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Muscle glutamine production in diabetic ketoacidotic rats

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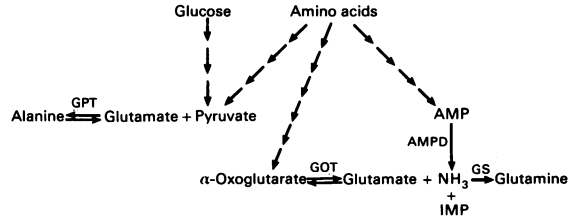
The mechanism of activation of glutamine production by the hindlimb during diabetic ketoacidosis (DKA) was investigated in rats. Muscle glutamine production was estimated to account for over 90% of the total glutamine produced by the hindlimb. DKA produced significant increases in the concentrations of NH_4^+ and IMP in hindlimb muscles, suggesting that AMP deaminase is activated by DKA. NH_4Cl- and HCl-induced acidosis did not produce these changes, indicating either that acidosis itself is not the stimulus for increased AMP deaminase activity or that the more severe degree of acidosis accompanying DKA is necessary for activation. Muscle glutamine concentrations were depressed in DKA. Experiments with isolated epitrochlearis muscle showed that the transport and permeability properties of the muscle cells (as judged by uptake and release of α-aminoisobutyrate and glutamine) were not altered by DKA. However, glutamine uptake by muscle cells was significantly inhibited by L-leucine, the concentration of which, along with other branched-chain amino acids, is markedly elevated in DKA.

In DKA, as in other forms of metabolic acidosis, large quantities of glutamine are taken up from the circulation by the kidneys and converted into ammonia (Goldstein *et al.*, 1980). The ammonia is excreted into the urine, where it aids in the elimination of protons and thereby in the renal compensation of metabolic acidosis. We have previously shown that the hindlimb can supply a significant fraction of the circulating glutamine taken up by the kidneys and that the production and release of glutamine by rat hindlimbs increases significantly in DKA (Schrock & Goldstein, 1981). The mechanism by which DKA increases glutamine synthesis in hindlimb is not known. The purpose of the present study was to determine the method by which glutamine synthesis is increased during DKA.

The scheme for glutamine synthesis in skeletal muscle is shown in Scheme 1. Previous studies in perfused isolated hindlimbs of rats have shown that glutamine is formed and released from tissue in the absence of a nitrogen source added to the medium and that the release in increased in diabetic rats (Ruderman & Berger, 1974). Under these conditions both the carbon and nitrogen atoms used in synthesizing glutamine would presumably come, in part at least, from amino acids derived from endogenous protein. In this regard it has been reported that protein degradation is increased in the muscles of diabetic rats (Dahlmann *et al.*, 1979). Nevertheless addition of amino acids such as leucine to the perfusion medium can accelerate the rate of synthesis of glutamine by rat hindlimb (Ruderman & Berger, 1974), and it is possible that circulating amino acids and ammonia are used as precursors for glutamine biosynthesis in the limbs. To gain some insight into the steps in the glutamine-biosynthetic pathways that are affected by DKA, we measured the concentrations of metabolites shown in Scheme 1 in freeze-clamped muscles from hindlimbs of normal



Scheme 1. *Hypothetical scheme for glutamine (and alanine) formation in skeletal muscle*
Abbreviations: GPT, glutamate–pyruvate transaminase; AMPD, AMP deaminase; GOT, glutamate–oxaloacetate transaminase; GS, glutamine synthetase.

Abbreviation used: DKA, diabetic ketoacidosis.

and DKA rats. We also made similar measurements of some of these metabolites in skin, since this tissue has been implicated in peripheral amino acid metabolism in both man (Pozefsky *et al.*, 1969) and experimental animals (Preedy & Garlick, 1981). In addition, the rates of glutamine synthesis by muscle and skin were assessed *in vitro*. Along with glutamine, muscle releases significant amounts of alanine into the circulation, and this release increases during DKA as well (Ruderman & Berger, 1974). Therefore, we measured the concentrations of alanine, as well as metabolites involved in alanine biosynthesis (Scheme 1), in both muscle and skin in normal and diabetic rats.

Methods

Animals and treatments

Male Sprague–Dawley rats weighing 150–250g were obtained from Charles River Laboratories and maintained on Purina Rat Chow and water supplied *ad libitum*, except where noted. Rats were made diabetic with streptozotocin and ketoacidotic by the method described previously (Goldstein *et al.*, 1980), except that food was withheld 12h before and 6h after injection with streptozotocin. Ketoacidosis was confirmed by arterial-blood pH and HCO_3^- analysis with a BMS 3 Mk2 Blood Micro System Radiometer. Animals were not used if the $[HCO_3^-]$ was above 20mM. Chronic NH_4Cl-acidotic rats had 1.5% NH_4Cl as their drinking-water source for 5–6 days. Chronic HCl acidosis was induced by stomach intubation with 10mmol of HCl/kg body wt. in a volume corresponding to 2.5% of body weight every 12h for 36h (three administrations). Muscle and blood samples were obtained 3h after the last intubation. Starved rats were deprived of food for 48h.

Muscle and skin sampling

Hindlimb muscle was sampled by freeze-clamping *in situ* with aluminium tongs precooled in liquid N_2. The rats were first anaesthetized with Nembutal (40mg/kg). The skin was cut around the 'ankle' and pulled back with forceps, exposing the hindlimb muscles. The exposed limb was wrapped with a gauze pad moistened with warm 0.9% NaCl and allowed to rest for 5min. The cooled tongs were then clamped over the biceps femoris, gastrocnemius and underlying muscles, and the muscle sample was cut away from the limb while still between the tongs. The sample was kept in liquid N_2 until ready for extraction. The frozen sample was weighed and ground to a powder in a precooled (−70°C) mortar and pestle. The sample was homogenized with 5vol. of 4% (w/v) $HClO_4$ as it warmed, centrifuged at about 10000*g* for 10min, and neutralized with KOH. For the enzymic analysis of AMP, ADP and IMP, the $HClO_4$ extract was neutralized with 1.4M-K_2CO_3/6.22M-triethanolamine instead of KOH. The $KClO_4$-free supernatant was frozen and later analysed for metabolite concentrations. Muscle extracts for amino acid analysis were prepared by homogenizing the frozen powder in 4% (w/v) sulphosalicylic acid (1ml/100mg of muscle). Blood for amino acid analysis was drawn from the abdominal aorta and centrifuged to remove erythrocytes, and the plasma was treated with sulphosalicylic acid to remove proteins. The supernatants were frozen and later adjusted to pH2.2 with LiOH and analysed with a Dionex D-556 automatic amino acid analyser modified as described by Lee (1974).

To confirm that the partial hindlimb muscle sample was representative of the whole hindlimb muscle, a series of DKA and control rats were assayed in which the entire hindlimb was freeze-clamped with large [4in (10cm) diameter] tongs. The limb was cut off at the upper femur, including most of the vastus muscle, and plunged into liquid N_2. With the limb placed in a mortar surrounded by liquid N_2, the skin was chiselled away and discarded, and the muscle chiselled off the bone. The muscle was then extracted in the same manner as the partial hindlimb muscle. Analysis of glutamine, glutamate, ammonia and alanine showed no statistical differences in whole leg compared with partial leg muscle. The same changes in control compared with diabetic ketoacidotic rats were noted in the whole-leg sampling.

To determine metabolite concentration in skin, a section of shaved hindlimb skin was freeze-clamped *in situ* and extracted with 4% $HClO_4$ in the same manner as for muscle. Ammonia, alanine, glutamine and glutamate were measured in the extracts.

Muscle water and chloride determinations

Muscle water and chloride space were determined by a modification of the method of Bergstrom *et al.* (1974). Muscle and plasma chloride were measured with a Buchler Digital Chloridometer. Large samples (0.5ml) were used for muscle chloride determinations on the low range of the chloridometer, but smaller samples (0.1ml) and the high range were used for plasma measurements. Plasma protein was measured by the method of Lowry *et al.* (1951). A normal membrane potential of (−)78mV for rat hindlimb muscle at 37°C (Albuquerque *et al.*, 1972) was assumed for the calculation of chloride distribution.

Muscle and skin incubation in vitro

The skin of the forelimb was cut and pulled back, exposing the ventral muscles, in Nembutal-anaesthetized rats. The epitrochlearis muscle was dissected free with a metal spatula and removed by cutting the tendons on each end. The muscle,

weighing about 30–40mg, was placed in a vial containing 1.5ml of modified Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) with 1.2mM-$CaCl_2$, 5mM-glucose and 5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH7.4. The vial was gassed with O_2/CO_2 (19:1) for 2min, capped and placed in a shaking water bath at 37°C. After preincubation for 30min, the muscle was transferred to 1.5ml of fresh buffer and again gassed for 2min. At the end of incubation for 1h the muscle was removed, blotted gently and freeze-clamped. Concentrated $HClO_4$ (0.64ml) was added to the incubation medium. The sample of medium was neutralized with KOH, centrifuged and later analysed for alanine, ammonia, glutamine and glutamate. The incubated muscle was ground and extracted as above. Since glutamine and alanine concentrations do not change in epitrochlearis during 1–2h incubations *in vitro* (Garber *et al.*, 1976), the rates of release of these amino acids into the incubation medium are measures of their rates of production.

Shaved hindlimb skin slices were cut free-hand on ice with a safety-razor blade as the skin was pressed between two glass slides. The slice (20–50mg) was incubated and extracted in a similar fashion as the muscle incubation. Release of alanine, glutamine and glutamate into the medium and tissue content were measured. Tissue contents did not change significantly during incubation.

Uptake and release of α-amino[^{14}C]isobutyric acid, [^{14}C]mannitol, [^{14}C]glutamine and [^{12}C]glutamine by epitrochlearis muscle

The epitrochlearis was removed and preincubated in Krebs–Ringer buffer for $\frac{1}{2}$h as described above. To measure uptake and subsequent release, paired muscles (from the same rat) were each incubated in two separate vials containing 1.5ml of Krebs–Ringer buffer with ^{14}C-labelled compound (1μCi/ml final concn.). The muscle used to measure uptake was removed after 1h, dipped briefly in three consecutive beakers each containing 5ml of buffer to remove excess label, blotted gently, weighed and placed in 1.0ml of Protosol (New England Nuclear) with shaking at 37°C for 24h. After digestion of the tissue, 15ml of scintillation fluid was added and the sample counted for radioactivity.

A preliminary experiment to measure release of [^{14}C]mannitol (New England Nuclear) was performed to distinguish extracellular efflux from intracellular efflux. After incubation for 1h with [^{14}C]mannitol, the muscle was removed, rinsed and placed sequentially in a series of vials each containing 1.5ml of buffer with no label for 1, 2, 4, 8, 16 and 32min. Samples from each time period were counted for radioactivity. A cumulative plot of released label showed a rapid efflux rate of mannitol for the first 15min, followed by an almost zero rate of release. Therefore measurement of release of α-amino[^{14}C]isobutyrate (New England Nuclear) was done after the first 15min of incubation. The muscle used to measure release was removed after incubation for 1h with the label, rinsed and placed in three consecutive vials for 5min each, then in four vials containing 1.5ml of buffer for 15min each. Samples were taken from the 15min periods to measure release rates. At the end the muscle was rinsed, blotted, weighed, digested in 1ml of Protosol and counted for radioactivity. Also 50μl of the original uptake medium and 100μl of the release medium were counted for radioactivity. All samples were counted for radioactivity with 1.0ml of Protosol and 15ml of scintillation fluid [4g of 2,5-diphenyloxazole and 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene] in a Packard Tri-Carb liquid-scintillation spectrometer. Corrections were made for quenching by using external standard ratios.

In [^{14}C]glutamine-uptake experiments, $^{14}CO_2$ was also measured to account for metabolism of glutamine during the uptake incubation. After incubation for 1h with label, the epitrochlearis was removed and digested as usual. The vial containing the medium was immediately re-stoppered with a tight-fitting rubber stopper containing a centre-well cup (Kontes Scientific Glassware). Protosol (0.2ml) was injected through the stopper into the centre well cup; 0.1ml of conc. $HClO_4$ was then injected into the medium. The vial was incubated for 1h further to allow the $^{14}CO_2$ to be released from the acidified medium and trapped in the Protosol. The stopper was removed and the centre well cup pushed into a scintillation vial. Then 0.8ml of Protosol and 15ml of scintillation fluid were added and the vial was counted for radioactivity as usual.

Glutamine release was not measured with the radio-isotope technique because of the metabolism of glutamine by the muscle. Instead one epitrochlearis was freeze-clamped at zero time, and its pair from the same animal was incubated for 15min (with no preincubation) and then freeze-clamped. The difference in tissue glutamine content between zero time and 15min was taken as the estimate of glutamine release. The glutamine in the medium was also measured to determine percentage recovery of released glutamine. A preliminary experiment showed 200 and 300% recovery at 30 and 45min incubation respectively, which suggested synthesis of glutamine. Therefore a brief time period of 15min, which gave 75–125% recovery, was chosen to minimize errors from metabolism and synthesis.

Metabolite analyses

Glutamine and glutamate (Lund, 1974), ammonia (Kun & Kearney, 1974), alanine (Williamson, 1974),

AMP and ADP (Jaworek *et al.*, 1974), and IMP (Grassl, 1974) were measured enzymically with a Gilford spectrophotometer. Pyruvate (Passonneau & Lowry, 1974) and α-oxoglutarate (Narins & Passonneau, 1974) were measured enzymically with a Turner fluorimeter. The alanine release by muscle and skin slices was assayed by the method of Williamson (1974) modified for the fluorimeter because of the small sample size and low concentration.

ATP, ADP, AMP and IMP were also determined by high-pressure liquid chromatography (Brown, 1970) on the Altex model 322 Advanced Gradient Research chromatograph. The values obtained for ATP, ADP and AMP by this method were similar and are averaged with those obtained by enzymic analysis. The values for IMP obtained by the chromatographic method were somewhat lower than with the enzymic analysis. However, since the directionality and magnitude of the changes in IMP concentrations observed were similar with both methods, all values were combined and used in calculating the means ± S.E.M. reported in the Tables.

All enzymes and substrates were purchased from Sigma Chemical Co., except for alanine dehydrogenase, which was obtained from Boehringer Mannheim. Statistical significance of the differences between group means was determined by Student's *t* test.

Results

Relative contributions of muscle, skin and adipose tissue to hindlimb glutamine and alanine production

Although muscle constitutes the bulk of the tissue in rat hindlimb (Ruderman *et al.*, 1971), we thought it worthwhile to evaluate the relative rates of glutamine and alanine synthesis in muscle and skin and used these data to assess the relative contribution of muscle, skin and adipose tissue to glutamine and alanine synthesis in rat hindlimb. Table 1 shows the glutamine and alanine synthesis (nmol/min per g of tissue) by muscle, thin slices of skin and pieces of adipose tissue. Epitrochlearis, a forelimb muscle, was chosen for its ability to maintain a normal physiological state *in vitro* (Garber *et al.*, 1976). Values for glutamine and alanine synthesis in adipose tissue were taken from Tischler & Goldberg (1980). As shown in Table 1, glutamine synthesis in muscle was approx. 5 times that in skin and 2–3 times that in adipose tissue per g of tissue. Alanine synthesis was also much greater in muscle than in either skin or adipose tissue.

Assuming that the production of glutamine and alanine by hindlimb muscles is of the same order as that in epitrochlearis (there is good evidence to support this assumption; Garber *et al.*, 1976), then the total production in hindlimb muscle can be calculated by multiplying glutamine and alanine production per g of muscle by the relative weight of muscle in hindlimb. The results of these calculations, with similar ones for skin and adipose tissue, are shown in Table 1. It is clear that muscle glutamine and alanine production accounts for 90% or more of the total production by hindlimb of these two amino acids. Therefore, in the studies described below, metabolite assays and other experiments were performed mainly on muscle, with a few assays done on skin as well.

Metabolite contents in freeze-clamped hindlimb tissues

The contents of key metabolites in the biosynthetic pathways for glutamine and alanine measured in freeze-clamped muscles of control and DKA rats are shown in Table 2. The blood bicarbonate concentration was 24.4 ± 0.8 mM (mean ± S.E.M. for 17 rats) in control rats and 8.7 ± 1.7 ($n = 18$) in DKA rats, indicating a severe degree of metabolic acidosis in the latter group.

Table 1. *Glutamine and alanine production in muscle, skin and adipose tissue of rat hindlimb*

Values are means ± S.E.M.; numbers of animals are shown in parentheses. 'Production' refers to the rate of glutamine (or alanine) formation by tissue incubated *in vitro* under the following steady-state conditions: muscle (epitrochlearis) and skin were incubated in Krebs–Ringer buffer containing 5 mM-glucose; adipose tissue was incubated in a similar solution containing 0.5 mM-L-leucine. For full details see the Methods section. Data on tissue weights are taken from Ruderman *et al.* (1971).

	Production *in vitro* (nmol/min per g)			Total hindlimb synthesis (nmol/min per 100 g of limb)	
Tissue	Glutamine	Alanine	Tissue wt. (g/100 g of hindlimb)	Glutamine	Alanine
Muscle	16.7 ± 2.0 (7)	12.2 ± 1.1 (9)	73	1219	891
Skin	2.6 ± 0.8 (8)	5.7 ± 0.7 (8)	12	31	68
Adipose	6.9 ± 1.4 (6)*	2.0 ± 0.0 (6)*	5	34	10

* Data taken from Tischler & Goldberg (1980).

Table 2. *Metabolite contents in hindlimb muscle and skin of normal and DKA rats*

For full details see the Methods section. Values are means ± S.E.M. for the numbers of animals shown in parentheses. Significance of differences from normal group: $^{*}P<0.001$; $^{**}P<0.005$; $^{***}P<0.01$.

		Metabolites (μmol/g of tissue)										Blood HCO_3^- (mM)
Tissue	Rats	Glutamine	Glutamate	Alanine	NH_4^+	Pyruvate	α-Oxo-glutarate	IMP	AMP	ADP	ATP	
Muscle	Normal	3.72 ±0.27 (17)	1.79 ±0.09 (17)	1.66 ±0.11 (16)	0.261 ±0.014 (17)	0.066 ±0.012 (12)	0.046 ±0.005 (12)	0.062 ±0.010 (10)	0.062 ±0.005 (10)	0.577 ±0.020 (10)	4.95 ±0.12 (10)	24.4 ±0.8 (17)
	DKA	2.44*** ±0.18 (18)	1.26*** ±0.17 (18)	1.57 ±0.09 (16)	0.442** ±0.057 (16)	0.042 ±0.007 (13)	0.026** ±0.004 (13)	0.128*** ±0.021 (12)	0.066 ±0.005 (12)	0.733*** ±0.032 (12)	6.76* ±0.14 (12)	8.7* ±1.7 (18)
Skin	Normal	1.98 ±0.13 (7)	2.56 ±0.23 (7)	3.17 ±0.24 (5)	1.21 ±0.14 (7)							
	DKA	1.54 ±0.21 (3)	1.37* ±0.17 (8)	2.69 ±0.13 (7)	1.10 ±0.11 (8)							

Muscle glutamine concentration was significantly lower in the DKA group, as was glutamate, but alanine concentration was relatively unaffected by DKA. Muscle ammonia concentration was elevated nearly 2-fold by DKA, which suggests that DKA might stimulate the activity of AMP deaminase, a key enzyme in the purine nucleotide cycle which is thought to be the major route of ammonia formation in skeletal muscle (Goodman & Lowenstein, 1977). The 2-fold increase in muscle IMP in DKA rats (Table 2) supports the idea that DKA activates AMP deaminase. The concentrations of the adenine nucleotides (ADP and ATP) were also somewhat elevated (25–35%) in muscle by DKA, but not to the same degree as IMP. The concentrations of the two keto acids involved in glutamine and alanine biosynthesis (α-oxoglutarate and pyruvate) tended to decrease in DKA, although only the former showed a statistically significant fall.

The unexpected fall in muscle glutamine concentration observed in DKA rats prompted us to measure the effect of DKA on the concentrations of other amino acids to determine whether there was a general effect of DKA on muscle amino acid contents. As shown in Table 3, there was a general tendency for amino acid concentrations to fall during DKA, with the notable exception of the branched-chain amino acids, valine, leucine and isoleucine, which were increased in muscles of DKA rats. The concentrations of three other amino acids, phenylalanine, α-aminoisobutyric acid and methionine, were significantly elevated too. Thus DKA does produce a general depression of the amounts of many amino acids in muscle, although the concentrations of some amino acids are elevated and those of others remain unchanged.

Muscle water and cell volume

To determine what role changes in muscle water content might have played in the increases and decreases in metabolite concentrations observed in DKA, we measured total tissue water content and chloride concentration and plasma chloride concentration. From these values we calculated intracellular and extracellular volumes by the method of Bergstrom *et al.* (1974). In eight normal rats, tissue water, intracellular and extracellular volumes were 756 ± 5 (mean ± S.E.M.), 606 ± 10 and 151 ± 12 ml/kg of muscle respectively. Muscle water content fell slightly (5%) but significantly ($P<0.05$) in eight DKA rats, to a value of 721 ± 3 ml/kg of muscle. This decrease was due to a significant ($P<0.02$) fall in extracellular water, to 114 ± 6 ml/kg of muscle, but intracellular water was unchanged (603 ± 13 ml/kg of muscle). Thus, since the changes in metabolite contents listed above were greater than 25% and can be assumed to be localized almost exclusively to the intracellular space, changes in muscle water content

Table 3. *Amino acid concentrations in arterial plasma and hindlimb muscle from control and DKA rats*
Values are means ± S.E.M. Significance of differences from control group: *$P < 0.001$; **$P < 0.01$; ***$P < 0.05$.

	Arterial plasma (nmol/ml)		Hindlimb muscle (nmol/g)	
	Control ($n = 9$)	Diabetic ($n = 8$)	Control ($n = 10$)	Diabetic ($n = 13$)
Taurine	89 ± 23	960 ± 263**	9040 ± 772	17820 ± 133*
Aspartate	18 ± 1	28 ± 5	823 ± 43	437 ± 45*
Hydroxyproline	107 ± 4	62 ± 5*	781 ± 67	210 ± 34*
Threonine	304 ± 29	482 ± 79	1050 ± 75	657 ± 64*
Serine	258 ± 10	289 ± 45	1330 ± 98	552 ± 44*
Asparagine	57 ± 5	118 ± 18**	224 ± 11	233 ± 19
Glutamate	75 ± 8	131 ± 14**	1960 ± 69	1220 ± 245*
Glutamine	512 ± 28	1164 ± 155*	4090 ± 217	2470 ± 203*
Proline	223 ± 19	281 ± 41	542 ± 48	484 ± 40
Glycine	470 ± 26	726 ± 101***	9920 ± 741	5350 ± 343*
Alanine	539 ± 26	494 ± 60	1880 ± 103	1890 ± 155
Citrulline	78 ± 3	169 ± 34***	280 ± 27	122 ± 32**
α-Aminobutyrate	—	321 ± 74*	—	295 ± 53*
Valine	169 ± 16	1030 ± 161*	183 ± 8	997 ± 102*
Cysteine	19 ± 7	18 ± 6	—	—
Methionine	41 ± 4	74 ± 8**	—	127 ± 10*
Isoleucine	65 ± 6	375 ± 54*	36 ± 16	395 ± 34*
Leucine	99 ± 11	713 ± 109*	48 ± 20	814 ± 78*
Tyrosine	65 ± 8	111 ± 13**	120 ± 11	164 ± 16***
Phenylalanine	44 ± 6	96 ± 9**	23 ± 11	142 ± 11*
Tryptophan	38 ± 4	20 ± 5**	—	—
Ornithine	67 ± 7	333 ± 64*	148 ± 18	125 ± 14
Lysine	244 ± 13	636 ± 93*	1100 ± 255	606 ± 72
Histidine	64 ± 2	303 ± 61**	195 ± 15	248 ± 28
Arginine	137 ± 16	99 ± 27	562 ± 118	283 ± 32***
Ethanolamine	52 ± 30	97 ± 15	—	—
β-Alanine	—	34 ± 11**	245 ± 33	188 ± 13
β-Aminoisobutyrate	—	14 ± 5**	—	—

or cell volume could not have played a significant role in bringing about the observed increases and decreases in metabolites during DKA.

Plasma amino acids

Although plasma amino acids have been measured previously in streptozotocin-treated rats (Bloxam, 1972), we repeated these measurements in our experimental animals, which had received a higher dose of the antibiotic (150 mg/kg). As shown in Table 3, striking changes occurred in the arterial plasma concentrations of several amino acids. In contrast with what we observed in muscle, there was a general tendency for amino acids to be increased in the plasma of DKA rats. Plasma glutamine concentration was markedly elevated by DKA and, as previously reported (Bloxam, 1972), the branched-chain amino acids were strikingly increased in DKA. The total plasma concentrations of valine, leucine and isoleucine rose from 0.33 mM in normal rats to 2.12 mM in DKA. However, not all plasma amino acids increased in DKA. For example, alanine concentration was relatively unchanged and tryptophan decreased.

Transport of α-aminoisobutyric acid and glutamine in isolated muscle from normal and DKA rats

The fall in concentration of glutamine and of other amino acids in the muscles of DKA rats could be due to an alteration in the transport or permeability properties of the muscle membrane in diabetic rats. It is known that insulin stimulates the uptake of certain amino acids by muscle (Castles *et al.*, 1965; Kipnis & Noall, 1958; Manchester & Young, 1960), and it is possible that in DKA the lack of insulin as well as other related metabolic factors may have contributed to the observed decrease in amino acids in muscle. Therefore we measured the rates of uptake and release of a non-metabolizable amino acid, α-aminoisobutyric acid, in strips of epitrochlearis muscle, isolated from control and DKA rats and incubated in Krebs–Ringer medium *in vitro*. In separate experiments (not shown) we found that insulin (0.4 unit/ml of medium) stimulated α-aminoisobutyric acid uptake in epitrochlearis just as it does in other muscle preparations.

Both uptake and release of α-aminoisobutyric acid were measured under conditions (see the Methods section) in which its movements into or out of the

muscle cells were linear with time. The rate of uptake of α-aminoisobutyric acid (Table 4) by epitrochlearis is similar to that observed in other skeletal muscles such as rat diaphragm (Kipnis & Noall, 1958). The rate of release of α-aminoisobutyric acid is rapid, with approx. 10% leaving the muscle cells during 15 min. As shown in Table 4, muscle from DKA rats took up α-aminoisobutyric acid at a rate similar to that observed in muscle from control rats. Muscle from DKA rats released α-aminoisobutyric acid at a somewhat lower rate than that observed in controls. Judging from experiments done on a single non-metabolizable amino acid, it appears that DKA does not produce a marked alteration in permeability or transport properties of the plasma membrane in skeletal-muscle cells.

To determine whether the conclusions derived from experiments with α-aminoisobutyric acid would apply to glutamine as well, we determined the rates of glutamine uptake and release by epitochlearis isolated from control and DKA rats. As shown in Table 4, glutamine uptake rates were essentially the same in muscles from normal and DKA rats. Approx. 15% of the glutamine taken up was metabolized to CO_2 by muscles from both control and DKA rats (results not shown).

The pattern of glutamine release from muscle was similar to that observed with α-aminoisobutyric acid (Table 4). There was a tendency (not statistically significant) for glutamine release to be somewhat lower from muscle of DKA rats than from controls. However, interpretation of these experiments is complicated by the fact that glutamine is degraded (as well as synthesized) during the incubation period used to assay for release. Only about 50% of the decrease in muscle glutamine (normal and diabetic) could be accounted for by glutamine appearing in the incubation medium.

Table 4. *Uptake and release rates for α-aminoisobutyrate and glutamine in isolated epitrochlearis muscle from normal and DKA rats*

Values are means ± S.E.M. *Significantly different from normal group, $P < 0.05$. Muscles were incubated in Krebs–Ringer bicarbonate solution with α-amino[^{14}C]isobutyric acid or [^{14}C]glutamine. After 1 h, half the muscles were analysed for radioactivity and uptake rates. In the α-aminoisobutyric acid experiments the remaining muscles were incubated in α-amino[^{14}C]isobutyric acid-free media and release rates were determined. In the glutamine experiments, release rates were determined on muscles incubated (without preincubation) in glutamine-free media. See the Methods section for details.

Amino acid	Group	*n*	Uptake (nmol/h per g)	Release (%/15 min)
α-Aminoisobutyric acid	Normal	10	18.0 ± 0.8	9.0 ± 0.5
	DKA	9	21.8 ± 1.6	7.0 ± 0.5*
Glutamine	Normal	6	7.2 ± 0.4	35.3 ± 1.9
	DKA	5	7.6 ± 0.3	28.8 ± 3.4

Effects of leucine on glutamine uptake by muscle

The branched-chain amino acids are known to be increased in the blood of diabetic humans (Felig *et al.*, 1970) and rats (Bloxam, 1972; Scharff & Wool, 1966; and Table 3). If these amino acids competed with glutamine for uptake by muscle, then the high blood concentrations found in DKA could lower muscle glutamine concentration by inhibiting its uptake into the cells. We found that both L-leucine and L-glutamine inhibited uptake of α-amino[^{14}C]isobutyric acid by epitrochlearis incubated in Krebs–Ringer bicarbonate medium; 1 mM of either amino acid in the incubation medium inhibited α-aminoisobutyric acid uptake by about 20%, indicating that leucine and glutamine may inhibit each other's transport in muscle, too.

In an experiment designed to test the direct effect of L-leucine on L-glutamine uptake by muscle, we measured the tissue content of ^{14}C and production of $^{14}CO_2$ from L-[^{14}C]glutamine by epitrochlearis incubated in Krebs–Ringer bicarbonate medium in the presence and absence of 1 mM-L-leucine. As shown in Table 5, the presence of L-leucine in the incubation medium significantly depressed the uptake of L-[^{14}C]glutamine by the muscle. The total amount of glutamine taken up by the tissue (tissue content plus metabolism) was inhibited by 21–27% by 1 mM-L-leucine. The percentage inhibition by leucine was relatively independent of the glutamine concentration. Approximately the same degree of inhibition was observed when either 3.8 μM- or 100 μM-glutamine was present in the incubation medium. Thus the inhibition of muscle glutamine transport by leucine appears to be non-competitive. These results support the idea that depressed content of glutamine in the muscle of diabetic ketoacidotic rats is due, in part at least, to the inhibitory action of the elevated blood concentrations of branched-chain amino acids on glutamine uptake by muscle.

Effects of acidosis and starvation on muscle metabolites

DKA in rats is accompanied by metabolic acidosis and decreased food intake (Goldstein *et al.*, 1980). In order to determine whether either of the latter conditions by themselves could have contributed to the metabolite changes observed in DKA, we measured the concentrations of those metabolites that had changed most in DKA, in rats made acidotic with NH_4Cl or HCl and in starved rats. In contrast with DKA, neither NH_4Cl- nor HCl-induced acidosis had significant effects on the concentration of any of the metabolites measured, except for glutamate, which decreased significantly

Table 5. *Leucine inhibition of glutamine uptake by epitrochlearis muscle*

Values are means ± S.E.M. for six experiments in which the uptake of glutamine by paired epitrochlearis muscles (25–35 mg each) was measured in the absence (control) and presence (experimental) of 1.0 mM-leucine. Two incubating concentrations of glutamine were used and are indicated as group A (3.8 μM-L-glutamine and 1.0 μCi of L-[^{14}C]glutamine/ml) and group B (100 μM-L-glutamine and 2.0 μCi of L-[^{14}C]glutamine/ml). Total glutamine uptake was calculated by summing the tissue glutamine ([^{14}C]glutamine in tissue) plus metabolized glutamine ($^{14}CO_2$) at the end of 1 h of incubation. *Control and experimental means are significantly different ($P < 0.05$).

	Glutamine uptake (nmol/h per g)		
Incubation	Control	Experimental (+1 mM-leucine)	Inhibition by leucine (%)
A [Glutamine] = 3.8 μM			
Tissue glutamine	12.4 ± 0.7	9.2 ± 0.8*	26
Metabolized glutamine	1.8 ± 0.2	1.2 ± 0.2	33
Total glutamine uptake	14.2 ± 0.9	10.4 ± 1.0*	27
B [Glutamine] = 100 μM			
Tissue glutamine	248 ± 18.1	197 ± 11.0*	21
Metabolized glutamine	23.9 ± 2.7	16.8 ± 1.3*	30
Total glutamine uptake	272 ± 20.3	214 ± 11.9*	21

Table 6. *Contents of key metabolites in hindlimb muscles from normal and starved rats, and from those with HCl- or NH_4Cl-induced acidosis*

For details see the Methods section. Values are means ± S.E.M. Significance of difference from untreated group: *$P < 0.005$; **$P < 0.01$; ***$P < 0.05$.

			Metabolites (μmol/g of tissue)				
Treatment	Duration (days)	n	Glutamine	Glutamate	NH_4^+	IMP	Blood HCO_3^- (mM)
None	—	6	4.09 ± 0.37	1.68 ± 0.09	0.23 ± 0.02	0.10 ± 0.01	26.5 ± 0.8
HCl-induced acidosis	1.5	7	3.93 ± 0.42	1.21* ± 0.09	0.21 ± 0.01	0.09 ± 0.01	18.5* ± 0.6
NH_4Cl-induced acidosis	6	6	4.14 ± 0.22	1.10** ± 0.15	0.28 ± 0.02	0.07 ± 0.01	
Starvation	2	6	4.04 ± 0.25	2.55*** ± 0.32	0.26 ± 0.03	0.10 ± 0.01	24.0 ± 0.8

in the muscles of rats with both NH_4Cl- and HCl-induced acidosis (Table 6). Similarly, starvation did not alter the concentration of any metabolite measured except for glutamate, which increased in concentration during the 2-day starvation. It appears therefore that decreased food intake does not contribute in any significant way to the alterations in muscle metabolite contents observed in DKA rats and that metabolic acidosis, to the degree induced in this study (blood $HCO_3^- = 18$ mM), is not a sufficient stimulus to bring about the metabolite changes seen in DKA.

Discussion

The aim of this study was to obtain information on metabolite concentrations in hindlimb muscle that would be helpful in determining the mechanism by which DKA causes increased production and release of glutamine by rat hindlimb (Schrock & Goldstein, 1981). We concentrated our attention on muscle, since this tissue constitutes about three-quarters of the metabolic activity of rat hindlimb (Ruderman *et al.*, 1971). Furthermore, we showed that muscle glutamine production can account for over 90% of the total (muscle, skin, adipose tissue) glutamine production by rat hindlimb (Table 1). However, some measurements were made in skin, too. The major pathway thought to be responsible for glutamine biosynthesis in muscle is shown in Scheme 1. One would expect that an increase in glutamine production in the hindlimb of DKA rats to be accompanied by an increase in concentration of glutamine in the muscle. Instead, however, a significant fall in glutamine concentration occurred in this tissue (Table 2). A similar fall (although not statistically significant) in glutamine concentration was observed in hindlimb skin of DKA rats.

Therefore the cause of this unexpected fall in muscle glutamine concentration during DKA was investigated further *in vitro*.

A fall in tissue concentration of metabolite whose production and release into the blood are elevated in the steady state can be due to a change in transport and/or permeability characteristics of the cell membrane to that metabolite. Insulin is known to enhance the uptake of amino acids by muscle cells (Castles *et al.*, 1965; Kipnis & Noall, 1958; Manchester & Young, 1960), and it is possible that the depressed concentration of glutamine in the muscle in DKA is due to an inhibition of uptake and/or accelerated release of the amino acids across the muscle cell membrane. However, using the non-metabolizable amino acid α-aminoisobutyric acid, which is transported across cell membranes by a system which it shares in part with glutamine (Christensen, 1975), and glutamine, we found no evidence to suggest that amino acid transport or permeability of muscle cells was altered in DKA. This finding was surprising, since insulin has been shown to stimulate α-aminoisobutyric acid uptake by isolated hemidiaphragm of rats, an effect that we duplicated in isolated epitrochlearis muscle (see the Results section).

If the glutamine-transport and permeability properties of the muscle cell membrane are not altered, then why does muscle glutamine concentration fall in DKA? The explanation may still have to do with transport changes that take place across the cell membrane. In DKA there are large increases in the concentrations of branched-chain amino acids in the extracellular fluid (Bloxam, 1972; Felig *et al.*, 1970; Scharff & Wool, 1966; see also Table 3). Since these amino acids inhibit the transport of glutamine across cell membranes (Christensen, 1975), it is possible that the markedly elevated concentrations of branched-chain amino acids in the extracellular fluid (Table 3) block the uptake of glutamine into muscle cells. In support of this idea, we found that leucine inhibited the uptake of glutamine by isolated epitochlearis muscle. Although this finding does not prove that the elevated concentrations of branched-chain amino acids in the blood of DKA rats are totally responsible for the depressed concentrations of glutamine in the muscle of these rats, it seems highly likely that they do contribute to the decrease.

As shown in Scheme 1, there are several potential sites of regulation in the pathway involved in the conversion of amino acids into glutamine. Insights into which of the steps might be affected by a stimulus to end-product formation can be gained by observing changes that take place along the pathway, particularly at steps that are irreversible. One such step is the deamination of AMP to NH_3 and IMP. We found that the concentrations of both NH_3 and IMP were significantly elevated in the muscles of DKA rats, with no significant increase in AMP concentration taking place. This observation suggests that AMP deaminase is activated during DKA (Goodman & Lowenstein, 1977). However, it should be pointed out that the conversion of [^{14}C]glutamine into $^{14}CO_2$ by muscle (Table 5) suggests the presence of a glutaminase in this tissue. If present, it is possible that, as in kidney, the enzyme could be activated by DKA, contributing to the increase in muscle ammonia. Such a process, of course, would be counter-productive to the supply of glutamine to the circulation by muscle. The extra ammonia formed in this reaction could increase glutamine production, since exogenous ammonia has been found to stimulate glutamine formation in perfused hindlimb (Ruderman & Berger, 1974). The fall in muscle glutamate concentration during DKA is consistent with an increase in activity of glutamine synthetase under these conditions. The flux through glutamine synthetase could be increased as a result of an increase in NH_3 concentration as well as other factors not measured in this study, such as synthesis of new enzyme or allosteric activation of pre-existing inactive enzyme. Hackenberg & Miller (1977) have reported a 2-fold increase in muscle glutamine synthetase activity in streptozotocin-treated rats. The fall in muscle α-oxoglutarate concentration during DKA could be related to an increase in glutamine synthetase activity, since glutamate (a substrate) would be consumed in the synthesis of glutamine, resulting in a 'pull' on α-oxoglutarate via the reversible glutamate dehydrogenase reaction in the direction of glutamate synthesis.

Alanine formation is determined mainly by the concentrations of substrates, pyruvate and glutamate, since its synthesis is catalysed by a highly active reversible enzyme, glutamate–pyruvate transaminase. Alanine formation is increased in the hindlimbs of DKA rats (Ruderman & Berger, 1974), but the concentrations of both pyruvate and glutamate are decreased in muscle, and glutamate concentration is decreased in skin as well. The concentration of alanine is not significantly altered by DKA in either tissue. These results are analogous to those found in starving rats, in which formation and release of alanine from skeletal muscle is increased, with no change occurring in the concentrations of alanine or glutamate in hindlimb muscle (Adibi, 1971). Thus the mechanism by which DKA (and starvation) increases alanine formation in skeletal muscle is not at all clear.

The changes in metabolite contents observed in the muscles of DKA rats could have been due to the accompanying acidosis, which is known to stimulate muscle glutamine biosynthesis (Schrock & Goldstein, 1981), or to the decreased food intake, which also stimulates glutamine (and alanine) release from muscle (Adibi, 1971), or a combination of both

effects. Therefore we determined the effects of acidosis (HCl- or NH_4Cl-induced) and starvation separately on the concentrations of those metabolites which showed the greatest changes in DKA. In contrast with DKA, neither acidosis nor starvation had a significant effect on the concentrations of glutamine, NH_4^+ or IMP. The concentration of glutamate, however, fell during both HCl- and NH_4Cl-induced acidosis, which is consistent with an activation of glutamine synthetase, but rose somewhat during starvation. Adibi (1971) also found no change in hindlimb-muscle glutamine concentration in starved rats, but he reported no significant change in muscle glutamate concentration during starvation.

The lack of effect of mineral acidosis and starvation on muscle NH_4^+ and IMP concentrations suggests that AMP deaminase is not activated under these conditions as it is in DKA. It should be noted, however, that the degree of acidosis was not nearly as great in HCl-induced acidosis (plasma $HCO_3^- = 18.5$ mM) as it was in DKA (plasma $HCO_3^- = 8.7$ mM), and no significant acidosis was present in the starved rats. This suggests that either the more severe degree of acidosis or possibly some other factor associated with DKA is necessary to activate AMP deaminase. The fall in muscle glutamate concentration in both HCl- and NH_4Cl-induced acidosis is consistent with the idea stated above, that glutamine synthetase is activated by a component of DKA such as metabolic acidosis. The rise in concentration of glutamate in starved rats indicates that either glutamine synthetase is not activated by this condition or that other compensating factors prevent the glutamate concentration from falling as it does during acidosis.

Increased glutamine release from muscle occurs in NH_4Cl- and HCl-induced acidosis as well as in DKA (Schrock & Goldstein, 1981). In the present study we found one metabolite change common to the three conditions: a significant fall in glutamate concentration. This change could reflect, among other possibilities, an activation of the glutamine synthetase reaction or an increased transamination of glutamate to aspartate, the latter compound serving as a nitrogen source for ammonia production via the purine nucleotide cycle. However, other metabolite changes found in muscle of DKA rats (e.g. increased NH_3 and decreased glutamine) were not observed in NH_4Cl- and HCl-induced acidosis. Although these differences might reflect qualitatively different metabolic patterns in mineral acidoses and DKA (e.g. markedly increased blood concentrations of branched-chain amino acids), it is just as likely that the greater degree of acidosis in DKA produced measurable changes in metabolite concentrations that went undetected in NH_4Cl- and HCl-treated rats. For example, in NH_4Cl-induced acidosis (Table 6) muscle ammonia was increased, but not sufficiently to be statistically significant at the $P < 0.05$ level.

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