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Bioluminescent and Fluorometric Techniques for Determinations of 19 Metabolites of ADP/ATP-Dependent Transformations in Energy Metabolism in 200 (or 400) mg Muscle

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Summary: A procedure is described for the determination of the following 19 metabolites in 200 mg gastrocnemius muscle from guinea pig: glucose, glucose 1-phosphate and 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, 3-phospho- and 2-phosphoglyceric acid, phosphoenolpyruvate, pyruvate, glycerol, glycerol 3-phosphate, AMP, ADP, ATP, inorganic phosphate, creatine, creatine phosphate. Bioluminometric and fluorometric techniques for mono- and dinucleotide determinations were used. In the case of fluorometric measurement of NADH, 400 mg tissue were necessary. The coefficient of variation for assays on the same sample was 0.04 for bioluminometric techniques and 0.10 for fluorometric techniques.

Biolurninometrische und fluorometrische Methoden zur Konzentrationsbestimmung von 19 Metaboliten ADP/ATP-abhängiger Umsätze des Energiestoffwechsels in 200 (oder 400) mg Muskel

Zusammenfassung: Es wird eine Methodik zur Konzentrationsbestimmung der folgenden 19 Metaboliten in 200 mg m. gastrocnemius des Meerschweinchen beschrieben:
Glucose, Glucose-1-phosphat und -6-phosphat, Fructose-6-phosphat, Fructose-1,6-bisphosphat, Glycerinaldehyd-3-phosphat, Dihydroxyacetonphosphat, 3-Phosphoglycerat, 2-Phosphoglycerat, Phosphoenolpyruvat, Pyruvat, Glycerin, Glycerin-3-phosphat, AMP, ADP, ATP, anorganisches Phosphat, Kreatin, Kreatinphosphat.
Dazu wurden bioluminometrische sowie fluorometrische Methoden angewandt. Falls NADH fluorometrisch bestimmt wurde, wurden 400 mg Gewebe benötigt. Der Variabilitätskoeffizient bei der Bestimmung einer und derselben Probe betrug 0,04 bei der bioluminometrischen und 0,10 bei der fluorometrischen Methodik.

Introduction

A knowledge of the concentrations of metabolites in tissues permits an assessment of the state of metabolism. In previous publications (1, 2), we demonstrated, by using certain criteria, that tissue concentrations of rat liver metabolites are rich sources of information concerning

(a) an eventual equilibrium state of the transformations implicating these metabolites,
(b) cosubstrate compartition, and
(c) stoichiometric relations between metabolite turnover in different cell compartments; this was true despite the fact that the metabolites may be compartmentalized within the cell. It was thereby shown in particular that tissue substrate concentrations of liver metabolites allow the metabolic status to be characterized under several physiological conditions (alloxan-diabetic, starved rats and rats fed on carbohydrate or fat diets).

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With a view to applying these same criteria of interpretation for cell metabolite concentrations to guinea pig gastrocnemius muscle, we have optimized determination methods for 16 phosphorylated and non-phosphorylated metabolites of energy metabolism. The limited sample material and the great number of assays created a requirement for high sensitivity; this was achieved with fluorometric methods, and especially by the use of bioluminescence for the assay of NADH and ATP, ADP and AMP. We could demonstrate that this method satisfies the precision requirements for determining metabolite concentrations in muscles.

Materials and Methods

Metabolites

Metabolite determinations were carried out as NADH or NADPH end point conversions which were then measured fluorometrically or by bacterial bioluminescence. The present procedure was derived from methods in the literature (3) with the aim of determining if possible several metabolites in one assay; it was optimized for determinations in muscle extracts. Two tris buffer solutions at different pH values were used: 0.30 mol · l^{-1} (final concentration in the assay) tris(hydroxymethyl)-aminomethane (tris) adjusted with HCl to pH = 7.0 or 8.0 containing 5.9 mmol · l^{-1} $MgCl_2$ and 0.5 mmol · l^{-1} ethylenediaminetetraacetic acid (EDTA).

The following assays were carried out in tris buffer at pH = 7.0:

a) *2-phosphoglyceric acid, 3-phosphoglyceric acid* and *phosphoenolpyruvate* were determined in the presence of 0.76 mmol · l^{-1} ADP, 0.21 mmol · l^{-1} NADH and *L*-lactate dehydrogenase (EC 1.1.1.27; 1.1 μmol · min^{-1}), by starting the individual reactions by addition of pyruvate kinase (EC 2.7.1.40; 0.80 μmol · min^{-1}), enolase (EC 4.2.1.11; 0.080 μmol · min^{-1}) and phosphoglycerate mutase (EC 2.7.5.3; 2.5 μmol · min^{-1}), in that order.

b) *dihydroxyacetone phosphate, glyceraldehyde 3-phosphate,* and *pyruvate* in the presence of 0.15 mmol · l^{-1} NADH, by starting the individual reactions by addition of glycerol 3-phosphate dehydrogenase (EC 1.1.1.8; 0.14 μmol · min^{-1}), triose phosphate isomerase (EC 5.3.1.1; 4.0 μmol · min^{-1}) and *L*-lactate dehydrogenase (1.1 μmol · min^{-1}), in that order.

c) *D-fructose 1,6-bisphosphate* was assayed in the same reaction solution of b) but with the extra addition of glycerol 3-phosphate dehydrogenase (0.14 μmol · min^{-1}) as well as triosephosphate isomerase (4.0 μmol · min^{-1}), and by starting the assay by addition of aldolase (EC 4.1.2.13; 0.09 μmol · min^{-1}).

d) *glycerol* was determined in the presence of 2.1 mmol · l^{-1} ATP, 1.4 mmol · l^{-1} phosphoenolpyruvate, 0.21 mmol · l^{-1} NADH, *L*-lactate dehydrogenase (1.1 μmol · min^{-1}) and pyruvate kinase (0.80 μmol · min^{-1}), and the assay was started by addition of glycerol kinase (EC 2.7.1.30; 0.034 μmol · min^{-1}).

e) The assay mixture for *creatine* was the same as that for glycerol, but the assay was started by addition of creatine kinase (EC 2.7.3.2; 7.6 μmol · min^{-1}).

Assays for the following metabolites were carried out in tris buffer at pH = 8.0:

f) *D-glucose 6-phosphate, D-glucose 1-phosphate, fructose 6-phosphate* in the presence of 6.0 μmol · l^{-1} glucose 1,6-bisphosphate and of 1.5 mmol · l^{-1} NADP by starting the reactions with glucose 6-phosphate dehydrogenase (EC 1.1.1.49; 0.056 μmol · min^{-1}), phosphoglucomutase (EC 2.7.5.1; 0.16 μmol · min^{-1}) and phosphoglucose isomerase (EC 5.3.1.9; 0.28 μmol · min^{-1}) in that order.

g) *D-glucose* in the presence of 0.70 mmol · l^{-1} ATP, 1.5 mmol · l^{-1} NADP and *D*-glucose 6-phosphate dehydrogenase (0.056 μmol · min^{-1}) by starting the assay with hexokinase (EC 2.7.1.1; 0.28 μmol · min^{-1}).

A further buffer solution (0.20 mol · l^{-1} imidazole adjusted with HCl to pH = 7.0; 0.60 mmol · l^{-1} $MgCl_2$, 1.0 mmol · l^{-1} EDTA) was used for:

h) *inorganic phosphate* assay and contained 6.0 μmol · l^{-1} *D*-glucose 1,6-bisphosphate, 4.9 mmol · l^{-1} glycogen (as glucosyl unit), 10 μmol · l^{-1} AMP, 0.50 mmol · l^{-1} *D,L*-dithiothreitol and 1.5 mmol · l^{-1} NADP, glucose 6-phosphate dehydrogenase (0.056 μmol · min^{-1}) and phosphoglucomutase (0.16 μmol · min^{-1}). The reaction was started with phosphorylase a (EC 2.4.1.1; 0.20 μmol · min^{-1}).

Enzymes for assays f) and h) were dialysed against the buffer solution for the corresponding assay.

For fluorimetric determinations, the total assay volume amounted to 145 μl, of which 120 μl were neutralized tissue extract and the remainder consisted of reagent and buffer mix. Enzymes for starting the reactions were added in sample tubes (3.0 mm inner diameter) made of selected optical glass (Hellma, Müllheim, Baden, Germany). The higher sensitivity of the bioluminescent determination allowed 20 μl tissue extract to be used.

All biochemicals were purchased from Boehringer, Mannheim, Germany; all chemicals from Merck, Darmstadt, Germany.

The *tissue extracts* were prepared by pulverizing one portion of frozen tissue and two portions of 0.60 mol · l^{-1} $HClO_4$ with 0.10 mmol · l^{-1} EDTA at the presence of liquid nitrogen in a pulverizer (Pulverisette, Fritsche, Idar-Oberstein, Germany). The thawed powder was centrifuged for 20 min at about 10^5 *g*; the supernatant was neutralized with 2 mol · l^{-1} K_2CO_3, centrifuged for 2 min as above and in part appropriately diluted for the assays. The samples in the different assays consisted of solutions of the neutralized tissue extract correspondingly diluted: 1 : 1 for b) and f); 1 : 2 for a); 1 : 3 for c); 1 : 5 for d); 1 : 10 for g); 1 : 20 for h) and 1 : 100 for the creatine assay (e).

Bioluminescence determinations of NADH concentration in the assay and in the blanks were carried out after dilution (1 : 100 or lower) and mixing 10.0 μl with 50.0 μl reconstituted NADH-monitoring reagent (LKB-Wallac, Turku, Finland) and 200 μl 0.100 mol · l^{-1} potassium phosphate buffer at pH = 7.00; the constant luminescence was measured in a 1251 Luminometer (LKB). In this same sample tube, 10.0 μl 0.5 μmol · l^{-1} NADH as internal standard were added twice; the luminescence was registered after every addition and the NADH-concentration calculated according to *Feraudi* et al. (4). The recovery ratio of NADH as an effect of metabolite conversion varied in the different assays a)—e). This ratio was therefore determined by addition of a metabolite standard to the assay (cf. l.c. (4)).

Calibration for direct fluorometric measurement of NAD(P)H concentrations in the assays was carried out by using a NAD(P)H sample as control before every measurement to correct light source inconstancy. A calibration curve for the NADH or NADPH fluorescence versus the concentration in the same assay solution was approximated with a polynome quotient (for algorithms see l.c. (5)). The sample concentration was then calculated from its fluorescence value. The coefficient of variation for the same sample amounted to 0.10, whereas in the bioluminescent method it amounted to 0.04.

i) *AMP, ADP* and *ATP* were determined according to *Feraudi* et al. (4); because of interferences from metabolites in the muscle extract, the recovery ratio for these adenine mononucleotides was specially determined for these samples and remained constant among them.

j) *Creatine phosphate* was determined bioluminescently after its conversion to ATP. 50.0 μl neutralized and diluted muscle extract which contained about 5 nmol creatine phosphate were mixed in micro test tubes with 50.0 μl 2.5 mmol · l^{-1} ADP and 144 μl of the following mixture:
1.44 ml 0.10 mol · l^{-1} tris adjusted to pH = 7.75 with acetic acid and 20 μl creatine kinase solution (EC 2.7.3.2; 1 mg · ml^{-1}, Boehringer, 25 U/mg protein). The closed tubes were incubated for 30 min at 37 °C. The reaction solution was diluted (1 : 10^4) and used for ATP determination according to i) (see above). An internal standard with creatine phosphate was also analysed for every sample to determine the recovery ratio.

Animals

The male guinea pigs used for the present metabolite determinations were the same as those in a previous study (6).

Calculations

Calculations were performed by means of a computer Commodore 3032 with printer 3022.
The coefficients of variation (CV) are given as $CV = \frac{S.D.}{\bar{x}}$.

Results

The recovery of each metabolite to be measured was calculated for fluorometric and bioluminescent metabolite determinations, using photometric calibration and assay mixtures of the composition given in the methods. Within the limits of experimental error, this recovery ratio was unity for fluorometry. Luminometric determinations allowed the measurement of recovery ratios different from unity. These recoveries were taken into consideration in the concentration calculations. With respect to recovery, similar behaviour was observed in the bioluminescent determination of AMP, ADP and ATP (4). Creatine phosphate was determined by means of an internal creatine phosphate standard.

Taking into account the above factors in calculating metabolite concentrations, we obtained the results shown in table 1 for gastrocnemius muscle of untreated guinea pig. Because of the singular feature of the results, i. e. that the large coefficient of variation (up to 0.56) of some metabolite concentrations contrasts with the reproducibility of the method, mean values may not describe the collective. A concentration of 10 μmol · kg^{-1} can still be measured with a coefficient of variation around 0.3. The question as to whether the broad distribution of the concentration values may be explained as an expression of the laws regulating muscle cell metabolism can be answered as follows: In recent studies (1, 2), we demonstrated that cell concentrations of metabolites in the liver allow constant relationships to be found between mass ratios of transformations which concern them. Consider the transformation from glycerate 2-phosphate to glycerate 3-phosphate:
The corresponding mass ratio glycerate 3-phosphate/glycerate 2-phosphate in table 1 shows a lower coefficient of variation (0.33) than that expected from the coefficients of variation of glycerate 3-phosphate and glycerate 2-phosphate (0.36 and 0.30 respectively). The mean 5.59 matches well with the equilibrium constant value 5.0 (7). Lower coefficients of variation (CV) were also found for the following mass quotients:

fructose 1,6-bisphosphate / [dihydroxyacetone phosphate × glyceraldehyde 3-phosphate] (CV = 0.24);
fructose 1,6-bisphosphate × pyruvate / [fructose 6-phosphate × phosphoenolpyruvate] (CV = 0.08);
glycerol × glucose 6-phosphate / [glycerol 3-phosphate × glucose] (CV = 0.28);
glucose 6-phosphate × creatine / [glucose × creatine phosphate] (CV = 0.51);
glucose 6-phosphate × pyruvate / [glucose × phosphoenolpyruvate] (CV = 0.24).
Also the energy charge (ATP + 0.5 ADP)/(ATP + ADP + AMP) shows a lower CV (0.09) than that expected. Besides glucose 1-phosphate and inorganic phosphate, all metabolites measured here seem to obey to relationships in which the mass ratios or energy charge may have nearly constant values. Similar results were also found for trained guinea pigs (*Feraudi* et al. in preparation). The low CV of the quotients above demonstrate that the single concentrations involved were determined precisely enough and that the broad scatter of values is to be attributed to the nature of interdependence relationships between muscle metabolite concentrations.

References

1. Feraudi, M. (1978) Arch. Internat. Physiol. Biochem. *86*, 487–507.
2. Feraudi, M. (1979) Arch. Intern. Physiol. Biochem. *87*, 915–923.
3. Bergmeyer, H. U. (ed.) (1974) Methoden der enzymatischen Analyse, 3rd ed. Verlag Chemie, Weinheim/Bergstraße, Germany.
4. Feraudi, M., Merkt, J. & Weicker, H. (1981) Int. J. Sports Medicine 2, 106–109.
5. Feraudi, M. & Glaser, W. (1977) Ital. J. Biochem. *26*, 22–26.
6. Feraudi, M. & Weicker, H. Submitted.
7. Grisolia, S. (1962) in Methods in Enzymology (Colowick, S. P. & Kaplan, N. O., eds.) Vol. 5, p. 236, Academic Press, New York.

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Tab. 1. Metabolite concentrations of freeze-clamped gastrocnemius muscle of untreated guinea pig. Concentrations in $\mu mol \cdot kg^{-1}$ (wet weight).

	Guinea pig number 8	9	10	11	12	13	18	19	20	21	24	25	26	$\bar{x} \pm SD$	$\frac{SD}{\bar{x}} = CV$
Substrates															
Glucose 1-phosphate	–	–	–	–	–	35	65	21	–	–	–	–	–	40.3 ± 22.5	0.56
Glucose 6-phosphate	–	–	–	–	–	13	–	15	42	17	–	13	–	20.0 ± 12.4	0.62
Glucose	631	1193	1261	1785	993	1024	–	817	1822	1322	987	890	731	1121 ± 379	0.34
Fructose 1,6-bisphosphate	507	669	521	1071	748	1388	903	1526	2420	1826	2105	1742	1678	1316 ± 627	0.48
Fructose 6-phosphate	–	–	–	–	–	–	10	–	–	16	–	10	–	12.0 ± 3.46	0.29
Dihydroxyacetone phosphate	98	105	102	124	–	44	86	79	122	110	84	111	189	104 ± 34.4	0.33
Glyceraldehyde 3-phosphate	–	–	–	–	10	11	–	–	9	–	–	–	5	8.75 ± 2.63	0.30
Phosphoenolpyruvate	268	509	270	388	–	244	134	251	454	–	278	273	177	293 ± 113	0.39
Pyruvate	29	23	–	–	47	23	39	19	23	26	–	37	–	29.7 ± 9.3	0.31
Glycerol 3-phosphate	–	–	–	–	126	118	90	71	82	106	69	63	75	88.9 ± 22.8	0.26
Glycerol	–	–	1012	1127	1537	1203	–	1027	804	1575	974	1196	1905	1236 ± 336	0.27
Creatine phosphate	6105	4901	5745	12984	3698	7267	4618	9142	4315	9269	2345	4721	5419	6194 ± 2845	0.46
Creatine	29637	31704	29240	41752	35615	47181	25396	21211	30404	43860	27604	37283	44066	34227 ± 8080	0.24
Glycerate 3-phosphate	612	412	–	617	–	784	423	645	968	–	1107	1139	1062	777 ± 276	0.36
Glycerate 2-phosphate	85	133	–	126	–	146	121	126	249	–	150	132	155	142 ± 42.3	0.30
AMP	2788	–	3118	3923	–	2406	–	2617	702	–	–	–	2714	2610 ± 975	0.37
ADP	9261	–	5530	8474	–	7943	–	6627	4084	6354	–	–	4611	6611 ± 1847	0.28
ATP	8440	–	10612	11956	–	5409	–	7145	8576	14335	–	–	7045	9190 ± 2932	0.32
Inorganic phosphate	–	–	–	–	18524	23021	15567	14327	15428	22637	11992	19342	22205	18116 ± 4007	0.22
Substrate quotients															
Glucose 6-phosphate/glucose	–	–	–	–	–	0.0127	–	0.0184	0.0231	0.0129	–	0.0146	–	0.015 ± 0.004	0.27
Fructose 1,6-bisphosphate/fructose 6-phosphate	–	–	–	–	–	–	90.3	–	–	114	–	174	–	126 ± 43.1	0.34
Phosphoenolpyruvate/pyruvate	9.24	22.1	–	–	–	9.74	3.44	13.2	19.3	–	–	7.38	–	12.1 ± 6.63	0.55
Glycerol 3-phosphate/glycerol	–	–	–	–	0.082	0.098	–	0.069	0.10	0.067	0.071	0.053	0.039	0.072 ± 0.020	0.28
Creatine phosphate/creatine	0.206	0.155	0.197	0.311	0.104	0.154	0.182	0.431	0.142	0.211	0.085	0.127	0.123	0.186 ± 0.093	0.50
ATP/ADP	0.911	–	1.9	1.41	–	0.681	–	–	2.10	2.26	–	–	1.5	1.48 ± 0.572	0.39

	Guinea pig number 8	9	10	11	12	13	18	19	20	21	24	25	26	$\bar{x} \pm SD$	$\frac{SD}{\bar{x}} = CV$
Mass ratios															
Fructose 1,6-bisphosphate x glycerol/ [fructose 6-phosphate x glycerol 3-phosphate]	–	–	–	–	–	–	–	–	–	1696	–	3307	–	2502 ± 1139	0.46
Fructose 1,6-bisphosphate x creatine/ [fructose 6-phosphate x creatine phosphate]	–	–	–	–	–	–	497	–	–	540	–	1376	–	804 ± 496	0.62
Phosphoenol pyruvate x glycerol/ [pyruvate x glycerol 3-phosphate]	–	–	–	–	–	99.0	–	191	186	–	–	140	–	154 ± 44.6	0.29
Phosphoenolpyruvate x creatine/ [pyruvate x creatine phosphate]	44.9	143	–	–	–	63.2	18.9	30.7	122	–	–	58.3	–	68.7 ± 41.5	0.60
Glucose 6-phosphate x fructose 6-phosphate/ [glucose x fructose 1,6-bisphosphate] x 10^6	–	–	–	–	–	–	–	–	–	113	–	83.9	–	98.4 ± 20.6	0.21
Glucose 6-phosphate x pyruvate/ [glucose x phosphoenolpyruvate] x 10^3	–	–	–	–	–	1.30	–	1.39	1.22	–	–	1.98	–	1.47 ± 0.35	0.24
Fructose 1,6-bisphosphate x pyruvate/ [fructose 6-phosphate x phosphoenolpyruvate]	–	–	–	–	–	0.129	–	0.266	0.266	0.191	–	0.277	–	0.217 ± 0.060	0.28
Glucose 6-phosphate x creatine/ [glucose x creatine phosphate]	–	–	–	–	–	0.0824	–	0.0426	0.162	0.609	–	0.115	–	0.092 ± 0.047	0.51
Creatine phosphate x glycerol/ [creatine x glycerol 3-phosphate]	–	–	–	–	1.27	1.57	–	6.23	1.39	3.14	1.20	2.40	3.12	2.54 ± 1.69	0.67
Glucose 6-phosphate/glucose 1-phosphate	–	–	–	–	–	0.371	–	0.714	–	–	–	–	–	0.542 ± 0.242	0.45
Fructose 1,6-bisphosphate/[dihydroxyacetone phosphate x glyceraldehyde 3-phosphate]	–	–	–	–	–	2.87	–	–	2.2	–	–	–	1.78	2.28 ± 0.549	0.24
Glycerate 3-phosphate/glycerate 2-phosphate	7.20	3.10	–	4.90	–	5.37	3.50	5.12	3.89	–	7.38	8.63	6.85	5.59 ± 1.85	0.33
Glycerate 2-phosphate/phosphoenolpyruvate	0.317	0.261	–	0.325	–	0.652	0.903	0.502	0.549	–	0.540	0.484	0.876	0.54 ± 0.22	0.41
Energy charge															
(ATP + 0.5 ADP)/(ATP + ADP + AMP)	0.638	–	0.695	0.665	–	0.595	–	0.638	0.795	–	–	–	0.651	0.668 ± 0.063	0.09