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INTRACELLULAR DISTRIBUTION OF PHOSPHOENOLPYRUVATE CARBOXYLASE AND (NADP) MALATE DEHYDROGENASE IN DIFFERENT MUSCLE TYPES

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SUMMARY

The intra- and extramitochondrial distribution of phosphoenolpyruvate carboxylase (GTP:oxaloacetate carboxylase (transphosphorylating), EC 4.1.1.32) and (NADP) malate dehydrogenase (L-malate:NADP+ oxidoreductase (decarboxylating), EC 1.1.1.40) were determined in white, red and heart muscle of different mammalian species, in flight and heart muscle of the pigeon and sea-gull, and in flight muscle of *Locusta migratoria*. The test system of phosphoenolpyruvate carboxylase, which was based on incorporation of [^{14}C]bicarbonate, was shown to be highly specific.

2. The extra- and intramitochondrial distribution of phosphoenolpyruvate carboxylase and (NADP) malate dehydrogenase were correlated at a high level of significance ($r = +0.84$). In red and heart muscle about 70% of the total activity of both enzymes was located in the mitochondria, whereas in white muscle up to 70% of the total activity of both enzymes was found in the cytosol. Absolute activities of phosphoenolpyruvate carboxylase in red and heart muscle were higher than in white muscle. It is suggested that in white muscles phosphoenolpyruvate carboxylase is involved in gluconeogenesis from lactate, a suggestion which is in accord with the relatively high activities of fructosediphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) and (NADP) malate dehydrogenase in the extramitochondrial compartment. Nevertheless the total gluconeogenetic capacity of whole skeletal muscle, as based on the total activity of phosphoenolpyruvate carboxylase, amounts to only 7% of the respective capacity of liver.

3. Activity levels of phosphoenolpyruvate carboxylase and (NADP) malate dehydrogenase were studied under different dietary and hormonal regimes in rat muscles.

4. In isolated rat heart mitochondria both synthesis and carboxylation of phosphoenolpyruvate were observed, suggesting that the inner membrane of rat heart mitochondria is permeable to phosphoenolpyruvate.

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INTRODUCTION

As described by Meyerhof[1,2], lactate formed during muscle work disappears when the muscle is allowed to recover in oxygen. But only 25% of this lactate is oxidized to CO_2 and water. It was suggested that a part of this lactate is converted to glycogen. Glycogen synthesis from lactate was demonstrated recently in frog sartorius and rabbit psoas muscle[3–5]. However, the rate of gluconeogenesis from lactate determined in these experiments is very low as compared with liver and kidney, and is restricted to white muscles[5]. The conversion of lactate into carbohydrate requires several specific enzymes, such as pyruvate carboxylase (pyruvate:CO_2 ligase (ADP), EC06.4.1.1), phosphoenolpyruvate carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) and fructosediphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11). Pyruvate carboxylase was found in only very small quantities in heart and skeletal muscle[6,7]. Activities of fructosediphosphatase were found to be present predominantly in white muscles, up to 1.2 munits/g fresh weight[8–11]. Determination of phosphoenolpyruvate carboxylase in muscles led to contradictory results. Whereas Keech and Utter[12] found very low activities, Opie and Newsholme[9] reported values higher than those of fructosediphosphatase. Determination of phosphoenolpyruvate carboxylase, especially in crude tissue extracts, is subject to several sources of error. For this reason, we have employed a highly specific assay, which is suitable for crude extracts, and used it to reinvestigate the activity distribution of phosphoenolpyruvate carboxylase in different muscles.

Activity levels of (NADP) malate dehydrogenase (L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40) were also determined in various muscles. This enzyme is of an interest because of the absence of pyruvate carboxylase. An interplay of (NADP) malate dehydrogenase may alternatively lead to production of oxaloacetate from pyruvate.

MATERIALS AND METHODS

Animals

Male FP 49 rats weighing 300 g, male guinea pigs (450 g body weight) (Thomae, Biberach, Germany) and male rabbits (Deutsche Widder) were used in the experiments. All animals were held on a standard diet obtained from Altrogge Lage-Lippe, Germany. Thyrotoxicosis was produced by daily intraperitoneal injection of 25 μg 3,3',5-triiodo-L-thyronine for 5 days. Prednisolone was administered subcutaneously every day for 4 days (10 mg per day and animal). Lipogenesis was induced after three days of starvation by feeding a diet low in fat and rich in carbohydrate (Altrogge, Lage-Lippe, Germany). In starvation experiments the animals were fasted for 3 days.

Preparation of extracts

The separation of intra- and extramitochondrial enzymes was achieved by the method of fractional extraction[10]. All media used were free of K^+. The extracts were concentrated 4- to 6-fold by vacuum dialysis.

Preparation of mitochondria

2 g of minced rat heart were stirred for 15 min with 20 mg collagenase (EC 3.4.4.19) at 30 °C in 2 ml of 0.3 M sucrose buffered with 10 mM triethanolamine to pH 7.4. After incubation the suspension was diluted to 40 ml by addition of cold sucrose medium and homogenized at 4 °C in a 50-ml teflon homogenizer with a clearance of 0.1 mm. The homogenate was spun for 5 min at 1200 × *g* in a Sorvall RC-2B centrifuge with rotor SS-34. The supernatant liquid was again centrifuged for 10 min in the same rotor at 10 000 × *g*.

The mitochondrial sediment was resuspended in 1 ml of 0.3 M sucrose buffered with 10 mM Tris to pH 8.0. To inactivate contaminating activity of pyruvate kinase (EC 2.7.1.40), enolase (EC 4.2.1.11) and lactate dehydrogenase (EC 1.1.1.27) the mitochondrial suspension was incubated for 20 min at 4 °C with subtilisin, 1 mg/mg of protein. After incubation the suspension was spun as described above for 10 min at 8000 × *g*. The pellet fraction was resuspended to a concentration of approx. 20 mg protein/ml in a medium containing 160 mM sucrose, 2 mM triethanolamine and 15 mM sodium phosphate (pH 7.4).

Chemicals

Inosine diphosphate was purchased from Calbiochem, U.S.A.; $NaH^{14}CO_3$ from NEN-Chemicals, U.S.A.; collagenase from Worthington, U.S.A.; subtilisin (Protease VII) from Sigma, U.S.A.; and all other biochemicals from Boehringer, Mannheim, Germany.

Enzyme determinations

Fructosediphosphatase: 50 mM Tris–HCl, 2.5 mM EDTA, 20 mM $MgSO_4$, 1 mM dithioerythritol, 0.3 mM NADP, 0.1 mM fructose diphosphate, 25 μg phosphoglucose isomerase, 25 μg glucose-6-phosphate dehydrogenase (EC 1.1.1.49), final pH 7.4. The reaction was started by the addition of fructose diphosphate. (NADP) malate dehydrogenase was determined by following the rate of $NADP^+$ reduction spectrophotometrically at 366 nm. The test system contained: 50 mM triethanolamine, 5 mM EDTA, 8 mM $MgCl_2$, 0.54 mM $NADP^+$, 2 mM L-malate (pH 7.6). Interference of (NAD) malate dehydrogenase and lactate dehydrogenase was excluded according to Brdiczka and Pette[13].

Phosphoenolpyruvate carboxylase. A modification of the test (carboxylating reaction) according to Chang and Lane[14] was used. 100 mM imidazole chloride, 3 mM phosphoenolpyruvate, 2 mM IDP, 2 mM $MgCl_2$, 1 mM dithioerythritol, 2.5 mM NADH, 50 mM $NaH^{14}CO_3$ (approx. 50 000 cpm/mmole), 50 μg malate dehydrogenase in 1.0 ml; final pH 6.8. To overcome phosphoenolpyruvate and ADP consumption by the pyruvate kinase reaction, K^+-free reagents were used. For the same reason, malate dehydrogenase was freed from $(NH_4)_2SO_4$ by extensive dialysis against quartz-distilled water. After incubation for 5, 10, 15 and 20 min at 30 °C the reaction was terminated by the addition of 0.5 ml 16% trichloroacetic acid. After centrifugation an aliquot of 0.5 ml was freed from $^{14}CO_2$ activity not incorporated by drying overnight in a desiccator over $CaCl_2$. The residue was dissolved in 0.5 ml distilled water and counted for acid-stable activity ([^{14}C]malate, and [^{14}C]fumarate) in a liquid scintillation spectrometer (Packard). Units of enzymic activity are defined as μmoles of bicarbonate fixed per min. Proportionality is found up to 0.003–0.004 unit[14]. Acid-

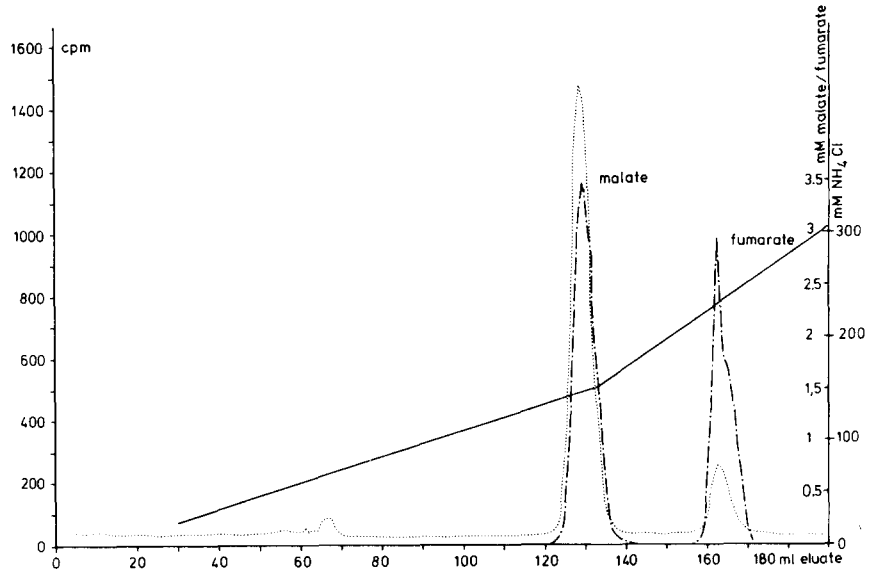

Fig. 1. Elution profile of acid-stable ^{14}C activity after incorporation of [^{14}C]bicarbonate in a standard phosphoenolpyruvate carboxylase test in crude extract from guinea-pig liver. Chromatography was performed on a Dowex 1X8 (Cl^-) column (70 cm × 0.9 cm) (H. W. Hofer, in preparation). The metabolites were eluted by a gradient of NH_4Cl and borate (0–10 mM). Malate and fumarate were added to the acid-stable ^{14}C-labelled material and were determined by photometric test systems in the eluted fractions: radioactivity was determined in a liquid scintillation counter. ·····, ^{14}C activity; —·—, malate, fumarate (mM); —— —, concentration of NH_4Cl (mM).

stable ^{14}C activity after incorporation in a standard test with crude guinea-pig liver extracts was analyzed by column chromatography on Dowex 1-X8 (Cl^- form) (H. W. Hofer, in preparation). As shown in Fig. 1, 86% of the radioactivity was found in the malate and 12% in the fumarate peak. The specificity of the phosphoenolpyruvate carboxylase test system is also evident from Table I, where activity measured in the complete assay system is compared with activity values obtained when phosphoenolpyruvate and IDP are deleted. Protein was determined by the biuret method.

TABLE I

SPECIFICITY OF THE PHOSPHOENOLPYRUVATE CARBOXYLASE TEST SYSTEM

Tests were performed with approximately 1.7 mg soluble protein of rat heart homogenate.

Test system	*munits/g wet wt*
Complete	12.0
Without phosphoenolpyruvate	0
Without IDP	0.9
Without phosphoenolpyruvate and IDP	0

Incubation of isolated mitochondria

Activity of heart muscle phosphoenolpyruvate carboxylase in intact mitochon dria in the forward and backward reactions was assayed in the following manner.

Decarboxylation reaction

Mitochondria (5–10 mg) were incubated in a total volume of 3 ml for 20 min at 30 °C in 160 mM sucrose, 2 mM triethanolamine, 17.5 mM $NaHPO_4$, 3 mM $MgCl_2$, 4 mM ATP, 2 mM malate, 2 mM fluorocitrate, 10 μg oligomycin, 2 mM ITP. Final pH 7.2. The reaction was terminated with 0.5 ml precooled 36 % $HClO_4$. After sonication (10 s at 5 A output of a Branson Sonifier) the samples were neutralized with 5 M K_2CO_3 and centrifuged at −5 °C. The supernatant liquid was lyophilized and resuspended in a small volume of double-distilled water. To eliminate unspecific fluorescence of tissue extracts the lyophilisate was treated batchwise with activated $Mg_2Si_3O_8$ (Florosil)[15]. Traces of oxalic acid and ketones were destroyed with H_2O_2 (0.06 % final concentration)[16]. The latter was then eliminated by addition of catalase (EC 1.11.1.6) (10 μg/ml). Phosphoenolpyruvate was determined fluorimetrically by the method of Lowry *et al.*[17].

Carboxylation reaction

Mitochondria (0.5–3 mg) were incubated in 1 ml for 5, 10, 15, 20 min at 30 °C in 160 mM sucrose, 2 mM triethanolamine, 17.5 mM sodium phosphate, 3 mM phosphoenolpyruvate, 2 mM IDP, 2 mM ADP, 1 mM dithioerythritol, 20 mM NaF, 2 mM fluorocitrate, 50 mM $NaH^{14}CO_3$ (approx. 50 000 cpm/mmole), 4 mM $MnCl_2$; final pH 7.0. The reaction was terminated with 0.5 ml 16 % trichloroacetic acid. The mixture was analyzed for acid-stable radioactivity as described above.

Absolute activity of phosphoenolpyruvate carboxylase within the mitochondrial fraction was determined after preincubation of the mitochondria in 0.1 M sodium phosphate buffer (pH 7.0). The standard assay as described above with 2 mM fluorocitrate was used.

RESULTS

Distribution of fructosediphosphatase and phosphoenolpyruvate carboxylase

Table II presents activity values of fructosediphosphatase in various rat and rabbit muscles. For purposes of comparison, activities of fructosediphosphatase in rat liver and kidney are also included. In both species the activity of this enzyme in muscle is mainly found in white type muscles. These results agree well with the

TABLE II

FRUCTOSEDIPHOSPHATASE ACTIVITIES IN RAT AND RABBIT MUSCLES AND IN RAT LIVER AND KIDNEY

Activities of fructosediphosphatase in red, white and heart muscle of rat and rabbit and in rat liver and kidney. The values (munits/g fresh weight) represent the means of 2 experiments. For each experiment the respective muscles of 2–3 animals were pooled.

		munits/g wet wt
Rat	*m. rectus fem.*	167
	m. soleus	< 15
	heart	< 15
	liver	4200
	kidney	4560
Rabbit	*m. psoas*	995
	m. semitendinosus	< 15
	heart	< 15

findings of several authors[8–11] and lead to the conclusion that gluconeogenetic capacity in muscles is low and is restricted mainly to white fibres.

Phosphoenolpyruvate carboxylase is present in white muscles of several animals. As shown in Table III, activity values vary from 3 munits/g (*rectus femoris*, guinea-pig) to 16 munits/g (*rectus femoris*, rat). These values are 10 times or more

TABLE III

INTRACELLULAR DISTRIBUTION OF PHOSPHOENOLYPRUVATE CARBOXYLYASE (PEP-CK) AND (NADP) MALATE DEHYDROGENASE (ME) IN DIFFERENT MUSCLE TYPES AND IN RAT LIVER AND KIDNEY

The values (munits/g fresh weight) represent the means of 4 experiments. In each experiment the respective muscles of 3 animals were pooled.

	Enzyme	*Extramitochondrial*		*Intramitochondrial*		*Total munits/g*
		munits/g	*%*	*munits/g*	*%*	
Rat						
m. rectus fem.	PEP-CK	11.7	72	4.5	28	16.2
	ME	337	82	76	18	413
m. soleus	PEP-CK	7.7	39	12.2	61	19.9
	ME	130	33	268	67	398
heart	PEP-CK	7	25	20.9	75	27.9
	ME	406	23	1416	77	1822
liver	PEP-CK	2260	76	700	24	2960
	ME	2488	94	151	6	2639
kidney cortex	PEP-CK	2680	75	870	25	3550
	ME	636	61	405	39	1041
Guinea pig						
m. rectus fem.	PEP-CK	2	60	1.3	40	3.3
	ME	76	89	9	11	85
m. soleus	PEP-CK	2.7	41	3.8	59	6.5
	ME	75	55	62	45	137
heart	PEP-CK	7.1	38	11.6	62	18.7
	ME	172	26	494	74	666
liver	PEP-CK	2400	21	8750	79	11150
	ME	74	100	0	0	74
Rabbit						
m. psoas	PEP-CK	3.3	83	0.7	17	4
	ME	305	100	0	0	305
m. semitend.	PEP-CK	26	76	8.3	24	34.3
	ME	105	80	26	20	131
heart	PEP-CK	10	31	22	69	32
	ME	236	35	443	65	679
Pigeon						
m. pectoralis	PEP-CK	2.3	70	1	30	3.3
	ME	189	90	21	10	210
heart	PEP-CK	12	90	1	10	13
	ME	82	87	13	13	95
Seagull						
m. pectoralis	PEP-CK	1.3	58	1.2	47	2.5
	ME	438	14	2745	86	3183
heart	PEP-CK	1.3	38	2.2	62	3.5
	ME	182	10	1791	90	1973
Locusta migratoria						
flight muscle	PEP-CK	25	15	142	85	167
	ME	825	77	265	23	1080

lower than the respective activities of fructosediphosphatase. Some 60–80% of the total activity of phosphoenolpyruvate carboxylase in white muscles is located in the extramitochondrial compartment, as is the activity of fructosediphosphatase. On the other hand, in red and heart muscle the activity of phosphoenolpyruvate carboxylase is higher than in the white muscles by as much as a factor of 8 (*e.g.* heart/*m. psoas*, rabbit). In heart and red muscles of rat and rabbit the activity of this enzyme is as high as the values for fructosediphosphatase. Note that (with the exception of the *m. semitendinosus* of rabbit) in these tissues of the rat, guinea-pig and rabbit, phosphoenolpyruvate carboxylase is not in the same compartment as is fructosediphosphatase: 60–70% is located within the mitochondria.

The highest absolute activity levels of phosphoenolpyruvate carboxylase were found in flight muscle of *Locusta migratoria*. This activity is concentrated almost exclusively within the mitochondria. In contrast with the finding in all other heart and red muscles, the heart muscle of pigeon is exceptional in that only 10% of the total activity is located in the mitochondrial fraction.

Distribution of (NADP) malate dehydrogenase

Absolute activities of (NADP) malate dehydrogenase are very much higher than those of phosphoenolpyruvate carboxylase (5–100-fold). The intracellular distribution of (NADP) malate dehydrogenase in the different muscles is similar to that of phosphoenolpyruvate carboxylase. This is obvious for pigeon heart where the intracellular percentage distribution of both enzymes is inversely related to that of other red and heart muscles listed. The correlation of the relative intracellular distribution of phosphoenolpyruvate carboxylase and (NADP) malate dehydrogenase is also well documented by a highly significant correlation coefficient ($r = +0.84$).

Dietary and hormonal influences

To learn more about the function of phosphoenolpyruvate carboxylase and (NADP) malate dehydrogenase in muscles, the activities of these enzymes were studied under different dietary and hormonal influences (Table IV). The activity of (NADP) malate dehydrogenase in different muscles of the rat remains unchanged during induced lipogenesis, thyrotoxicosis, prednisolone application, and starvation. The activity of phosphoenolpyruvate carboxylase decreases under the influence of starvation, prednisolone and induced lipogenesis up to a factor of 2 in all muscles investigated. A different response of phosphoenolpyruvate carboxylase in red and white muscles is also observed in thyrotoxicosis. Application of thyroid hormone causes a slight increase of this enzyme in red muscle and heart, whereas activity in white muscle is decreased.

Phosphoenolpyruvate and oxaloacetate synthesis by isolated mitochondria

Isolated rat heart mitochondria are able to synthesize phosphoenolpyruvate from added malate and ATP (Table V). The reverse reaction of phosphoenolpyruvate carboxylase is also demonstrable in intact mitochondria. In this reaction, [^{14}C]bicarbonate is incorporated half as fast as the maximal activity of phosphoenolpyruvate carboxylase in ruptured mitochondria. The labelled substrates were analyzed by chromatography on Dowex 1-X8 (Cl^- form) (Fig. 2). Besides lactate, we found malate, fumarate, aspartate, citrate and probably other citric acid cycle intermediates.

TABLE IV

HORMONAL AND DIETARY INFLUENCES ON PHOSPHOENOLPYRUVATE CARBOXYLASE (PEP-CK) AND (NADP) MALATE DEHYDROGENASE (ME) IN MUSCLES OF THE RAT

Intracellular distribution of (NADP) malate dehydrogenase and phosphoenolpyruvate carboxylase in *m. rectus fem.*, *m. soleus* and heart muscle of the rat under different dietary and hormonal conditions. The values (munits/g wet wt) represent the means of 2 experiments. In each experiment the respective muscles of 3 animals were pooled.

	Enzyme	*Extramitochondrial*		*Intramitochondrial*		*Total munits/g*
		munits/g	*%*	*munits/g*	*%*	
Lipogenesis						
m. rectus	PEP-CK	4.0	68	1.9	32	5.9
	ME	395	83	79	17	474
m. soleus	PEP-CK	5.6	46	7.8	54	13.4
	ME	164	49	170	51	334
heart	PEP-CK	4.6	41	6.5	59	11.1
	ME	515	25	1580	75	2095
Triiodothyronine						
m. rectus	PEP-CK	4.5	53	3.9	47	8.4
	ME	360	80	92	20	452
m. soleus	PEP-CK	18.3	69	8.2	31	26.5
	ME	182	46	226	54	408
heart	PEP-CK	16.3	40	24.1	60	40.4
	ME	644	31	1155	69	1799
Prednisolone						
m. rectus	PEP-CK	5.5	90	0.6	10	6.1
	ME	304	83	64	17	368
m. soleus	PEP-CK	4.2	50	4.2	50	8.4
	ME	128	42	176	58	304
heart	PEP-CK	4.0	40	6.2	60	10.2
	ME	256	18	1152	82	1408
Starved						
m. rectus	PEP-CK	7.3	78	2.0	22	9.3
	ME	284	89	47	11	331
m. soleus	PEP-CK	4.6	58	3.3	42	7.9
	ME	126	42	173	58	299
heart	PEP-CK	1.6	18	7.7	82	8.3
	ME	379	19	1659	81	2038

Probably lactate dehydrogenase was not completely inactivated by preincubation of the isolated mitochondria with subtilisin. Thus lactate may have been produced by activity of intramitochondrial (NADP) malate dehydrogenase and lactate dehydrogenase.

DISCUSSION

Phosphoenolpyruvate carboxylase and fructosediphosphatase are both required for the conversion of lactate to glucose. Because of the absence of pyruvate carboxylase[6,7] it is suggested that the (NADP) malate dehydrogenase pathway is the probable route of oxaloacetate synthesis from pyruvate in muscle. This suggestion is supported by the observed correlation of the intracellular distribution of (NADP) malate dehydrogenase and phosphoenolpyruvate carboxylase. The rather low activity levels of phosphoenolpyruvate carboxylase as compared with fructosediphosphatase and

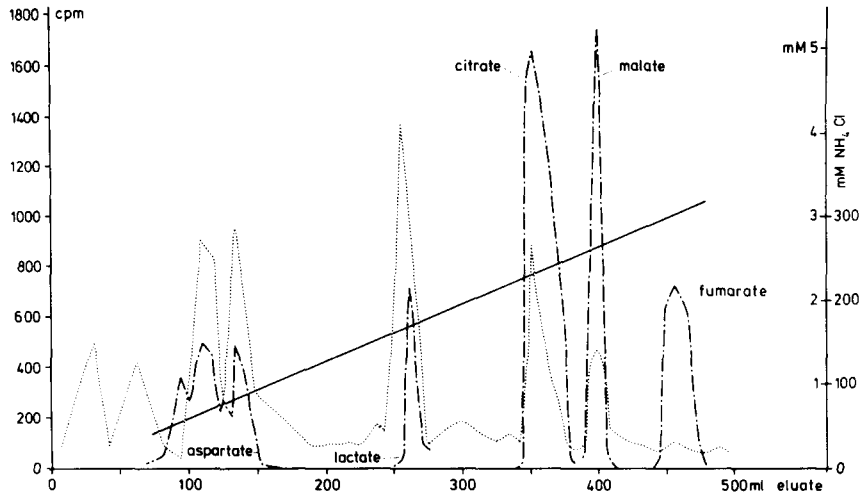

Fig. 2. Elution profile of acid-stable ^{14}C activity after incubation of heart muscle mitochondria with [^{14}C]bicarbonate (approx. 250 000 cpm/mmole) and phosphoenolpyruvate. Conditions are as described in Table V. Chromatography was performed on a Dowex 1-X8 (Cl^- form) column (70 cm × 0.9 cm) (H. W. Hofer, in preparation). The metabolites were eluted by a gradient of NH_4Cl and borate (0–10 mM). Malate, fumarate and lactate were added to the acid-stable ^{14}C-labelled material and were determined by photometric tests in the eluted fractions. Radioactivity was determined in a liquid scintillation counter. ·····, ^{14}C activity; — · —, aspartate, lactate, citrate, malate, fumarate (mM); ———, concentration of NH_4Cl (mM).

(NADP) malate dehydrogenase might suggest this enzyme to be rate limiting in the overall reaction of gluconeogenesis in muscle. Opie and Newsholme[9] reported much higher activities of phosphoenolpyruvate carboxylase in muscle. This discrepancy may result from the fact that these authors used a spectrophotometric test system which is perhaps not satisfactory for assay in crude tissue extracts.

When red and white muscles are compared, gluconeogenetic capacities might be ascribed mainly to the latter in the light of the high activities of fructosediphosphatase and since fructosediphosphatase as well as phosphoenolpyruvate carboxylase are present predominantly within the extramitochondrial compartment. The activity of phosphoenolpyruvate carboxylase measured in rabbit psoas muscle (4 nmoles phosphoenolpyruvate used per min and per g muscle) would also correlate well with the rate of glycogen synthesis from lactate in the same muscle (12 nmoles, expressed as glucose units, per min and per g muscle) as reported by Bendall and Taylor[5]. The above activity value refers to the carboxylation reaction of phosphoenolpyruvate carboxylase and would be 3–8 times higher in the reverse reaction relevant for gluconeogenesis according to Chang and Lane[14].

The gluconeogenetic capacity based on phosphoenolpyruvate carboxylase activity per g of muscle is obviously very low in white muscle. This conclusion, however, is misleading since it does not take into account the large percentage of muscle tissue in the whole organism. Assuming that 40% of the body weight is represented by skeletal muscle, this amounts to approximately 100 g per 250 g rat body weight. As to the activity measured in white muscle (Table III), a total activity of phosphoenolpyruvate carboxylase of 1600 munits per animal may be estimated. This value should

be compared with the respective total activities in kidney and liver. Using organ weights of 8 g for liver and 0.8 g for kidney cortex, the following total activities of phosphoenolpyruvate carboxylase are obtained: 24 000 munits/liver and 2700 munits/kidney cortex. Thus, it is evident that the total activity of muscle amounts to only approx. 7% of that of liver, but is in the same range as that of kidney cortex.

It is questionable whether the function of phosphoenolpyruvate carboxylase in muscle is exclusively related to gluconeogenesis. In view of the predominantly intramitochondrial localization of the enzyme in red muscle and heart, and the finding that in these muscles absolute activities are higher than in white muscle, other functions may be indicated. Phosphoenolpyruvate is transported across the inner mitochondrial membrane[18], and according to our findings, this transport is not rate limiting in phosphoenolpyruvate synthesis (Table V, Fig. 2). Under the conditions used in

TABLE V

DECARBOXYLATION AND CARBOXYLATION REACTION OF PHOSPHOENOLPYRUVATE CARBOXYLASE IN RAT HEART MITOCHONDRIA

(a) Decarboxylation reaction: mitochondria (5–10 mg) were incubated in 3 ml for 20 min at 30 °C in 160 mM sucrose, 2 mM triethanolamine, 17.5 sodium phosphate buffer, 3 mM $MgCl_2$, 4 mM ATP, 2 mM malate, 2 mM fluorocitrate, 10 μg oligomycin, 2 mM ITP. Final pH 7.2. The reaction was terminated with 0.5 ml 36% trichloroacetic acid. Phosphoenolpyruvate was determined as described in Methods. (b) Carboxylation reaction: mitochondria (0.5–3 mg) were incubated in 1 ml for 20 min at 30 °C in 160 mM sucrose, 2 mM triethanolamine, 17.5 mM sodium phosphate, 3 mM phosphoenolpyruvate, 2 mM IDP, 1 mM dithioerythritol, 20 mM NaF, 2 mM fluorocitrate, 4 mM $MnCl_2$, 50 mM $NaH^{14}CO_3$ (approx. 50 000 cpm/nmole). Final pH 7.0. Mitochondria were swollen by incubation with 0.1 M phosphate buffer (pH 7.0). The activity of phosphoenolpyruvate carboxylase in the swollen mitochondria was determined by standard assay as described in Methods.

	μmole phosphoenolpyruvate formed · mg⁻¹ protein · 20 min⁻¹	*μmole [¹⁴C]bicarbonate incorporated · mg⁻¹ protein · 20 min⁻¹*
Intact mitochondria	$1.6 \cdot 10^{-2}$	$4.4 \cdot 10^{-3}$
Swollen mitochondria		$9.7 \cdot 10^{-3}$

these experiments, synthesis of phosphoenolpyruvate is correlated with the absolute activity levels of phosphoenolpyruvate carboxylase. It may also be that phosphoenolpyruvate is taken up into the mitochondria and there metabolized: this could represent an important mechanism in regulating the malate–aspartate shuttle (Fig. 3). As indicated by the experiments of LaNoue and Williamson[19] the rate of utilization of cytosolic reducing equivalents *via* the malate–aspartate shuttle by mitochondria of rat heart is determined by the rates of α-ketoglutarate and aspartate efflux. The efflux of these metabolites is subject to a variety of controls: (1) the activity of α-ketoglutarate dehydrogenase, which is decreased by a high GTP/GDP ratio; (2) the dependence of aspartate efflux on an energy-linked step; and (3) the intramitochondrial oxaloacetate concentration which is itself regulated by affecting both α-ketoglutarate and aspartate efflux. The two latter control mechanisms may be responsible for oligomycin decreasing the rate of flux through the malate–aspartate shuttle.

The utilization of phosphoenolpyruvate by phosphoenolpyruvate carboxylase would provide GTP and oxaloacetate. Thus it seems possible that in the hypoxic state of heart muscle the inhibition of the malate–aspartate shuttle (analogous to the effect

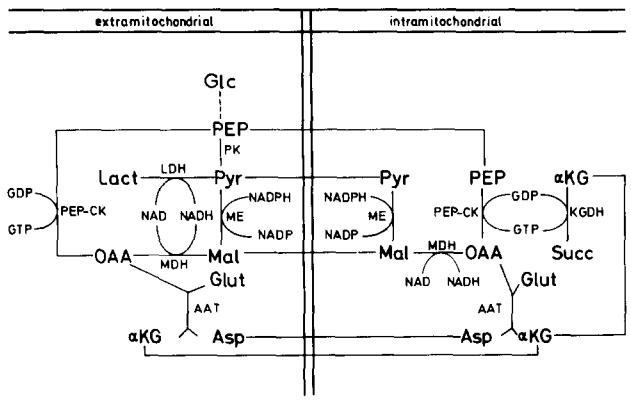

Fig. 3. Proposed relationship of phosphoenolpyruvate carboxylase and (NADP) malate dehydrogenase reactions with the malate–aspartate shuttle. Abbreviations: Glc, glucose; PEP, phosphoenolpyruvate; Pyr, pyruvate; Lact, lactate; Mal, malate; OAA, oxaloacetate; Glut, glutamate; α-KG, α-ketoglutarate; Asp, aspartate; Succ, succinate; PK, pyruvate kinase; LDH, lactate dehydrogenase; PEP-CK, phosphoenolpyruvate carboxylase; MDH, (NAD) malate dehydrogenase; ME, (NADP) malate dehydrogenase; AAT, aspartate aminotransferase; KGDH, α-ketoglutarate dehydrogenase.

obtained by the addition of oligomycin) is abolished by GTP and oxaloacetate supply by the action of phosphoenolpyruvate carboxylase. The observation that aspartate accumulates when heart muscle mitochondria are incubated with phosphoenolpyruvate and IDP is consistent with this suggestion (Fig. 2).

Another of the findings of LaNoue and Williamson[19] was that accumulation of α-ketoglutarate by isolated rat heart mitochondria is dependent on the concentration of extramitochondrial malate. (NADP) malate dehydrogenase ultimately regulates the extramitochondrial concentration of α-ketoglutarate by providing malate.

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