glutamine can be stimulated in skeletal muscle by a number of added amino acids (29–32). However, it is unclear as to the mechanisms by which alanine and glutamine formation are stimulated by amino acids, such as leucine and cysteine, which do not appear to be metabolized

TABLE IV  
Effect of Chronic Uremia on Alanine Utilization  
by Skeletal Muscle

Treatment	[U- <sup>14</sup> C]Alanine	
	Oxidation to [ <sup>14</sup> C]CO <sub>2</sub>	Incorporation into TCA-precipitable protein
	<i>nmol/min/per g muscle, wet wt</i>	
Control	0.155±0.064	0.606±0.086
Chronic uremia	0.173±0.012	0.673±0.039

Epitrochlearis preparations from chronically uremic and control rats were obtained and incubated in Krebs buffer (pH 7.4) containing 100 mU/ml insulin and 5 mM glucose. In those experiments determining the rate of alanine oxidation, 10 μCi/ml; 0.4 mM [U-<sup>14</sup>C]alanine was added to the incubation media, and for studies of alanine incorporation into protein, 1.0 μCi/ml; 0.4 mM [U-<sup>14</sup>C]alanine was added to the incubation media together with a mixture of 17 essential and nonessential amino acids. After incubation, the muscles were rapidly removed from the incubation media, rinsed, blotted, and homogenized in hot 12% TCA. The acid insoluble pellet was washed and subsequently counted in a liquid scintillation spectrometer. For calculations of alanine flux, mean alanine specific activities were determined after enzymatic analysis of the before and after incubation media. Values given are the means (±SEM) for at least five experiments.

to alanine or its precursors in muscle. To assess maximal capacities of alanine, glutamate, and glutamine formation in muscle from chronically uremic rats, amino acids such as aspartate, cysteine, leucine, and valine were added individually (10 mM each) to the incubation media and the rates of alanine, glutamate, and glutamine release were determined (Table V). Each of the amino acids studied increased the release of alanine and glutamine from skeletal muscle of uremic as well as control rats. Maximally stimulated rates of alanine and glutamine release by each added amino acid were not significantly different in uremic preparations as compared with control preparations.

Muscle proteins were partially labeled *in vivo* using pulse-chase administration of 50 μCi [guanido-<sup>14</sup>C]-arginine followed 6 h later by a second intraperitoneal injection of 20 μmol of unlabeled arginine. 24 h after the injection of [<sup>14</sup>C]arginine label, equal amounts of label were found *in vivo* in the acid-precipitable protein fractions of skeletal muscle (Fig. 1). By 48 h, the amount of <sup>14</sup>C-label in trichloroacetic acid precipitable protein was less in muscle from uremic animals compared with control animals. However, the rate of decrease of protein-precipitable label after 24 h was nonlinear over the period studied. The

TABLE V  
Effect of Added Amino Acids on Alanine, Glutamate, and  
Glutamine Release from Epitrochlearis Muscle  
Preparations of Normal and Chronically  
Uremic Rats

Amino acid added	Animal pretreatment	Alanine	Glutamate release	Glutamine
<i>nmol/min per g muscle, wet wt</i>				
None	Control	20.4±1.3	10.6±1.0	30.1±2.2
	Uremia	28.2±1.4	16.9±2.3	39.1±2.3
Aspartate	Control	53.5±4.2	25.2±2.2	37.4±2.3
	Uremia	53.3±3.6	27.3±2.3	41.7±2.0
Cysteine	Control	44.5±2.8	10.0±1.3	41.2±1.4
	Uremia	42.8±3.4	17.1±1.9	42.5±2.6
Leucine	Control	34.9±1.6	10.9±1.1	44.9±2.7
	Uremia	37.2±0.9	15.1±2.2	47.0±2.6
Valine	Control	37.0±2.1	11.0±1.1	45.4±2.3
	Uremia	39.4±1.9	16.8±2.0	49.5±4.2

Epitrochlearis preparations were obtained from chronically uremic and control rats were incubated in Krebs-Henseleit buffer containing 5 mM glucose and 100 mU/mg insulin. To this incubation media, aspartate, cysteine, leucine, or valine (10 mM each) were added singly. The release of alanine, glutamate, and glutamine in the incubation media were determined using fluorometric microtechniques. Values given are the means (±SEM) for at least six experiments.

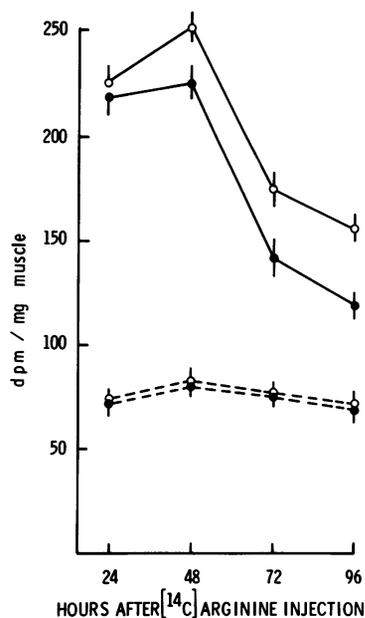


FIGURE 1 The incorporation of [*guanido*-<sup>14</sup>C]arginine in skeletal muscle of control and chronically uremic rats. A 50- $\mu$ Ci intraperitoneal injection of [*guanido*-<sup>14</sup>C]arginine was administered to control (○), and chronically uremic rats (●), 6 h later a 20- $\mu$ mol injection of unlabeled arginine was administered. After the [<sup>14</sup>C]arginine, animals were sacrificed and the radioactivity was determined in the acid soluble (○---○) and acid insoluble (○—○) fractions of muscle as described in Methods. Values shown are the means ( $\pm$ SEM) for at least 12 experiments.

decrement in <sup>14</sup>C-labeling of protein in uremic muscle appeared to be accentuated with an increasing period between the time of the injection of [<sup>14</sup>C]arginine label and the time of sampling of muscle. Experi-

ments were then performed *in vitro* to assess the rate of clearance of <sup>14</sup>C-label in endogenous skeletal muscle proteins in uremia. Using similar pulse-chase isotope labeling protocols, epitrochlearis preparations from control and chronically uremic rats were obtained in which partial labeling of at least some proteins with [*guanido*-<sup>14</sup>C]arginine had been produced. Muscles were isolated 24 h after the injections. After sacrifice of the animals, muscles of one extremity were dissected and incubated and muscles from the other extremity were freeze-clamped *in situ* (Table VI). Labeling of acid-precipitable protein in freeze-clamped muscles was similar in both control and uremic animals. On incubation, the amount of acid-precipitable <sup>14</sup>C-label decreased and this increased 62.7% ( $P < 0.05$ ) in muscles of uremic animals as compared with control animals. Similarly, there were proportional increments in nonprecipitable <sup>14</sup>C-label. Total nonprecipitable <sup>14</sup>C-label was increased on incubation 55% with uremic muscle ( $P < 0.05$ ), from 18.5  $\pm$  2.3 dpm/mg muscle with control preparations, to 28.6  $\pm$  2.5 dpm/mg muscle with uremic preparations.

Because insulin insensitivity or resistance has been postulated in chronic uremia (33–38), the effects of added insulin were studied on muscle alanine and glutamine formation, and release in uremia. As shown in Figs. 2 and 3, increasing concentrations of insulin were associated with an  $\approx$ 10% decrease in the rate of alanine and glutamine release from control preparations, but with preparations from chronically uremic rats, insulin produced a 30% suppression of amino acid release. Tissue levels of alanine and glutamine were unaltered. Significant suppression of amino acid release was observed with insulin concentrations of  $\approx$ 1–10 mU/ml in uremic preparations. Adrenergic

TABLE VI  
Effect of Incubation on the Distribution of [<sup>14</sup>C]Arginine in Skeletal Muscle from Normal and Chronically Uremic Rats Previously Labeled *in vivo*

Treatment	Muscle TCA		Released to media	Total nonprecipitable label	Increment in nonprecipitable label after incubation	Decrement in precipitable label after incubation
	Insoluble	Soluble				
<i>dpm/mg muscle, wet wt</i>						
Control						
Frozen, <i>in situ</i>	227.2 $\pm$ 7.5	67.4 $\pm$ 5.2	—	67.4 $\pm$ 5.2	—	—
Incubated	209.5 $\pm$ 5.9	32.8 $\pm$ 2.4	53.4 $\pm$ 5.3	85.9 $\pm$ 4.9	18.5 $\pm$ 1.4	17.7 $\pm$ 1.6
Uremic						
Frozen, <i>in situ</i>	221.6 $\pm$ 9.4	73.2 $\pm$ 5.0	—	73.2 $\pm$ 5.0	—	—
Incubated	192.8 $\pm$ 7.9	35.6 $\pm$ 1.4	66.3 $\pm$ 5.9	101.8 $\pm$ 5.8	28.6 $\pm$ 2.0	28.8 $\pm$ 2.3

Control and chronically uremic rats were injected with 50  $\mu$ Ci [*guanido*-<sup>14</sup>C]arginine, followed 6 h later by 20  $\mu$ mol of unlabeled arginine. 24 h later, epitrochlearis preparations were obtained and incubated. Preparations from the contralateral extremity were immediately freeze-clamped *in situ*. At the conclusion of the 2-h incubation, preparations were removed, frozen in liquid nitrogen, homogenized in 12% trichloroacetic acid, and the acid insoluble pellet washed and subsequently solubilized. Samples of the incubation media, the acid soluble extract of skeletal muscle, and of the acid insoluble pellet were counted in a liquid scintillation spectrometer. Values shown below are the means ( $\pm$ SEM) for at least eight experiments.

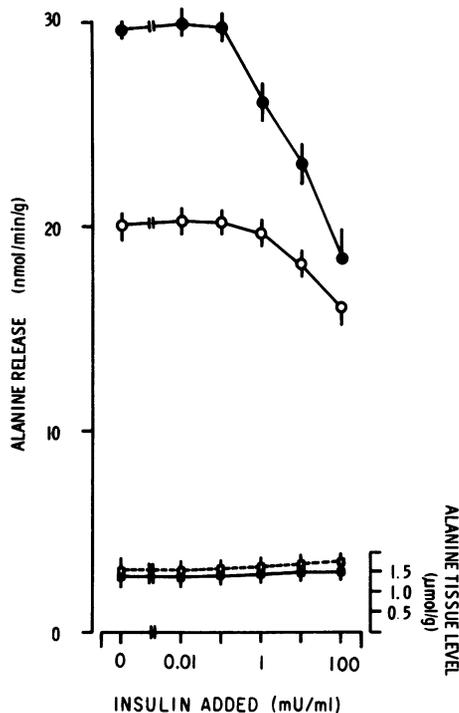


FIGURE 2 The effects of added insulin on alanine release and tissue levels in skeletal muscle of chronically uremic and control rats. Epitrochlearis preparations were obtained from chronically uremic (●) and control (○) rats and were incubated as described in detail in Methods in modified Krebs-Henseleit buffer (pH 7.4) containing crystalline glucagon-free pork insulin added to a final concentration as indicated. After a 1-h incubation, alanine was determined in the media using enzymatic techniques. Values shown for alanine release are the means ( $\pm$ SEM) for at least six experiments. Tissue levels of alanine were determined in acid extracts of muscle. Values shown are the means ( $\pm$ SEM) for at least six experiments.

agonists inhibit alanine and glutamine formation and release in skeletal muscle (39, 40). The extent of this inhibition has been shown to be generally proportional to the increments in muscle cAMP levels produced by  $\beta$ -adrenergic agonists. Accordingly, the influence of exogenous insulin on steady-state levels of cAMP in skeletal muscle of chronically uremic and control animals was also determined. A variety of incubation periods ranging between 2 and 60 min were investigated. Increasing concentrations of insulin caused no significant alteration of steady-state cAMP levels in skeletal muscle preparations from either control or chronically uremic animals.

Other actions of insulin on skeletal muscle metabolism in uremia were also investigated. As shown in Fig. 4, exogenous insulin produced a threefold increase in the rate of glucose uptake by skeletal muscle preparations from control animals. In preparations obtained from chronically uremic animals, basal

rates of glucose uptake were  $\approx$ 50% greater than rates obtained in preparations from control animals ( $P < 0.05$ ), and insulin produced a proportionally greater increase in the rate of glucose uptake in uremic muscles as compared with control muscles. At each concentration of exogenous insulin studied, rates of insulin-stimulated glucose uptake were 50–100% greater in uremic muscle as compared with control muscle. Other insulin-stimulated processes in muscle, such as protein synthesis, were also studied (Fig. 5). Basal rates of [4,5- $^3$ H]leucine incorporation into trichloroacetic acid precipitable protein were decreased  $\approx$ 25% in muscle from uremic animals as compared with control animals ( $P < 0.02$ ). With increasing concentrations of insulin, rates of leucine incorporation increased in both uremic and control preparations. At the lower concentrations of insulin studied (1–100 mU/ml), protein synthesis rates in uremic muscles remained somewhat lower than in control muscles. But, at the higher insulin levels studied (0.1–1 U/ml), rates of protein synthesis were equal with both preparations. In parallel studies, intracellular leucine pool size was found not to be significantly different in uremic preparations as compared with control preparations, either before or after incubation. Calculated intracellular leucine specific

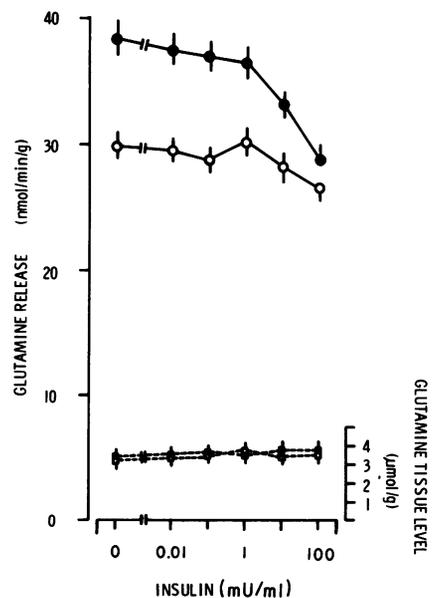


FIGURE 3 The effect of added insulin on glutamine release and tissue levels in skeletal muscle of chronically uremic and control rats. Epitrochlearis preparations were obtained from chronically uremic (●) and control (○) rats and were incubated for 1 h as detailed in Methods with crystalline glucagon-free pork insulin at concentrations indicated. The values shown are the means ( $\pm$ SEM) for at least six experiments.

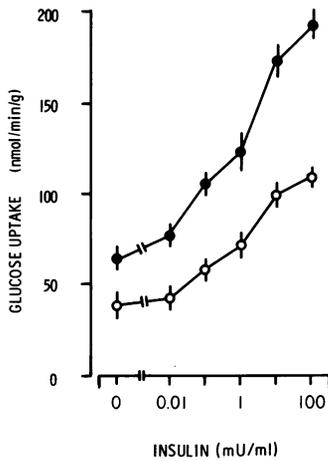


FIGURE 4 The effect of exogenous insulin on glucose uptake by skeletal muscle from control and chronically uremic rats. Epitrochlearis preparations from control (○) and chronically uremic (●) rats were obtained and incubated at 37°C for 1 h in a modified Krebs-Henseleit buffer containing 5 mM glucose, and insulin added to the concentrations indicated. As described in Methods, glucose concentrations were determined in the media before and after incubation. Glucose uptake was calculated by difference and the values shown are the means ( $\pm$ SEM) for at least eight experiments.

activities were not different in the preparations studied.

## DISCUSSION

The isolated intact epitrochlearis preparation of rat skeletal muscle, which we have described previously (21, 31, 32, 29), appears to provide an excellent model system for *in vitro* studies of potential derangements of muscle metabolism in uremia. A broad range of metabolic criteria were used to determine the suitability of the epitrochlearis preparation for *in vitro* studies of skeletal muscle metabolism, including (a) maintenance of linear rates of protein synthesis for periods up to 6-h duration, (b) a substantial facilitation by exogenous insulin of muscle glucose uptake, and (c) the preservation on incubation of muscle phosphocreatine and ATP levels which closely approximate those levels found in muscles freeze-clamped *in vivo*. Phosphocreatine and ATP levels in the incubated muscle preparations were approximately equal to levels observed in skeletal muscles of uremic animals freeze-clamped *in situ* (Table I), and insulin facilitation of glucose uptake was readily apparent (Fig. 4). Using these criteria, we can therefore conclude that the epitrochlearis preparation is a suitable model for studies of skeletal muscle metabolism in chronic uremia.

Although epitrochlearis preparations from chronically uremic rats demonstrated an increased rate of release of all amino acids studied, the observed in-

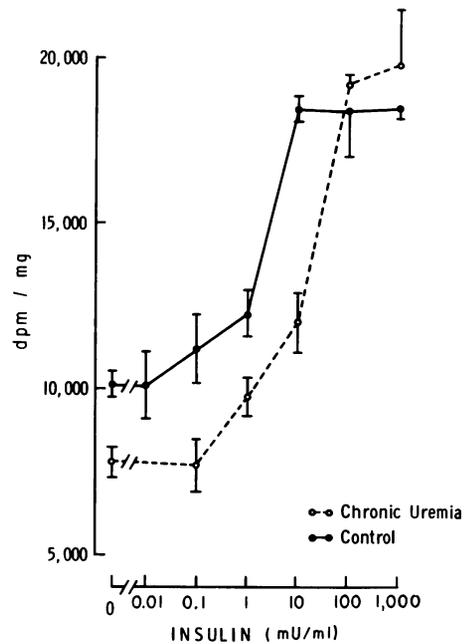


FIGURE 5 The effect of insulin on [4,5- $^3$ H]leucine incorporation into protein in skeletal muscle of control and chronically uremic rats. Epitrochlearis preparations from control (●) and chronically uremic (○) rats were obtained and incubated with 10  $\mu$ Ci [4,5- $^3$ H]leucine and insulin at the concentrations indicated as detailed in Methods. Protein was isolated from trichloroacetic acid homogenates, washed, solubilized, and counted. Values shown are the means ( $\pm$ SEM) for at least five experiments and are expressed as dpm of  $^3$ H-label incorporated per milligram muscle, wet weight.

creases in alanine and glutamine release were quantitatively the most substantial (Table II). This increased release of alanine and glutamine appears to result from an increased net formation of these two amino acids in uremic muscle because tissue levels of alanine and glutamine were essentially unaltered with incubation as compared with levels observed in muscle freeze-clamped *in situ*. These levels in uremic muscle were virtually identical to levels observed in muscle of control rats (Table III, Fig. 3). Although it is possible that uremia might induce compensatory decreases in pathways of muscle alanine reutilization which were not detected in studies with U- $^{14}$ C added to the media (Table IV), it seems more reasonable to conclude that the increased rates of alanine and glutamine release from skeletal muscle of uremic rats derive primarily from an increased formation of these two amino acids.

The stimulation of alanine and glutamine formation by a variety of amino acids has been well documented in a number of different rat skeletal muscle preparations (31-33), although the precise mechanism for this stimulation has not been established. The rate of alanine and glutamine formation in muscle has been related to the rate at which endogenous skeletal mus-

cle proteins are degraded, because it is the latter process which in part maintains the availability of endogenous amino acids for alanine and glutamine formation within the muscle cell (32). The observations of the present study on alanine and glutamine formation and release in skeletal muscle of chronically uremic animals suggest that the enhanced rate of alanine and glutamine formation in uremia may derive from an accelerated net protein degradation which may be caused by an increased breakdown of one or more classes of endogenous skeletal muscle proteins, or by an inhibition of protein synthesis, or both. Support for this conclusion is provided by the finding that the stimulated rates of alanine and glutamine formation produced by added amino acids were not substantially enhanced in uremic muscle as compared with control muscle (Table V). Because the maximum capacity of uremic skeletal muscle to form alanine and glutamine may not be altered, it seems possible that an increased availability of amino acids may be an important regulatory factor accounting for the increased alanine and glutamine formation in uremia. Experiments in which at least some of the proteins of the epitrochlearis preparations were partially labeled with [*guanido*-<sup>14</sup>C]-arginine in vivo suggest that an accelerated net degradation can also be observed in uremic skeletal muscle incubated in vitro (Table VI). Additionally, patterns of <sup>14</sup>C-labeling of muscle protein in vivo with [*guanido*-<sup>14</sup>C]arginine also suggest either enhanced pattern degradation or diminished protein synthesis or both in uremic animals (Fig. 1). As a consequence of either or both effects, the resultant increase in the availability of amino acids for alanine and glutamine formation may account in part for the increased rate of alanine and glutamine formation in skeletal muscle of uremic animals.

The precise biochemical pathogenesis for the increased muscle alanine and glutamine formation and release in uremia is not fully explained by the results of this study. Insulin has been shown to be a major hormonal factor modulating protein synthesis and protein degradation in skeletal muscle. Insensitivity or resistance to the metabolic actions of insulin in peripheral tissues such as skeletal muscle have been postulated in uremia (33–38). For the most part, these conclusions derive from observations of hyperinsulinism and carbohydrate intolerance or even overt fasting hyperglycemia in patients with chronic uremia. That the phenomenon of insulin insensitivity or resistance appears not to account for the increased formation and release of alanine and glutamine is shown by the data of Figs. 2 and 3. The extent of suppression of alanine and glutamine release by exogenous insulin was quantitatively greater in muscle from chronically uremic rats as compared with control rats. For example, the decrement produced in alanine release from uremic

muscle at the highest concentration of insulin studied was more than threefold greater than the decrement produced with muscles from control rats. These findings of heightened insulin sensitivity in uremic muscle were not limited to insulin effects solely on amino acid release. Other aspects of muscle metabolism also appeared to retain normal sensitivity to exogenous insulin. For example, rates of insulin-stimulated glucose uptake by skeletal muscle from uremic animals were equal to or greater than rates observed in muscle from control rats, and the magnitude of the insulin stimulation was approximately equal in both preparations. On the other hand, insulin-stimulated leucine incorporation into protein did appear to show some element of diminished sensitivity to exogenous insulin (Fig. 5). In this instance, lower basal rates of protein synthesis were observed in muscle from chronically uremic rats, although maximally stimulated rates of amino acid incorporation into protein with high concentrations of insulin were equal in uremic and control muscles. It seems reasonable to infer from these data that a unitary mechanism solely involving abnormalities of insulin action cannot account for the observed derangements of muscle metabolism in uremia. Insofar as muscle protein and amino acid metabolism are concerned, disturbances of insulin action do not appear to account for data of this study.

The results of the present study have important implications for an understanding of the abnormal carbohydrate metabolism in patients with chronic uremia. Although virtually every abnormality of glucose homeostasis has been reported in uremia, carbohydrate intolerance and even overt fasting hyperglycemia are most frequently encountered (36). Hyperglycemia may be associated with abnormalities of hepatic gluconeogenesis. Alanine, the principal amino acid precursor for hepatic glucose production (41), is quantitatively the most important substrate other than lactate for hepatic gluconeogenesis. Alanine and glutamine account for the bulk of amino acids released by muscle (21). Studies performed in vitro using an isolated perfused liver model for hepatic gluconeogenesis have demonstrated that uremia is associated with an increased maximal capacity for hepatic glucose production from amino acids (42, 43). Because studies performed in vivo as well as in vitro have demonstrated that the rate of alanine delivery can be rate limiting for gluconeogenesis (7, 44), the findings of the present study demonstrating an increased rate of alanine production and release from skeletal muscle in uremia, suggest that an increased rate of hepatic gluconeogenesis from alanine may indeed occur in vivo. Thus, accelerated gluconeogenesis in liver and accelerated substrate delivery from skeletal muscle may be combined to produce a chronically expanded blood glucose pool in uremia. In support of this concept, it should be

noted that increased hepatic gluconeogenesis, together with an increased rate of alanine delivery from skeletal muscle, have been observed in other hyperglycemic states such as diabetes mellitus (32, 45, 46).

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