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ON THE TRIPLE LOCALIZATION OF CREATINE KINASE IN HEART AND SKELETAL MUSCLE CELLS OF THE RAT: EVIDENCE FOR THE EXISTENCE OF MYOFIBRILLAR AND MITOCHONDRIAL ISOENZYMES

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SUMMARY

1. In order to assess the intracellular localization of creatine kinase (EC 2.7.3.2) in muscle, a fractionation prodecure was developed in which (a) 1 mM EDTA was used to prevent the Ca^{2+}-dependent binding of cytosolic creatine kinase to myosin A; (b) 0.2 mM dithiothreitol was used to prevent binding of the cytosolic marker enzyme pyruvate, kinase (EC 2.7.1.40) to the heavy particulate fraction; (c) the myofibrils were solubilized by ultrasonic treatment in 1 M KCl; (d) the washing of the minced tissue was omitted since it was found that this gave rise to the loss of up to 40% of pyruvate kinase. The tissues were fractionated in three fractions enriched in the myofibrillar marker enzyme myosin ATPase (EC 3.6.1.3.), the mitochondrial marker enzyme cytochrome *c* oxidase (EC 1.9.3.1) or the cytosolic marker enzyme pyruvate kinase.

2. Creatine kinase was found to be located in the myofibrils, the mitochondria and the cytosol of both rat heart and rat masseter muscle. With the aid of the marker enzymes mentioned above, it was calculated that in heart 33% of the total cellular creatine kinase activity (measured with 25 mM creatine at 25° C) resides in the myofibrils, 19% in the mitochondria and 48% in the cytosol. For rat masseter muscle these values were 4.4, 2.3 and 93% respectively. The total creatine kinase activity was in heart 145 units/g wet weight, and in masseter muscle 432 units/g wet weight.

3. The electrophoretic mobility at pH 9 on cellulose acetate was different for the three isoenzymes. The mobility towards the cathode increased in the following order:cytosolic (MM-type), myofibrillar and mitochondrial isoenzyme, extracted from mitochondria. Mitochondrial creatine kinase, when bound to the outside of the inner membrane, where it resides *in situ*, was not mobile.

4. The apparent K_m values for creatine for reactions catalyzed by rat-heart myofibrillar, mitochondrial and cytosolic subcellular fractions were 4.3, 13 and 20 mM, respectively. In the corresponding fractions of masseter muscle these values were 18, 13 and 28 mM, respectively. For creatine phosphate as the substrate the values were for heart 0.9, 1.0 and 1.8 mM, and for masseter muscle 3.0, 1.6 and 2.6 mM, respectively.

5. It is proposed that the physiological function of the mitochondrial and cytosolic creatine kinases is the synthesis of creatine phosphate, when fatty acids or

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carbohydrates, respectively, are the main source of energy. As the function for myofibrillar creatine kinase is proposed the degradation of creatine phosphate to yield ATP for muscular contraction.

INTRODUCTION

The literature data about the intracellular localization of creatine kinase in heart and skeletal muscle are summarized in Table I. Most of the creatine kinase activity is located in the cytosol[1–10]. There are three forms of cytosolic creatine kinase: the MM-, MB- and BB-types[11–15]. The MM-type, which in muscle is the most abundant type present, moves usually towards the cathode at pH 8.7–9, while the other enzymes move towards the anode. The BB-type, which occurs in brain, moves faster than the MB-type. The BB-enzyme has, as far as striated muscle is concerned, only been demonstrated in foetal or pathological tissue[16–18].

The mitochondrial isoenzyme, immobile during electrophoresis, was discovered by Jacobs *et al.*[4]. They found that mitochondria from rat-skeletal muscle, rat-heart and pigeon-breast muscle contained 7, 48 and 25% of the total cellular activity, respectively. Other workers, however, did not find any significant activity in mitochondria isolated from human, rabbit, rat and mouse skeletal muscle, nor from human heart[1,6,8,10].

Other particulate localizations of creatine kinase have been described. Yagi and Mase[9] showed that in rabbit muscle part of the supernatant creatine kinase could be bound to myosin A in the presence of 0.1 M KCl and 5 mM $CaCl_2$. Ottaway[2] purified cytosolic creatine kinase from beef-heart myofibrils, which were washed with EDTA. Also Alievskaia and Chetverikova[5] found after several washes with EDTA creatine kinase activivity in the myofibrils of rat heart and rat skeletal muscle. The enzyme was solubilized by destruction of the myofibrillar integrity by an 18-h treatment with 0.9 M KCl.

Keto and Doherty[3] found in pig heart a heavy, particulate, but not mitochondrial, creatine kinase with an absolute requirement for a thiol protecting agent. The enzyme moved faster towards the cathode than the cytosolic MM-enzyme. Baskin and Deamer[10] found 1% of the cellular creatine kinase activity in rabbit-skeletal muscle microsomes, and no activity in the mitochondria.

The purpose of the present study was to reconcile the conflicting reports on the localization of creatine kinase in heart and skeletal muscle, by assessing the exact distribution, and studying the possibility of isoenzymes by means of electrophoresis and the study of some kinetic parameters. As a model rat heart and rat masseter muscle were chosen.

METHODS

Animals

For the experiments normally fed male Wistar rats were used (weight 200–250 g). The animals were anaesthetized by a mixture of ether and air, and killed by decapitation.

TABLE I

LITERATURE DATA ON THE INTRACELLULAR LOCATION OF CREATINE KINASE IN HEART AND SKELETAL MUSCLE

Fraction N + F is the nuclear *plus* myofibrillar fraction, Fraction M is the mitochondrial fraction, Fraction P is the microsomal fraction, and Fraction S is the particle-free supernatant

Tissue	*Medium*	*Location* (%)				*Reference*
	Fraction:	*N+F*	*M*	*P*	*S*	
Heart muscle						
Human	0.88 M sucrose	0.1	0.02	0.7	98	Kleine[1]
Ox	0.25 M sucrose, 20 mM TRA*, 2 mM EDTA	part (28%) of supernatant enzyme binds to myofibrils				Ottaway[2]
Pig	0.1 M phosphate, 1 mM EDTA, 10 μM dithiothreitol	particulate creatine kinase, different from the mitochondrial and the cytosolic ones				Keto and Doherty[3]
Rat	0.3 M sucrose, 10 mM TRA*, 1 mM EDTA		48		52	Jacobs *et al.*[4]
Rat	20 mM Tris, 2 mM EDTA	13				Alievskaia and Chetverikova[5]
Skeletal muscle						
Human	0.88 M sucrose	13	1	0.2	82	Laudahn and Heyck[6]
Human	0.88 M sucrose	9	0.2	0.2	91	Kleine[7]
Human	0.88 M sucrose	14	0.2	0.2	86	Kleine[1]
Human	0.3 M sucrose	0.5–2.8% particle-bound				Park and Pennington[8]
Rabbit	0.1 M KCl, 5 mM Ca^{2+}	part (1/3) of supernatant enzyme binds to myosin A				Yagi and Mase[9]
Rabbit	0.1 M KCl		0	1		Baskin and Deamer[10]
Rat	0.3 M sucrose, 10 mM TRA*, 1 mM EDTA		7.0		93	Jacobs *et al.*[3]
Rat	0.88 M sucrose		0.4	2.4	97	Kleine[1]
Rat	20 mM Tris, 2 mM EDTA	1				Alievskaia and Chetverikova[5]
Mouse	0.3 M sucrose	0.4–0.6% particle-bound				Park and Pennington[8]
Pigeon	0.3 M sucrose, 10 mM TRA*, 1 mM EDTA		25		75	Jacobs *et al.*[4]

* TRA = triethanolamine–HCl buffer.

"Classical" fractionation of muscle (Method A)

The medium used was 0.25 M sucrose, 10 mM tricine–KOH and 1 mM EDTA (pH at 0 °C, 7.4). The EDTA was used to prevent the Ca^{2+}-dependent binding of cytosolic creatine kinase to myosin A reported by Yagi and Mase[9]. Two rat hearts or two masseter muscles were thoroughly minced with a pair of scissors, and washed. The mince was taken up in 10 ml of medium and 0.3 ml of a trypsin solution (2 mg/ml medium) was added. After 5 and 10 min at 0 °C the mince was homogenized by one stroke in an electrically driven Potter–Elvehjem homogenizer. 15 min after the trypsin addition the digestion was stopped with 0.4 ml of soy-bean trypsin inhibitor (Calbiochem, 3 times crystallized, 2 mg/ml medium), followed by homogenization. The homogenate was filtered through fine-mesh nylon cloth. The residue was extracted for 5 h with 0.9 M KCl, the connective tissue was removed by centrifugation for 10 min at $200 \times g_{av}$, and the supernatant was taken as the myofibrillar Fraction F.

The filtrate was fractionated as described in earlier work[19] into a nuclear fraction (Fraction N) and a nuclear-free homogenate (Fraction E). Fraction E was further separated into a mitochondrial (Fraction M), a microsomal fraction (Fraction P) and a particle-free supernatant (Fraction S).

Fractionation of muscle by differential extraction (Method B)

This method is based on that of Delbrück *et al.*[20] (*cf.* refs 2, 4, 5 and 21). As medium was used: 0.25 M sucrose, 10 mM tricine–KOH, 1 mM EDTA, 0.2 mM dithiothreitol (pH at 0 °C 7.4). The dithiothreitol was used in order to stabilize the heavy particulate, but not mitochondrial, creatine kinase, as described by Keto and Doherty[3]. Two rat hearts or two masseter muscles were thoroughly minced, but not washed, since it was found that up to 40% of the cytosolic marker enzyme pyruvate kinase was lost by the washing procedure. The mince was homogenized in the sucrose medium with a Potter–Elvehjem homogenizer, and centrifuged for 10 min $27\,500 \times g_{av.}$ (all centrifugations of this partition were run for 10 min at $27\,500 \times g_{av.}$). This extraction with sucrose medium was repeated twice. The cytosolic extract, consisting of the collected supernatants, is Fraction S. The remaining sediment was suspended in 40 mM potassium phosphate buffer (pH 7.2) *plus* 0.2 mM dithiothreitol, incubated for 15 min at 0 °C and centrifuged. This extraction with phosphate was repeated twice. By this treatment the mitochondrial creatine kinase, which is located on the outside of the inner mitochondrial membrane, is extracted[21]. The mitochondrial extract, consisting of the collected supernatants, is Fraction M. The remaining sediment was suspended in 0.9 M KCl *plus* 0.2 mM dithiothreitol and incubated for 1 h at 0 °C and centrifugated. This extraction with KCl was repeated twice. The myofibrillar extract, consisting of the collected supernatants, is Fraction F. The remaining sediment was homogenized in 1% Lubrol WX (I.C.I.) *plus* 0.2 mM dithiothreitol, and incubated for 15 min at 0 °C. The sediment is Fraction X and the supernatant Fraction Y.

Rapid fractionation of muscle into myofibrillar plus nuclear, mitochondrial, and microsomal plus supernatant fractions (Method C)

The medium of Method B was used. Two rat hearts or two masseter muscles were thoroughly minced in 10 ml medium and homogenized in a Potter–Elvehjem

homogenizer. The homogenate was centrifuged for 10 min at 200 × $g_{av.}$. The pellet was three times resuspended and centrifuged twice for 10 min at 110 × $g_{av.}$ and finally for 10 min at 200 × $g_{av.}$ (to reduce the volume of the gelatinous pellet). To the pellet the same volume 2 M KCl *plus* 0.2 mM dithiothreitol were added, and pellet *plus* liquid were sonicated for 15 s/ml at 21 kHz, amplitude 5 μm from peak to peak, 0 °C. The sonicate was sieved through fine-mesh nylon cloth. The residue, connective tissue, was washed with 1 M KCl *plus* 0.2 mM dithiothreitol, and discarded. The extract is Fraction F + N (myofibrillar *plus* nuclear fraction). The supernatants from the four centrifugations were combined and centrifuged for 10 min at 24000 × $g_{av.}$. The sediment was again homogenized and centrifuged. The pellet is the mitochondrial Fraction M, and the combined supernatants Fraction P + S (microsomal *plus* supernatant fraction).

Electrophoresis

Electrophoresis was carried out for 2 h at 240 V (5 °C) on cellulose acetate strips (width 2.5 cm; Mikrophor, Sartorius) with 0.1 M Tris–HCl (pH 9.0), 0.2 mM dithiothreitol and unless otherwise stated 1% fatty acid-free bovine serum albumin (1 g/l)[2]. Usually 1–2 μl and maximally 5 μl of sample was applied with creatine kinase activities of more than 1 munits (measured with 25 mM creatine before the chromatography). After electrophoresis another strip was wetted with the incubation mixture, the excess of fluid was removed with filter paper, and the wetted strip was placed on the electrophoresis strip. Both strips were "sandwiched" between two microscope slides, and the ends were clamped. The strips were then incubated in a damp chamber for 10–60 min at 37 °C.

The NADH disappearance assay (incubation mixture 1): 83 mM Tris–HCl, 170 μM dithiothreitol, 31 mM $MgSO_4$, 12 mM ATP (dipotassium–disodium salt), 19 mM NADH, 12 mM phosphoenolpyruvate (tricyclohexylammonium salt), 20 μg/ml oligomycin, 3 μM rotenone, 20 μg/ml lactate dehydrogenase, 40 μg/ml pyruvate kinase (pH 9.0). To the mixture for the test strips 75 mM creatine was added (*cf.* ref. 11).

The NADPH appearance assay (incubation mixture 2): 0.2 M triethanolamine–HCl, 20 mM magnesium acetate, 20 mM glucose, 2.92 mM ADP (free acid), 1.02 mM AMP, 0.74 mM $NADP^+$, 16 μg/ml oligomycin, 2.4 μM rotenone, 8 μg/ml glucose-6-phosphate dehydrogenase, 8 μg/ml hexokinase (pH 6.9). To the mixture for the test strips 9 mM creatine phosphate (disodium salt) was added (*cf.* ref. 22).

After the incubation the spots were visualized under ultraviolet light (350 nm), and marked with a ballpoint pen.

Assays

Cytochrome *c* oxidase (expressed in units of μatoms O consumed per min at 25 °C), pyruvate kinase (μmoles NADH oxidized per min at 25 °C), creatine kinase (μmoles NADH oxidized per min at 25 °C, [creatine] = 25 mM), myosine ATPase (μmoles phosphate produced per min at 25 °C in the presence of 5 mM ATP (dipotassium–disodium salt), 1 M KCl, 50 mM Tris–HCl, 5 mM EDTA and 2.5 μg/ml oligomycin) and NADPH–cytochrome *c* reductase (μmoles of cytochrome *c* reduced per min at 25 °C) were determined as described in refs 23, 24, 25, 26 and 27, respectively.

Creatine kinase and pyruvate kinase were measured in the presence of 7.5 μg/ml

oligomycin and 1.5 μM rotenone in order to inhibit mitochondrial ATPase and the NADH oxidation by the respiratory chain respectively.

The K_m of creatine kinase for creatine phosphate was determined in the assay of ref. 28. Creatine phosphate was determined in the same reaction with added creatine kinase.

To abolish the latency of cytochrome *c* oxidase, the fractions were pretreated with 0.025% Lubrol WX for 12 min at 0 °C.

Protein was determined according to Lowry *et al.*[29].

RESULTS

Fractionation of muscle according to Method A

The results of this fractionation are shown in Table II and Fig. 1. In rat heart homogenate the mitochondrial marker enzyme cytochrome *c* oxidase is completely particulate, while in rat masseter muscle a part of the activity has been recovered in the supernatant fraction. Obviously the skeletal muscle mitochondria are more damaged by the fractionation procedure; nevertheless the enzyme has been consider-

TABLE II

THE PARTITION OF CREATINE KINASE AND MARKER ENZYMES IN HEART AND MASSETER MUSCLE AFTER FRACTIONATION BY METHOD A

The marker enzymes used were cytochrome *c* oxidase for the mitochondria, rotenone-insensitive NADPH–cytochrome *c* reductase for the microsomes and pyruvate kinase for the cytosol. Fraction F is the myofibrillar fraction, Fraction N is the nuclear fraction and Fraction E the nuclear-free homogenate, Fraction M is the mitochondrial fraction, Fraction P is the microsomal fraction and Fraction S is the final supernatant. The corresponding "De Duve-plots" are given in Fig. 1.

	Absolute values	*Percentage values*						
Fraction:	*F+N+E*	*F+N+E*	*F*	*N*	*M*	*P*	*S*	*Recovery*
Rat masseter muscle								
Protein (mg)	75.8	100	29.7	27.8	6.7	2.6	33.1	99
Cytochrome *c* oxidase (units)	46.9	100	11.7	10.2	51.9	12.7	24.5	111
Rotenone insensitive NADPH–cytochrome *c* reductase (munits)	120	100	17.4	19.6	9.7	5.6	64.5	117
Pyruvate kinase (units)	149	100	12.0	4.8	0.1	0.8	71.0	88
Creatine kinase (units)	355	100	10.3	5.3	1.7	0.5	73.6	91
Rat heart								
Protein (mg)	100	100	12.2	31.5	18.4	4.3	24.9	91
Cytochrome *c* oxidase (units)	305	100	6.3	22.3	63.3	6.2	0.4	98
Rotenone insensitive NADPH–cytochrome *c* reductase (munits)	329	100	5.8	22.8	40.8	13.9	29.7	113
Pyruvate kinase (units)	44.3	100	7.4	9.6	0.4	5.6	84.9	108
Creatine kinase (units)	126	100	7.7	18.1	22.3	4.2	35.2	87

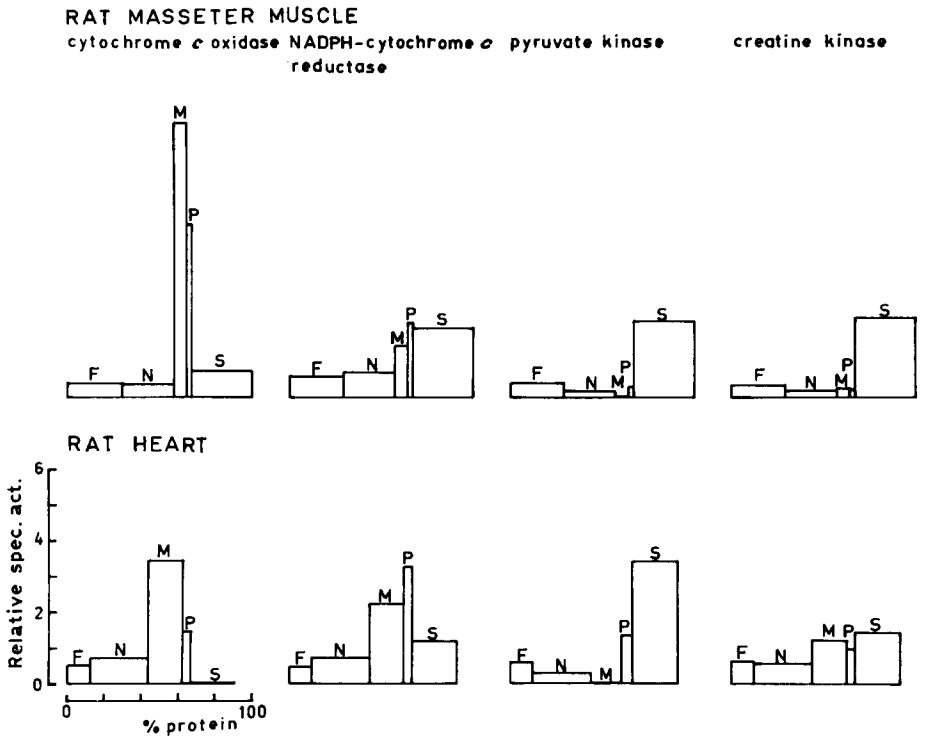

Fig. 1. The partition of creatine kinase and marker enzymes in rat heart and masseter muscle by Method A. For conditions see Table II. relative spec. act. = percentage of total activity/percentage of total protein.

ably purified in the mitochondrial fraction. The microsomal marker enzyme NADPH–cytochrome *c* reductase is present in every subfraction, and possesses the highest activity in the microsomal Fraction P. A considerable part of the activity has been recovered in the supernatant Fraction S. This is only in part due to the action of trypsin on the membrane-bound enzyme (see ref. 30), since omission of trypsin changes the distribution pattern hardly or not. Trypsin was used to facilitate the homogenization, but is not essential for the partition. The cytosolic marker enzyme pyruvate kinase has been highly purified in Fraction S, but there appears to be a binding of this enzyme to heavy subcellular components. Probably this can be explained by the ability of F-actin to bind various glycolytic enzymes, including pyruvate kinase[31]. The distribution of creatine kinase is clearly different from one of the marker enzymes. Especially in heart the pattern is intermediate between those of cytochrome *c* oxidase and pyruvate kinase. In skeletal muscle this is less clear, because this tissue possesses more cytosolic creatine kinase and less mitochondrial creatine kinase than heart.

Figs 2 and 3 show the results of electrophoresis of Fractions F, M and S of masseter muscle and heart, respectively. The NADH disappearance assay (incubation mixture 1) shows the myofibrillar and cytosolic (MM) isoenzymes. In this assay the membrane-bound mitochondrial isoenzyme cannot be detected because of

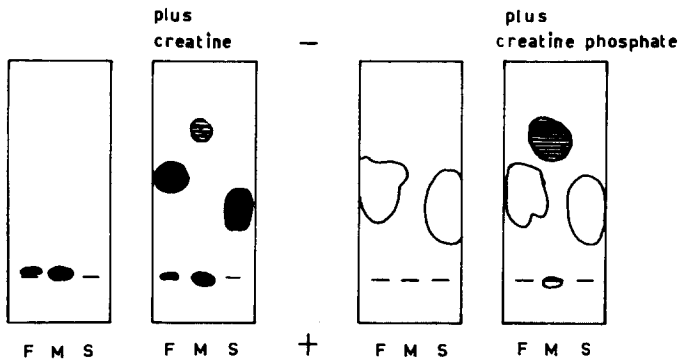

Fig. 2. The electrophoresis of intracellular fractions derived from rat masseter muscle by Method A. No albumin was present in the electrophoresis medium. The strips were assayed for creatine kinase by the two incubation media described in Methods. The striped spots were visible after 30 min of incubation, the others became visible after 10 min.

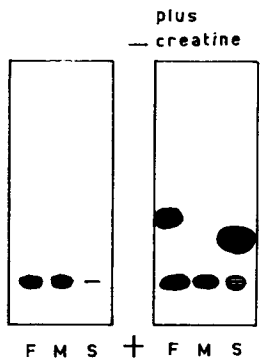

Fig. 3. The electrophoresis of intracellular fractions derived from rat heart by Method A. Incubation medium I was used.

the rotenone-insensitive NADH oxidation, due to the presence of mitochondrial membranes on the origin. The NADPH appearance assay (incubation mixture 2) can be used to detect membrane-bound mitochondrial creatine kinase, while the myofibrillar and cytosolic isoenzymes are masked by adenylate kinase (EC 2.7.4.3). After prolonged incubation (30 min) by both methods a very fast moving iso-enzyme became visible in the patterns of Fraction M. This reflects probably that a small part of the mitochondrial isoenzyme has been released from the mitochondrial inner membranes by the salt of the electrophoresis medium (see ref. 21). The myofibrillar isoenzyme resembles the particle-bound non-mitochondrial isoenzyme found in pig heart[3], since it moves faster to the cathode than the cytosolic (MM) isoenzyme. Ottaway[2] purified creatine kinase from ox-heart myofibrils, and showed the identity with cytosolic creatine kinase by comparing the patterns obtained by polyacrylamide gel electrophoresis. These patterns however were not exactly the same.

With the assumptions that mitochondrial creatine kinase is partitioned as cytochrome *c* oxidase, that cytosolic creatine kinase does not bind to the heavy particulate fraction as does pyruvate kinase (since by electrophoresis no cytosolic

creatine kinase could be detected in these fractions), and finally that all myofibrillar creatine kinase is extracted in Fraction F, it could be calculated that in rat masseter muscle 11% of the total creatine kinase was myofibrillar, 4% was mitochondrial and 76% was cytosolic. In rat heart these values were 6, 40 and 40%, respectively.

By electrophoresis of Fraction P (not shown) it was clear that the creatine kinase activity of this fraction was due to cytosolic and mitochondrial contamination. No evidence for a separate microsomal enzyme, as reported by Baskin and Deamer[10], could be found.

These partitions indicate that there exists a myofibrillar isoenzyme of creatine kinase in rat muscle, which differs from the cytosolic and mitochondrial ones in localization and in electrophoretic mobility. This enzyme resembles the heavy, particulate pig-heart enzyme discovered by Keto and Doherty[3]. Since last enzyme was dependent upon a thiol protecting agent, 0.2 mM dithiothreitol was used in the following partitions. Myofibrillar ATPase was used as myofibrillar marker enzyme.

Fractionation of muscle by Method B

The results of the preceding fractionations were checked by another fractionation procedure, based upon the successive extraction of cytosolic creatine kinase (with isotonic sucrose), mitochondrial creatine kinase (with 40 mM phosphate buffer of pH 7.2; ref. 21), and myofibrillar creatine kinase (with 0.9 M KCl; ref. 5). The remaining sediment was extracted with Lubrol WX. In this partition the washing of the minced tissue was omitted, since it was found that this treatment caused a considerable loss of cytosolic enzymes.

The results of the fractionation of masseter muscle and heart are shown in Table III. The partition of creatine kinase over the subcellular fractions is again completely different from one of the marker enzymes. In this fractionation it cannot be expected that cytochrome *c* oxidase and mitochondrial creatine kinase are equally distributed over the fractions. In contrast to mitochondrial creatine kinase, cytochrome *c* oxidase is not released from the mitochondria by the phosphate treatment (see ref. 21). In fraction S, however, it can be expected that the two enzymes have the same distribution. If we assume for the calculation that myofibrillar creatine kinase is partitioned as myosin ATPase, cytosolic creatine kinase as pyruvate kinase, and that the recovery of the 4 enzymes is 100%, the following set of equations can be obtained from this fractionation of masseter muscle (Fractions "F", "X" and "Y" were taken together):

$$0.941\,f + 0.004\,s = 1.6\%$$
$$0.028\,f + 0.643\,m + 0.053\,s = 7.1\%$$
$$0.031\,f + 0.357\,m + 0.945\,s = 95.4\%$$

f = percentage of myofibrillar creatine kinase, m = percentage of mitochondrial creatine kinase and s = percentage of cytosolic creatine kinase. Solution of these equations yields: $f = 1.3\%$, $m = 3.1\%$ and $s = 95\%$. The rat-heart partition gives: $f = 20\%$, $m = 23\%$ and $s = 57\%$.

These results differ from those obtained with Method A, mainly because in the partition with Method B no cytosolic enzymes have been discarded. It can be seen in Table III that after the extractions with 0.9 M KCl, still some creatine kinase

TABLE III

THE PARTITION OF CREATINE KINASE AND MARKER ENZYMES IN HEART AND MASSETER MUSCLE AFTER FRACTIONATION BY METHOD B

The marker enzymes used were myosin ATPase for the myofibrils, and the others are indicated in Table I. Fraction S is the cytosolic extract, Fraction M is the mitochondrial extract, Fraction F is the myofibrillar extract, Fraction Y is Lubrol extract and Fraction X the final sediment.

	Absolute values	*Percentage values*					
		Fraction:					
	Total	*Total*	*S*	*M*	*F*	*X*	*Y*
Rat masseter muscle (1.04 g)							
Protein (mg)	100	100	36.8	9.1	31.7	16.3	6.4
Myosin ATPase (units)	16.9	100	3.1	2.8	52.9	25.5	15.6
Cytochrome *c* oxidase (units)	44.4	100	35.7	12.5	5.4	43.6	2.8
Pyruvate kinase (units)	133	100	94.5	5.3	0.2	0.0	0.2
Creatine kinase (units)	994	100	91.3	7.1	1.1	0.3	0.2
Rat heart (1.25 g)							
Protein (mg)	165	100	32.5	6.9	36.0	19.0	5.7
Myosin ATPase (units)	9.24	100	2.6	1.6	83.4	12.3	0.1
Cytochrome *c* oxidase (units)	292	100	17.5	3.2	12.7	63.4	3.3
Pyruvate kinase (units)	80.1	100	96.4	2.5	0.8	0.0	0.2
Creatine Kinase (units)	104	100	59.6	20.6	16.8	1.0	2.1

TABLE IV

THE FRACTIONATION OF CREATINE KINASE AND MARKER ENZYMES IN HEART AND MASSETER MUSCLE AFTER FRACTIONATION BY METHOD C

Fraction F+N is the myofibrillar *plus* nuclear fraction, Fraction M is the mitochondrial fraction and Fraction P+S is the microsomal *plus* supernatant fraction. The corresponding "De Duve-plots" are given in Fig. 4.

	Absolute values	*Percentage values*			
		Fraction:			
	Total	*Total*	*F+N*	*M*	*P+S*
Rat masseter muscle (0.62 g)					
Protein (mg)	104	100	66.1	5.9	28.1
Myosin ATPase (units)	22.5	100	93.8	6.2	0.0
Cytochrome *c* oxidase (units)	46.0	100	20.6	48.0	31.3
Pyruvate kinase (units)	75.0	100	1.8	0.1	98.1
Creatine kinase (units)	268	100	6.2	1.5	92.3
Rat heart (1.01 g)					
Protein (mg)	182	100	52.0	17.7	30.3
Myosin ATPase (units)	5.58	100	89.8	9.2	1.0
Cytochrome *c* oxidase (units)	204	100	7.8	78.8	13.4
Pyruvate kinase (units)	47.7	100	1.4	0.4	98.3
Creatine kinase (units)	147	100	31.3	18.4	50.2

activity is present, which is only partly released by a treatment with Lubrol WX. It was subsequently found that myofibrillar creatine kinase was completely solubilized by sonic treatment in 1 M KCl. This finding was used in the following experiment.

Fractionation of muscle by Method C

This method was used to obtain rapidly three subfractions, each containing the bulk of one of the marker enzymes. The results are shown in Table IV and Fig. 4.

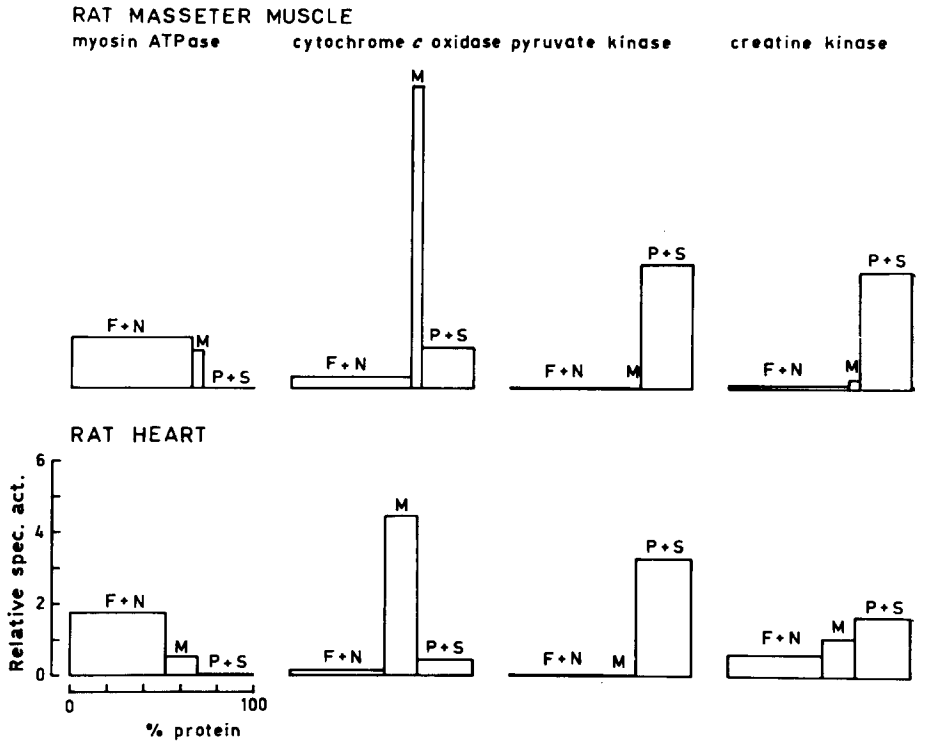

Fig. 4. The partition of creatine kinase and marker enzymes in rat heart and masseter muscle by Method C. For conditions see Table IV.

Surprisingly in this partition less pyruvate kinase has been bound to the myofibrillar *plus* nuclear fraction than in the partition with Method A (Table II, Fig. 1). Since trypsin has no marked effect on the partitions, the cause of the difference must be due to the use of dithiothreitol. The binding of various glycolytic enzymes, including pyruvate kinase, to F-actin has been described[31]. In these studies no sulfhydryl compounds were added.

With the assumption that each of the creatine kinase isoenzymes is distributed as the respective marker enzyme, and that the recovery for each enzyme is 100%, it was calculated that in rat masseter muscle 4.4% of the creatine kinase is myofibrillar, 2.3% mitochondrial and 93% cytosolic. In rat heart these values were 33, 19 and 48%, respectively.

Fig. 5 shows the electrophoresis of the fractions. Note the resemblance of

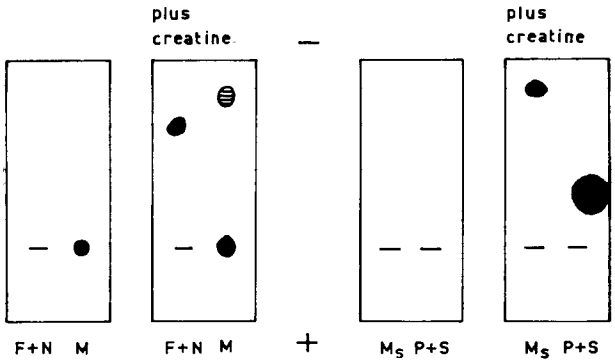

Fig. 5. The electrophoresis of intracellular fractions derived from rat heart by Method C. Fraction M_s is the 30 min, 159 000 × g_{av} supernatant of a pellet of fraction M incubated with 40 mM phosphate (pH 7.2) *plus* 0.2 mM dithiothreitol for 15 min. Incubation medium 1 was used. The strips were incubated for 1 h. The striped spot is a spot of a low intensity after an incubation of 1 h. The absence of dithiothreitol gave rise to a decrease in intensity of the myofibrillar and mitochondrial creatine kinase spots, but not to a change in the position of the spots.

the mobility of the fast moving material in Fraction M and a phosphate extract of the mitochondrial Fraction: M_s.

Table V shows the apparent K_m values which were found for creatine and creatine phosphate in the three fractions. In Lineweaver–Burk plots straight lines were obtained. Even in these crude fractions the differences between the three isoenzymes is striking. As far as the tissue is concerned there appears to be a difference between the myofibrillar enzyme of heart and skeletal muscle. The myofibrillar creatine kinase of heart was less stable during electrophoresis and assay than that of masseter muscle (not shown). The mitochondrial isoenzymes show about the same kinetics, and so do the supernatant enzymes. When mitochondrial creatine kinase

TABLE V

THE APPARENT K_m VALUES FOR CREATINE AND CREATINE PHOSPHATE IN FRACTIONS OF RAT HEART AND MASSETER MUSCLE

The fractions were obtained by Method C. Fractions F+N_s, M_s and P+S_s are the supernatants (30 min, 159 000 × g_{av}) of Fraction F+N, of the Fraction M pellet treated for 15 min with 40 mM phosphate *plus* 0.2 mM dithiothreitol and of Fraction P+S, respectively. The K_m values are determined from fractions obtained by separate partitions, and are expressed in mM.

Fraction:	*F+N$_s$*	*M*	*M$_s$*	*P+S$_s$*
Masseter muscle				
Creatine	18; 18	–	15; 11	31; 25
Creatine phosphate	3.1; 2.9	–	1.6; 1.7	2.4; 2.9
Heart				
Creatine	4.7; 4.0	17; 17	14; 12	21; 19
Creatine phosphate	0.8; 1.0	2.2; 2.0	1.1; 1.0	1.6; 2.1

is bound to the inner membrane the K_m values for creatine and creatine phosphate are somewhat higher. The difference of the kinetic behaviour of the myofibrillar enzymes is not accompanied by a striking difference in electrophoretic behaviour (Fig. 6).

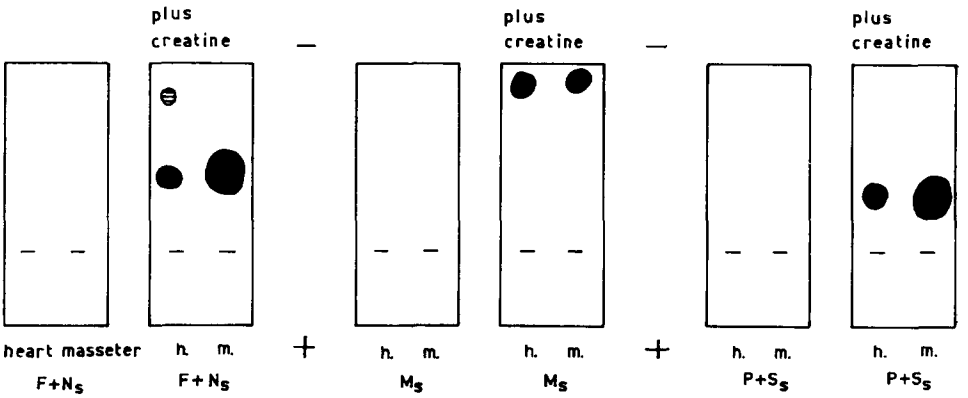

Fig. 6. The electrophoresis of intracellular fractions derived from rat heart and masseter muscle by Method C. Fractions F+N$_S$ and P+S$_S$ are 30 min, 159000×g_{av} supernatants of Fractions F+N and P+S, respectively. For Fraction M$_S$ see the legends to Fig. 5. Incubation medium I was used. A striped spot is a spot of low intensity after an incubation for 1 h.

DISCUSSION

The many isoenzymes of creatine kinase, which were observed in the past after electrophoresis (refs 11, 13–15, 32), and which were separated recently on DEAE-Sephadex A-50 (ref. 33), can be identified. In the direction of the anode the MB and the BB cytosolic isoenzymes move at pH 9 with increasing velocities. Mitochondrial creatine kinase, which is bound to the inner membranes, remains at the origin. In the direction of the cathode, the MM cytosolic isoenzyme, the myofibrillar isoenzyme and the liberated mitochondrial isoenzyme move with increasing velocities, in the order given.

The reason that many investigators were not able to detect the mitochondrial isoenzyme in skeletal tissues of various species (see Table I), is probably due to the fact that the mitochondrial isoenzyme has, especially in white muscle, a low activity, since there are only few mitochondria. Note the constant ratio between cytochrome *c* oxidase and mitochondrial creatine kinase in rat-heart and rat-masseter muscle (Table IV, see also ref. 4). That Kleine[1,7] was not able to detect the mitochondrial isoenzyme in human heart is probably due to the fact that the tissue was partitioned relatively late after death had occurred. When rat heart mitochondria were isolated from a rat which was decapitated 4 h before the isolation, and stored at room temperature, the mitochondria contained 32% of the creatine kinase activity of freshly isolated mitochondria. When the rat was stored in the cold room the mitochondria possessed the normal activity.

Baskin and Deamer[10] found 1% of the total cellular creatine kinase activity in rabbit-muscle microsomes. This can possibly be explained by the presence of some myofibrillar contamination. The zero activity in the mitochondrial fraction, found

by these authors, was previously (ref. 21) explained by the fact that creatine kinase is extractable from the outside of the mitochondrial inner membranes by salt.

The physiological meaning of the mitochondrial and cytosolic isoenzymes will probably be the synthesis of creatine phosphate when fatty acids and carbohydrates are the main source of energy, respectively. The function of myofibrillar creatine kinase is probably the degradation of creatine phosphate to yield ATP for muscular contraction. Probably the two particulate creatine kinase isoenzymes are of vital importance for the energy metabolism in the muscle cell.

Future work will be devoted for a study of the properties of these enzymes. Preliminary experiments have indicated the existence of the particulate creatine kinase isoenzymes in human skeletal muscle (Scholte, H. R., Busch, H. F. M. and Hülsmann, W. C., unpublished work).

Part of this paper has been presented elsewhere[34].

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REFERENCES

1 Kleine, T. O. (1965) *Nature* 207, 1393–1394
2 Ottaway, J. H. (1967) *Nature* 215, 521–522
3 Keto, A. I. and Doherty, M. D. (1968) *Biochim. Biophys. Acta* 151, 721–724
4 Jacobs, H., Heldt, H. W. and Klingenberg, M. (1964) *Biochem. Biophys. Res. Commun.* 16, 516–521
5 Alievskaia, L. L. and Chetverikova, E. P. (1971) *Dokl. Akad. Nauk. S.S.S.R.* 197, 216–218
6 Laudahn, G. and Heyck, H. (1963) *Klin. Wochenschr.* 41, 493–500
7 Kleine, T. O. (1965) *Clin. Chim. Acta* 11, 85–87
8 Park, D. C. and Pennington, R. J. (1966) *Clin. Chim. Acta* 13, 694–700
9 Yagi, K. and Mase, R. (1962) *J. Biol. Chem.* 237, 397–403
10 Baskin, R. J. and Deamer, D. W. (1970) *J. Biol. Chem.* 245, 1345–1347
11 Burger, A., Richterrich, R. and Aebi, H. (1964) *Biochem. Z.* 339, 305–314
12 Eppenberger, H. M., Dawson, D. M. and Kaplan, N. O. (1967) *J. Biol. Chem.* 242, 204–209
13 Eppenberger, H. M., Eppenberger, M. E. and Scholl, A. (1970) in *Enzymes and Isoenzymes, Properties and Function* (Shugar, D., ed.), *FEBS 5th Meeting, Prague, 1968*, pp. 269–279, Academic Press, London
14 Van der Veen, K. J. and Willebrands, A. F. (1966) *Clin. Chim. Acta* 13, 312–316
15 Dawson, D. M. and Fine, I. H. (1967) *Arch. Neurol.* 16, 176–180
16 Cao, A., de Virgilliis, S., Lippi, C. and Coppa, G. (1971) *Enzyme* 12, 49–62
17 Tzvetanova, E. (1971) *Enzyme* 12, 279–288
18 Cao, A., Trabalza, N., de Virgiliis, S., Furbetta, M. and Coppa, G. (1971) *Biol. Neonate* 17, 126–134
19 Wit-Peeters, E. M., Scholte, H. R. and Elenbaas, H. L. (1970) *Biochim. Biophys. Acta* 210, 360–370
20 Delbrück, A., Zebe, E. and Bücher, Th. (1959) *Biochem. Z.* 331, 273–296
21 Scholte, H. R., Weijers, P. J. and Wit-Peeters, E. M. (1973) *Biochim. Biophys. Acta* 291, 764–733
22 Khan, M. A., Holt, P. G., Knight, J. O. and Kakulas, B. A. (1971) *Histochemie* 26, 120–125
23 Schnaitman, C., Erwin, V. G. and Greenawalt, J. W. (1967) *J. Cell Biol.* 32, 719–735
24 Bücher, Th. and Pfleiderer, G. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, pp. 435–440, Academic Press, New York

25 Bücher, Th., Luh, W. and Pette, D. (1964) in *Handbuch der Physiologische, Pathologische und Chemische Analyse*, 10th edn, Vol. 6A, pp. 292–239, Springer-Verlag, Berlin
26 Seidel, J. C. (1969) *Biochim. Biophys. Acta* 189, 162–170
27 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) *J. Cell Biol.* 32, 415–438
28 Forster, G., Bernt, E. and Bergmeyer, H. U. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), 2nd. edn, Vol. 1, pp. 750–759, Verlag Chemie, Weinheim
29 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
30 Takesue, S. and Omura, T. (1970) *J. Biochem. Tokyo* 67, 259–266
31 Arnold, H., Henning, R. and Pette, D. (1971) *Eur. J. Biochem.* 22, 121–126
32 Sjövall, K and Voigt, A. (1964) *Nature* 202, 701
33 Takahashi, K., Ushikubo, S., Oimomi, M. and Shinko, T. (1972) *Clin. Chim. Acta* 38, 285–290
34 Scholte, H. R. (1972) *Abstr. Commun. Meet. FEBS, Amsterdam*, Vol. 8, p. 1179, North-Holland, Amsterdam