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Relationship Between Plasma and Muscle Concentrations of Ketone Bodies and Free Fatty Acids in Fed, Starved and Alloxan-Diabetic States

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1. Concentrations of ketone bodies, free fatty acids and chloride in fed, 24–120h-starved and alloxan-diabetic rats were determined in plasma and striated muscle. Plasma glucose concentrations were also measured in these groups of animals. 2. Intracellular metabolite concentrations were calculated by using chloride as an endogenous marker of extracellular space. 3. The mean intracellular ketone-body concentrations (±S.E.M.) were 0.17±0.02, 0.76±0.11 and 2.82±0.50μmol/ml of water in fed, 48h-starved and alloxan-diabetic rats, respectively. Mean (intracellular water concentration)/(plasma water concentration) ratios were 0.47, 0.30 and 0.32 in fed, 48h-starved and alloxan-diabetic rats respectively. The relationship between ketone-body concentrations in the plasma and intracellular compartments appeared to follow an asymptotic pattern. 4. Only intracellular 3-hydroxybutyrate concentrations rose during starvation whereas concentrations of both 3-hydroxybutyrate and acetoacetate were elevated in the alloxan-diabetic state. 5. During starvation plasma glucose concentrations were lowest at 48h, and increased with further starvation. 6. There was no significant difference in the muscle intracellular free fatty acid concentrations of fed, starved and alloxan-diabetic rats. Mean free fatty acid intramuscular concentrations (±S.E.M.) were 0.81±0.08, 0.98±0.21 and 0.91±0.10μmol/ml in fed, 48h-starved and alloxan-diabetic states. 7. The intracellular ketosis of starvation and the stability of free fatty acid intracellular concentrations suggests that neither muscle membrane permeability nor concentrations of free fatty acids *per se* are major factors in limiting ketone-body oxidation in these states.

During catabolic states such as starvation and diabetes free fatty acids and the ketone bodies, acetoacetate and 3-hydroxybutyrate, compete as fuels for muscle metabolism. After prolonged starvation in man free fatty acids displaced ketone bodies as the preferred fuels for oxidation in striated muscle (Owen & Reichard, 1971; Hagenfeldt & Wahren, 1971). Studies *in vitro* and *in vivo* have also shown impairment of ketone-body oxidation in preparations from diabetic animals (Beatty *et al.*, 1959; Balasse & Havel, 1971; Goodman & Ruderman, 1972). The biochemical background for the diminished utilization of ketone bodies by muscle in these states is unknown. The activities of the key enzymes of ketone-body utilization do not appear to limit the oxidation of these fuels (Williamson *et al.*, 1971). Weidemann & Krebs (1969) concluded from studies *in vitro* that preference for free fatty acids or ketone bodies by rat kidney cortex depends on their relative concentrations. In prolonged starvation or diabetic ketoacidosis the blood concentrations of ketone bodies exceed those of free fatty acids (Cahill *et al.*, 1966; Wieland, 1968; Owen *et al.*, 1969; Stephens *et al.*, 1971; Hawkins *et al.*, 1971). However, the concentration of competitive substrates at the intracellular sites of metabolism may not be reflected by their blood concentration. Harrison & Long (1940) reported large concentration gradients for ketone bodies between plasma and muscle. Hawkins *et al.* (1971*b*) similarly reported large blood–brain concentration gradients for these substrates and suggested that relative impermeability of ketone bodies into tissues may be a factor limiting the rate of their metabolism. In contrast Schonfeld & Kipnis (1968) showed that the free fatty acid concentration in the rat diaphragm muscle exceeded that in plasma after 96h of starvation. It is therefore possible that the intracellular free fatty acid concentrations exceed that of acetoacetate and/or 3-hydroxybutyrate during prolonged starvation or diabetic ketoacidosis. The relative intramuscular availability of ketone bodies compared with free fatty acids may be an important factor in controlling oxidation of acetoacetate and 3-hydroxybutyrate.

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The present paper reports rat plasma and muscle concentrations of free fatty acids, acetoacetate and 3-hydroxybutyrate in various nutritional and alloxan-diabetic states.

Materials and Methods

Rats

Male Sprague–Dawley rats weighing 250–350g were used. The animal room was maintained at a temperature of 20–22.5°C. The lights were turned on from 06:00h to 18:00h. The rats were well adapted to their animal quarters. Starved animals were allowed only water *ad libitum*. Alloxan-diabetes was produced by a modification of procedures employed as a routine in the Fels Research Institute. After 48h starvation, the rats were given 2.0units of regular insulin (Eli Lilly and Co., Indianapolis, Ind., U.S.A.)/kg body wt. and 30min later 175mg of alloxan monohydrate (J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.; freshly purified by triple recrystallization from glass-distilled water)/kg body wt. was administered subcutaneously as a 3% solution (w/v) in 0.125M-citrate–sodium phosphate buffer, pH4.0 (Kass & Waisbren, 1945; Arteta *et al.*, 1954; Klebanoff & Greenbaum, 1954). During the first 24h after treatment 5% (w/v) dextrose in water was substituted for drinking water. By 48h after the alloxan treatment all surviving animals had glucosuria. Lente insulin (Eli Lilly and Co.) was then administered in amounts sufficient to promote weight gain. After at least 2 weeks of maintenance, insulin was withdrawn for 48h and those rats with glucosuria and ketonuria were killed (Prahl & Steenrod, 1965). Glucosuria and ketonuria was evaluated by Keto-Diastix™ (Ames Co., Elkhart, Ind., U.S.A.). In the alloxan-diabetic animals studied plasma glucose concentrations were in excess of 19μmol/ml (350mg/100ml) and ketone-body concentrations were 8.2±1.3μmol/ml (range 0.52–19.0μmol/ml).

Preparation of muscle and blood specimens

Fed, starved and alloxan-diabetic animals were treated so that nearly simultaneous procurement of muscle and blood was obtained. All animals were anaesthetized by an intraperitoneal injection of 50mg of pentobarbital (Abbott Laboratories, North Chicago, Ill., U.S.A.)/kg body wt. The skin was rapidly separated from the hind leg and the calf muscle was mobilized with blunt dissection. Rupture of major blood vessels was avoided. A portion of muscle, mainly gastrocnemius, was rapidly frozen *in situ* with aluminium clamps pre-cooled in liquid N_2 (Wollenberger *et al.*, 1960); the frozen portion was cut free and transferred to liquid N_2. The frozen tissue was powdered in a liquid-N_2-cooled percussion mortar. Weighed portions of the frozen-powdered muscle were transferred to pre-cooled Potter–Elvehjem homogenizers placed in a solid-CO_2–acetone bath.

After removal of frozen muscle from the hind limbs, blood was drawn from the inferior vena cava with a heparinized syringe. There was approximately a 1min lag between obtaining muscle and blood samples. The blood was centrifuged at 4°C and the plasma used to determine ketone bodies, free fatty acids and chloride. A portion (1ml) of whole blood was immediately added to a tube containing 7.5mg of KF, and after centrifugation at 4°C plasma was obtained for glucose determination. Two or more analyses were done on each sample.

Analytical procedures

Tissue for ketone-body measurements was rapidly mixed with ice-cold 0.3M-$HClO_4$ (5ml/g of tissue) and homogenized, care being taken that the tissue remained frozen until in contact with the $HClO_4$. After homogenization and centrifugation, the protein-free supernatant was neutralized with 1M-KOH–3M-K_2CO_3, left for 15min in an ice-water bath and the $KClO_4$ was removed by centrifugation. Samples of the supernatant were taken for determination of acetoacetate and D-(–)-3-hydroxybutyrate by a modification of the enzymic method of Mellanby & Williamson (1965). A 0.1M-potassium phosphate–hydrazine buffer (pH8.5) was used to determine D-(–)-3-hydroxybutyrate and a 0.1M-potassium phosphate buffer (pH7.0) was used to determine acetoacetate. Recovery from tissue of acetoacetate was 84±2% and of 3-hydroxybutyrate was 92±0.5%. Plasma samples were mixed with equal volumes of 1M-$HClO_4$ and the neutralized protein-free filtrate was analysed for acetoacetate and 3-hydroxybutyrate.

Free fatty acids were extracted from approx. 0.3g of weighed tissue, by the method of Dole (1956). After homogenization in 2ml of water and 10ml of Dole's extraction mixture, an additional 4ml of water plus 6ml of heptane were added. Portions (2ml) of the heptane phase were analysed for free fatty acids by a modification (Mahadevan *et al.*, 1969) of the colorimetric method of Duncombe (1963). The heptane samples were dried and the residue was taken up in 2.0ml of chloroform. Phospholipids were then removed by the addition of 0.4g of silicic acid followed by 0.4ml of water. The extracted chloroform phase was then mixed with 1ml of copper reagent. After removal of the aqueous copper reagent, 1ml of chloroform was mixed with 0.18ml of colour reagent and the E_{440} of the samples was read (Duncombe, 1963). Recovery of palmitic acid was 87±0.5% and there was no interference by up to 200μmol of 3-hydroxybutyrate or acetoacetate/ml.

Plasma samples, 0.2–0.4 ml in a final volume of 0.4 ml, were extracted with 2 ml of Dole's extraction mixture; then 1.2 ml of heptane and 0.8 ml of water were added. A portion (1 ml) of the heptane phase was analysed for free fatty acids as described above with omission of the silicic acid extraction.

Chloride concentrations were measured by the Aminco–Cotlove (American Instrument Co., Silver Spring, Md., U.S.A.) automatic titration method (Cotlove, 1961). Approx. 0.5 g of weighed frozen-powdered muscle was homogenized with 4 ml of deionized–distilled water, centrifuged and the supernatant analysed for chloride. Recovery of exogenous chloride was 90±0.4% and the analysis gave results equivalent to those obtained for muscle that had been ashed and then dissolved in boiling water. Plasma chloride was determined directly by the Aminco–Cotlove method.

Plasma was deproteinized with $Ba(OH)_2$ and $ZnSO_4$, and glucose was determined by the Technicon (Technicon Corp., Tarrytown, N.Y., U.S.A.) auto-analyser glucose oxidase–peroxidase procedure (Hill & Kessler, 1961).

Calculations

The concentrations of acetoacetate and 3-hydroxybutyrate in μmol/ml of plasma water (c_p) or muscle water (c_m) were calculated by assuming a water content of 93% (w/w) for plasma (Altman, 1961) and 77% (w/w) for muscle (Kobayashi & Yonemura, 1967). The muscle water is the sum of the intracellular (c_i) and extracellular (e) water. The distribution of chloride (c_m/c_p) was used as a measure of extracellular water, since chloride is predominately excluded from the intracellular compartment of rat skeletal muscle (Manery & Hastings, 1939; Muntwyler, 1968). We found that the chloride space was comparable in size with the raffinose space (Kipnis *et al.*, 1959). The chloride ion was therefore used as an endogenous marker for extracellular space. It was assumed that a ketone-body distribution greater than the chloride space indicated intracellular accumulation.

Water concentrations of ketone bodies were calculated by using the expressions:

(1) μmol/ml of plasma water (c_p) = (μmol/ml of plasma)/0.93

(2) μmol/ml of muscle water (c_m) = (μmol/g of muscle)/0.77

(3) μmol/ml of intracellular muscle water:

(a) $(\mu\text{mol})_{\text{intracellular}} = (\mu\text{mol})_{\text{muscle}} - (\mu\text{mol})_{\text{extracellular}}$

(b) $(H_2O)_{\text{intracellular}} = (H_2O)_{\text{muscle}} - (H_2O)_{\text{extracellular}}$

(c) $c_i = \dfrac{(\mu\text{mol})_{\text{intracellular}}}{(H_2O)_{\text{intracellular}}} = \dfrac{c_m - c_p \cdot e}{1-e}$

At physiological concentrations approx. 99% of plasma free fatty acids are bound to albumin (Spector, 1971). Albumin comprises 4% (w/w) of rat plasma (Albritton, 1952). Therefore, the concentration of free fatty acids in the albumin–water complex (c'_p) was calculated assuming that free fatty acids are distributed in 97% of plasma [this represents the sum of 93% (w/w) water plus 4% (w/w) albumin]. Plasma free fatty-acid albumin–water complex concentrations were computed by using the expression:

$$(4)\ c'_p = (\mu\text{mol/ml of plasma})/0.97$$

A highly specific and soluble intracellular free fatty acid-binding protein has been identified by Ockner *et al.* (1972). This protein provides transportation of free fatty acids in the aqueous cytoplasmic milieu. Protein comprises about 20% (w/w) of striated muscle, at least two-thirds of which is insoluble contractile elements (Fenn, 1946). Of the remaining 7% (w/w) of striated muscle it seems unlikely that any more than one-twentieth can be estimated as the free fatty acid-binding protein (Fleischner *et al.*, 1972; Ockner *et al.*, 1972). This minute amount of soluble, free fatty acid-binding protein can be disregarded in calculating free fatty acid concentrations in muscle water (c_m).

For free fatty acids, extracellular water (e') was defined as the chloride distribution (c_m/c'_p). As with ketone bodies, it was assumed that a free fatty acid distribution greater than the chloride space indicated intracellular free fatty acids. Thus free fatty acid concentrations in intracellular water were computed by using the expression:

$$(5)\ c'_i = \frac{c_m - c'_p \cdot e'}{1-e'}$$

To avoid errors in the mathematical treatment of the data, c_i and c'_i were calculated from the individual e and e' values.

The (intracellular muscle water concentration)/(plasma water concentration) ratios (c_i/c_p or c'_i/c'_p) were calculated to determine the gradients between extracellular and intracellular water spaces.

Statistics

Student's *t* test (small-sample method), correlation coefficient (*r*), and least-square regression line were used for statistical analyses (Hoel, 1960).

Results

Chloride distribution

The values obtained for fed, starved and alloxan-diabetic animals are presented in Table 1. By the third day of progressive starvation there was a small

Table 1. *Chloride distribution and substrate concentrations in fed, starved and alloxan-diabetic rats*

Concentrations are expressed as μmol/ml of plasma and μmol/g of muscle. Values are means ± S.E.M., with the number of animals in parentheses. Values that are statistically different from those for fed rats are indicated by *(P<0.001), †(P<0.005) and ‡(P<0.05). For other details see the Materials and Methods section.

		Total ketone bodies			Free fatty acids			
State of rats	Chloride distribution	Plasma	Muscle	Muscle/plasma ratio	Plasma	Muscle	Muscle/plasma ratio	Glucose in plasma
Fed	0.21 ± 0.01 (10)	0.34 ± 0.02 (10)	0.17 ± 0.02 (10)	0.50	0.43 ± 0.07 (7)	0.55 ± 0.04 (7)	1.3	9.5 ± 0.6 (10)
24h-starved	0.21 ± 0.01 (6)	1.28 ± 0.11 (6)*	0.58 ± 0.06 (6)*	0.45	0.70 ± 0.04 (5)‡	0.77 ± 0.13 (5)	1.1	6.8 ± 0.3 (7)†
48h-starved	0.21 ± 0.01 (5)	2.20 ± 0.26 (6)*	0.92 ± 0.08 (6)*	0.42‡	0.72 ± 0.05 (5)‡	0.71 ± 0.04 (5)‡	1.0	5.9 ± 0.2 (6)*
72h-starved	0.17 ± 0.01 (6)*	1.08 ± 0.15 (6)*	0.44 ± 0.06 (6)*	0.41‡	0.68 ± 0.08 (6)‡	0.75 ± 0.07 (6)‡	1.1	7.1 ± 0.4 (6)‡
96h-starved	0.18 ± 0.004 (6)*	1.44 ± 0.19 (6)*	0.60 ± 0.07 (6)*	0.42‡	0.59 ± 0.07 (6)	0.59 ± 0.03 (6)	1.0	6.8 ± 0.4 (6)†
120h-starved	0.16 ± 0.002 (5)*	1.31 ± 0.25 (5)*	0.54 ± 0.08 (5)*	0.41‡	0.59 ± 0.05 (5)	0.57 ± 0.05 (5)	1.0	—
Alloxan-diabetic	0.23 ± 0.01 (17)	8.20 ± 1.27 (16)*	3.39 ± 0.54 (16)*	0.41†	1.17 ± 0.12 (17)†	0.77 ± 0.05 (17)‡	0.7‡	27.5 ± 1.7 (17)*

decrease in the volume of chloride distribution in the muscle. The chloride distribution was not changed significantly in muscle obtained from alloxan-diabetic rats.

Ketone-body concentrations

Plasma ketone-body concentration increased sixfold after starvation for 48h (Table 1). Thereafter total plasma ketone bodies decreased to an amount approximately threefold higher than controls. The mean ketone-body concentrations in alloxan-diabetic rats were more than 21-fold higher than in fed animals. Plasma ketone bodies ranged from 0.21 μmol/ml in a fed animal to 19.0 μmol/ml in the most ketotic rat.

The mean (muscle concentration, per g wet wt.)/(plasma concentration, per ml) ratio, 0.42 remained comparable ($r = 0.99$, $P < 0.001$) over a 90-fold increase in circulating ketone-body concentrations (Fig. 1). The individual group results for muscle/plasma concentration gradients were lower ($P < 0.05$) in starved and alloxan-diabetic than in fed rats, being 0.43 ± 0.01, 0.42 ± 0.01 and 0.51 ± 0.03 respectively.

Since the volume of ketone-body distribution (c_m/c_p) was larger than the chloride space, e (Table 2), striated muscle obtained from fed, starved and alloxan-diabetic rats contained intracellular quantities of ketone bodies. Calculated in terms of plasma and muscle water, the mean intramuscular (c_i) ketone-body concentrations rose from 0.17 μmol/ml of water in fed animals to a maximum of 0.76 μmol/ml of water on the second day of starvation and then declined. In the muscle of alloxan-diabetic rats the mean intracellular concentration was 2.82 μmol/ml of water with a maximal accumulation of 7.22 μmol/ml of water in the most ketotic animal.

When the results are corrected for extracellular ketone bodies it becomes clear that increased plasma concentrations were not directly paralleled by increased intracellular concentrations. There was a tendency for the (intracellular muscle water concentration)/(plasma water concentration) ratio to decrease with increasing plasma ketone-body concentrations. Thus, the gradient between intracellular muscle water and plasma water increased as the animals become more ketotic.

Free fatty acid concentrations

Plasma free fatty acid concentrations increased 1.7-fold after starvation for 48h and decreased slowly thereafter (Table 1). The mean plasma free fatty acid concentrations in the alloxan-diabetic rats were 2.7-fold higher than those in fed animals. A scatter plot of the individual free fatty acid concentrations in whole muscle and plasma (Fig. 2) demonstrated

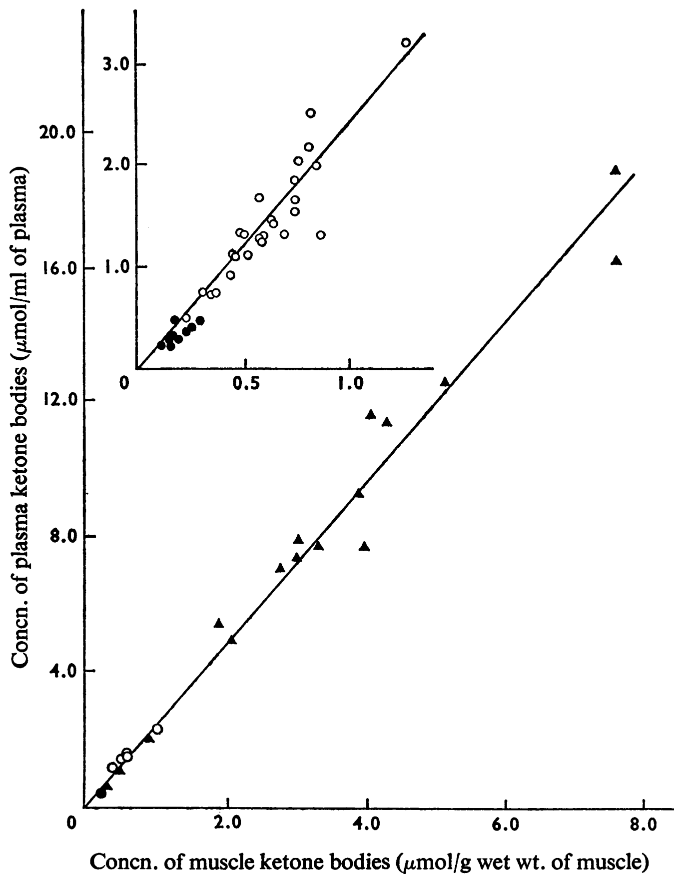

Fig. 1. *Relationship between total muscle and plasma ketone-body concentrations*

For experimental details see the Materials and Methods section. Animal preparation: ●, fed; ○, 24–120h-starved; ▲, alloxan-diabetic. Individual values were plotted for alloxan-diabetic rats and mean concentrations for fed, 24h-, 48h-, 72h-, 96h- and 120h-starved rats: for values for individual fed and starved animals see the inset. The line indicates the regression equation of the plasma concentration on the muscle concentration, $y = 0.02+2.38\,x$ ($r = 0.99$; $P < 0.001$).

that there was no constant relationship between the concentrations in these two compartments ($r = 0.4$). Whole muscle free fatty acid concentrations tend to cluster between 0.4 and 0.9μmol/g whereas plasma concentrations range from 0.2 to 2.5μmol/ml. When free fatty acid concentrations are expressed in terms of their aqueous milieu, the calculated concentration differences between the albumin–water complex and the intracellular binding-protein–water complex become more obvious (Table 3).

Since the volume of free fatty acid distribution (c_m/c'_p) was larger than the chloride space (e'), muscle from fed, starved and alloxan-diabetic rats contained intracellular free fatty acids. When the free fatty acid concentrations of whole muscle are corrected for circulating free fatty acids and the values expressed in terms of aqueous milieu $(c)'_i$ there were no significant changes in the intracellular concentrations of free fatty acids between the fed, starved and alloxan-diabetic states (Table 3). However, mean free fatty acid concentrations in the albumin–water complex (c'_p) varied from 0.45μmol/ml in the fed rats to 0.74μmol/ml in the 48h-starved rats and to a maximum of 1.21μmol/ml in the alloxan-diabetic rats. The independence of the intracellular free fatty acids to the fluctuation of the plasma free fatty acids

Table 2. *Extracellular space (e) and ketone-body concentrations in plasma water (c_p), muscle water (c_m) and intramuscular water (c_i)*

Concentrations are expressed as μmol/ml of water, and are the means±S.E.M. with the number of animals in parentheses. Results were calculated by means of the equations derived in the Materials and Methods section. Values that are statistically different from those for fed rats are indicated by *(P<0.001), †(P<0.005) and ‡(P<0.05).

State of rats	e	c_p	c_m	c_m/c_p	c_i	c_i/c_p
Fed (10)	0.26±0.01	0.36±0.02	0.22±0.02	0.61	0.17±0.02	0.47
24h-starved (6)	0.25±0.01	1.37±0.11*	0.75±0.08*	0.55	0.54±0.07*	0.39
48h-starved (6)	0.25±0.01	2.55±0.26*	1.20±0.13*	0.47‡	0.76±0.11*	0.30‡
72h-starved (6)	0.20±0.01†	1.29±0.13*	0.63±0.06*	0.48‡	0.47±0.04*	0.36
96h-starved (6)	0.21±0.01†	1.54±0.20*	0.79±0.10*	0.51‡	0.57±0.07*	0.37
120h-starved (5)	0.20±0.001†	1.40±0.27*	0.70±0.10*	0.50†	0.54±0.07*	0.39
Alloxan-diabetic (17)	0.27±0.01	8.81±1.37*	4.40±0.70*	0.50†	2.82±0.50*	0.32†

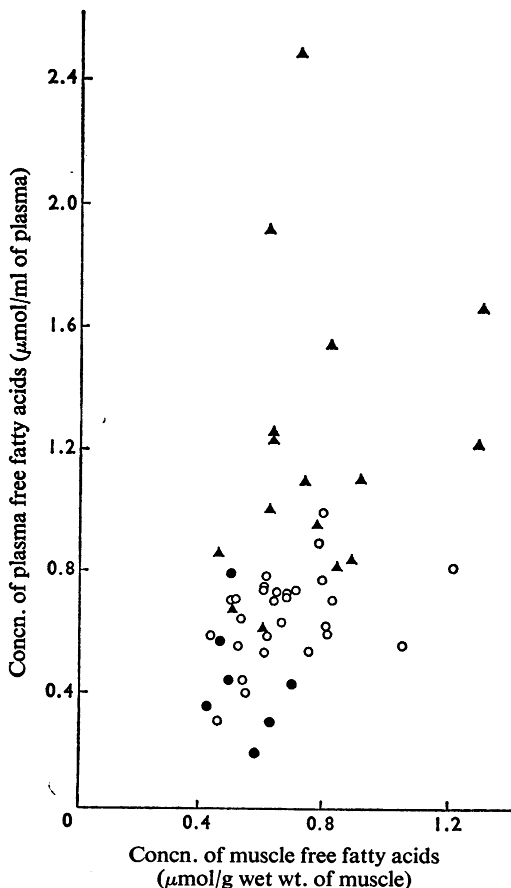

Fig. 2. *Relationship between total muscle and plasma free fatty acid concentrations*

For experimental details see the Materials and Methods section. Animal preparation: ●, fed; ○, 24–120h-starved; ▲, alloxan-diabetic; (r = 0.4).

resulted in a maximum c_i'/c_p' ratio of 1.8 in the fed animals and a minimum ratio of 0.7 in the hyperlipidaemic diabetic animals.

Glucose concentrations

Plasma glucose concentration (Table 1) fell from 9.5±0.6μmol/ml in fed rats to a minimal value of 5.9±0.2μmol/ml after starvation for 48h (P<0.001). With continued starvation glucose concentrations rose and approached the values for fed animals. In the alloxan-diabetic rat plasma values ranged from 19.5 to 49.5μmol/ml (350–891mg/100ml).

Discussion

Ketone-body oxidation by tissues requires the availability of acetoacetate and 3-hydroxybutyrate and the presence of the key enzymes 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), 3-oxo acid CoA-transferase (EC 2.8.3.5) and acetoacetyl-CoA thiolase (EC 2.3.1.9). Undefined enzyme activators or inhibitors, competitive substrates and the hormonal milieu may also modulate ketone-body oxidation. Since the activities of degrading enzymes are not thought to limit the rate of ketone-body oxidation (Krebs *et al.*, 1971) the present study explored the intracellular availability of ketone bodies and free fatty acids in striated muscle.

The results presented here show that intracellular ketone-body concentrations in striated muscle increased as plasma concentrations rose during starvation and alloxan-diabetic states (Table 2). However, at high circulating concentrations the ketone-body concentration gradients between plasma and muscle increased. Thus, the relationship between acetoacetate and 3-hydroxybutyrate in the aqueous plasma and intracellular compartments appeared to diverge as the circulating concentrations increased. Our study differs from the finding of Harrison & Long (1940),

Table 3. *Extracellular space (e') and free fatty acid concentrations in the plasma albumin–water complex (c'_p) in the muscle (c_m) and in the intramuscular protein-bound water complex (c'_i)*

The values are expressed as μmol/ml of water and are means±S.E.M. with the number of animals in parentheses. Results were calculated by the equations in the Materials and Methods section. Values that are statistically different from those for fed rats are indicated by *($P<0.005$), †($P<0.025$) and ‡($P<0.05$).

State of rats	e'	c'_p	c_m	c_m/c'_p	c'_i	c'_i/c'_p
Fed (7)	0.26±0.01	0.45±0.07	0.71±0.05	1.6	0.81±0.08	1.8
24h-starved (5)	0.26±0.02	0.72±0.04†	1.00±0.17	1.4	1.09±0.21	1.5
48h-starved (5)	0.26±0.01	0.74±0.06†	0.92±0.05†	1.2	0.98±0.07	1.3
72h-starved (6)	0.21±0.01†	0.70±0.08‡	0.98±0.10†	1.4	1.05±0.12	1.5
96h-starved (6)	0.22±0.01‡	0.60±0.07	0.76±0.04	1.3	0.81±0.04	1.4
120h-starved (5)	0.20±0.002†	0.61±0.05	0.74±0.06	1.2	0.77±0.08	1.3
Alloxan-diabetic (17)	0.28±0.01	1.21±0.13*	1.00±0.07‡	0.8‡	0.91±0.10	0.7‡

who only detected intracellular ketone bodies in striated muscle at high plasma concentrations. The discrepancy may be due to the greater sensitivity and specificity of the analytical methods currently employed.

The alloxan-diabetic rats used in this study were severely diabetic and were dependent on exogenous insulin for survival. When these animals were deprived of exogenous insulin for 48h severe diabetic ketosis developed. In this hypoinsulinaemic state the concentration gradient between the intracellular and plasma compartments was large. Nevertheless, the mean ketone-body concentration was 2.8μmol/ml of intracellular water (Table 2), with a maximal concentration in the most ketotic animal of 7.2μmol/ml. Ketosis caused by starvation also resulted in a significantly high intracellular concentration of ketone bodies. Therefore, it seems unlikely that muscle permeability of ketone bodies is a major factor in limiting the rate of acetoacetate or 3-hydroxybutyrate oxidation in muscle during hyperketonaemia secondary to either starvation or diabetic ketosis.

The increased accumulation of intracellular ketone bodies in ketotic animals was not necessarily the result of impaired utilization by muscle. However, accumulation implied that the rates of delivery from the bloodstream exceeded the rates of metabolism. Only intracellular 3-hydroxybutyrate increased during starvation, whereas concentrations of both acetoacetate and 3-hydroxybutyrate were elevated in alloxan diabetes. In starvation ketosis there is a direct relationship between circulating concentrations of ketone bodies and the rates of appearance and disappearance of these substrates in the bloodstream (Bates *et al.*, 1968; McGarry *et al.*, 1970). Further measurements of arteriovenous differences of ketone bodies across the extremities of rats starved for 48h (Ruderman *et al.*, 1971) showed that these substrates are major oxidative fuels for striated muscle. Their study demonstrated that acetoacetate is extracted in preference to 3-hydroxybutyrate even though 3-hydroxybutyrate concentration exceeded that of acetoacetate. The lack of intracellular accumulation of acetoacetate during starvation adds evidence that this fuel is rapidly utilized by the muscle of starved rats. In contrast, in man fasted for several weeks (Owen & Reichard, 1971; Hagenfeldt & Wahren, 1971), in depancreatized dogs (Basso & Havel, 1970), and in alloxan-diabetic rats (Goodman & Ruderman, 1972) the extraction and utilization of ketone bodies by muscle were markedly depressed. In the present study the results for alloxan-diabetic rats showed elevated intracellular concentrations of both acetoacetate and 3-hydroxybutyrate. Although the results do not imply impaired oxidation, they do demonstrate that muscle from the diabetic rat cannot utilize ketone bodies fast enough to prevent intracellular hyperketosis.

There is no known cause for depressed ketone-body metabolism by muscle from severely diabetic animals, from man after prolonged fast, and perhaps, from decompensated diabetic man. We previously suggested the possibility that intracellular free fatty acid concentrations relative to those of acetoacetate and/or 3-hydroxybutyrate may directly influence the preference for one oxidative substrate over the other in various catabolic states (Owen & Reichard, 1971). The results presented here do not support this proposal.

Intracellular free fatty acid concentrations in rat hind-limb striated muscle, calculated in terms of their aqueous milieu, were not significantly different in the fed, starved or alloxan-diabetic states (Table 3). These results are similar to the findings of Kraupp *et al.* (1967) that there were no differences in free fatty acid concentrations in heart muscle obtained from fed, starved or diabetic rats. Our results showed that the plasma concentration was lower than the intracellular concentration in the fed state, approached the intracellular concentration in the starved states, and exceeded the intracellular concentration in the alloxan-diabetic state. These results support the contention of Schonfeld & Kipnis (1968) that free fatty

acid pools in plasma and rat diaphragm muscle vary independently. Our results suggest that this changing relationship was due to a relatively constant intracellular free fatty acid concentration in contrast to the fluctuating plasma free fatty acid concentrations. The stability of the intracellular concentration of fatty acids may reflect the affinity and saturation of the recently identified fatty acid-binding protein which permitted these poorly water-soluble substrates to traverse the cytosol (Ockner *et al.*, 1972).

When ketone-body extraction and presumptive oxidation were maximal during starvation, especially at 48h, the intracellular free fatty acid concentration exceeded those of acetoacetate or acetoacetate plus 3-hydroxybutyrate. Conversely, when ketone-body oxidation was decreased in severe diabetes the intracellular concentrations of acetoacetate or acetoacetate plus 3-hydroxybutyrate were equal to or greater than those of free fatty acids (Tables 2 and 3). Thus, simple intracellular availability of acetoacetate and/or 3-hydroxybutyrate in relationship to the availability of free fatty acids is not the modulating factor for ketone-body oxidation.

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