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GLYCOLYSIS AND KREBS CYCLE METABOLITES IN MOSQUITOFISH, *GAMBUSIA HOLBROOKI*, GIRARD 1859, EXPOSED TO MERCURIC CHLORIDE: ALLOZYME GENOTYPE EFFECTS

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Abstract—Concentrations of glycolysis and Krebs (citric acid) cycle metabolites were measured in the tail tissues of mosquitofish (*Gambusia holbrooki*, Girard 1859) in response to exposure to 0.86 mg/L Hg (as $HgCl_2$) for 28 h. Substrate and product concentrations were compared between allozyme genotypes at two loci (glucosephosphate isomerase-2 and malate dehydrogenase-1) to determine whether allozyme genotypes in mosquitofish were differentially inhibited by mercury. Mercury treatment, regardless of allozyme genotype, caused decreased concentrations of glucose-6-phosphate (−27%) and lactate (−27%). Mercury treatment led to increased concentrations of malate (+33%) and oxaloacetate (+28%). Increased Krebs cycle activity could have been a response to greater energy needs associated with maintaining homeostasis under stressful conditions. There was no evidence of differential inhibition of allozymes at either locus. Fish of genotype *Gpi-2*$^{38/38}$ exhibited an overall increase in glycolytic activity in response to mercury treatment.

Keywords—Heavy metal Poeciliidae Allele Metabolism Stress

INTRODUCTION

Measurement of allozyme genotype frequencies is receiving increased attention as a method for identifying populations affected by pollution events. Allozymes, allelic variants of an enzyme, can be used to monitor the genetic characteristics of a population. One explanation for the observed changes in allozyme frequency associated with pollution is that allozymes differ in their sensitivity to inhibition by the pollutants. Lavie and Nevo [1] suggested that copper and zinc differentially inhibited the activity of glucose phosphate isomerase (GPI) allozymes in a marine gastropod, resulting in differential survival under acute exposure. Chagnon and Guttman [2] suggested a similar mechanism to explain differential survival of mosquitofish (*Gambusia affinis*) exposed to copper and cadmium.

Diamond et al. [3] observed differential survival of mosquitofish exposed to inorganic mercury that they related to allozyme genotypes at three loci: glucosephosphate isomerase-2, *Gpi-2*; isocitrate dehydrogenase-1, *Icd-1*; and malate dehydrogenase-1, *Mdh-1*. Specifically, fish expressing any of the following genotypes exhibited significantly shorter times to death than other genotypes: *Gpi-2*$^{38/38}$, *Icd-1*$^{134/134}$, *Mdh-1*$^{100/100}$, and *Mdh-1*$^{118/118}$. Diamond et al. [3] suggested that differential inhibition of these allozyme forms could have caused the early demise of fish expressing these forms. However, in a related study, Newman et al. [4] demonstrated that mosquitofish expressing the *Gpi-2*$^{38/38}$ genotype were also most susceptible to poisoning by arsenate, suggesting that this genotype is sensitive to stress, regardless of the toxicant.

This study tested the hypothesis that differential inhibition of GPI and malate dehydrogenase (MDH) allozymes occurred in mosquitofish exposed to inorganic mercury. A physiological approach was used in which the concentrations of the substrate and product of each enzyme were measured in the tail tissues. This approach allowed an evaluation of the allozyme response to the toxicant stress both in relation to other biochemical pathways and in relation to the whole-animal response to mercury exposure. If specific inhibition of one of these allozymes occurs, then a greatly increased concentration of allozyme substrate should result. For example, in the case of aconitase inhibition by monofluoracetate, up to a 350% increase in citrate

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(the substrate of aconitase) was observed in mouse brains after monofluoracetate injection with little or no increase in isocitrate, the product [5].

Two null hypotheses were tested in this study: (a) the concentrations of the substrate and product of the GPI and MDH allozymes are the same in control and mercury-treated fish, and (b) other metabolite concentrations are the same in control and mercury-treated fish. In this study, it was shown that, although metabolite concentrations did shift in response to mercury stress, the patterns of response were not indicative of direct differential inhibition.

MATERIALS AND METHODS

Chemicals

Enzymes, substrates, cofactors, and buffers were purchased from U.S. Biochemical, Cleveland, Ohio; Calbiochem, La Jolla, California; Sigma Chemical, St. Louis, Missouri; and Aldrich Chemical, Milwaukee, Wisconsin. A 1,000-mg/L Hg (±1% as $HgCl_2$) mercury reference solution was purchased from Fisher Scientific, Fairlawn, New Jersey. Reagent-grade mercuric chloride and concentrated perchloric acid ($HClO_4$) were obtained from Baker Chemical, Phillipsburg, New Jersey.

Fish collection and maintenance

Female mosquitofish (1,409, May 10–25, 1990) were collected by dip-netting from Risher Pond, a 1.1-ha abandoned farm pond on the U.S. Department of Energy Savannah River Site, Barnwell County, South Carolina. Fish were transported at ambient temperatures to the laboratory in tanks of aerated water containing a stress treatment (Stress-coat®, Aquarium Pharmaceuticals, Chalfont, PA). The fish were held in an artificial recirculating stream (Living Stream™ model LSW-700, Frigid Units Inc., Toledo, OH) and fed approximately 5 g commercially prepared flake food twice daily (Tetramin® tropical fish food, Tetrawerke, Germany) until the beginning of the experiment, at which time feeding was terminated.

Mercuric chloride exposure

On May 27, 1990, 1,220 female mosquitofish were placed in four glass aquaria holding 140 L water (305 fish per tank) maintained at 16 ± 1°C by a circulating water jacket and constantly mixed by immersible water pumps. The water was renewed at a flow-through rate of 0.21 ± 0.05 L/min.

The following day, the first of two mercury exposure tanks (Hg-1) was spiked to achieve a maximum concentration of 1 mg/L Hg (as $HgCl_2$). The second mercury exposure tank (Hg-2) was spiked 6 h later to allow time to harvest fish separately from each tank while keeping the total exposure time of both groups constant. Fish in the mercury exposure and control tanks (C-1 and C-2) were harvested 28 h after exposure began. Mercury-laden water was purified by passage through a cation exchange resin (Rexyn® 101H, Fisher Scientific) before discharge.

Water quality

Specific conductance, dissolved oxygen, pH, and temperature were measured with a Hydrolab Environmental Data Systems probe (model SVR 2-SU, Hydrolab Corp., Austin, TX). Water samples for anion, cation, and total alkalinity determinations were stored in acid-washed polyethylene bottles at 4°C until analysis (within 24 h for alkalinity or two weeks for anions and cations). A Dionex 4020i ion chromatography module (Dionex Corp., Sunnyvale, CA) with conductivity detection was used in the following analyses: chloride, nitrate, sulfate on an AS5A column; sodium, potassium, magnesium, and calcium on a CS3 column. Total alkalinity was measured by potentiometric titration [6], using an Orion (Boston, MA) research ionalyzer model 901 and a Ross combination electrode model 81-02. Total reactive mercury concentration was determined by cold vapor atomic absorption (hot acid digestion omitted [6]) with a Perkin Elmer (Norwalk, CT) 50A atomic absorption spectrophotometer.

Metabolite extraction

Fish tail tissues, 0.089 to 0.555 g wet weight, were cut from live fish (total mass of fish ranged from 0.308–3.418 g wet weight) along a dorso-ventral line from the anterior of the dorsal fin to just caudad of the anus. The tissue was immediately plunged into liquid nitrogen. Freezing time, determined as the time required for gaseous nitrogen to cease its release from the tissue, was 5 to 15 s. Frozen tail tissues were stored in liquid nitrogen until analysis (less than four months). Tissues anterior to the pectoral fins were placed in 1.5 ml microcentrifuge tubes and stored at −70°C until electrophoresis (less than two weeks).

Metabolites were extracted from the tail tissues by using a modification of the method described by Burlina [7]. The frozen fish tail tissues were ground to a homogeneous powder with an agate mortar and pestle cooled in liquid nitrogen. The powder was placed in a tared 1.5-ml microcentrifuge tube, cooled in liquid nitrogen, and closed with a vented cap. The tube containing the frozen tissue was

chilled in liquid nitrogen while an equal volume to weight of 3 M $HClO_4$ in a 12-ml conical polystyrene centrifuge tube was chilled in a dry ice/propanol bath at −25°C. The frozen tissue was placed in the chilled $HClO_4$, and an additional equal volume of chilled 3 M $HClO_4$ was immediately mixed into the tissue. After a 20-min extraction, the mixture was brought to 4°C in an ice water bath and diluted with double deionized water, 3.33 volume:tail tissue weight. After centrifugation for 15 min at 4°C (1,100 *g*, Dynac II swinging bucket rotor, Clay Adams, Parsippany, NJ), the decanted supernatant was neutralized by mixing with a volume of 3 M K_2CO_3, 0.5 M triethanolamine equal to the volume of acid previously added. The neutralized extract was chilled at −15°C for 10 min to enhance the precipitation of potassium perchlorate ($KClO_4$). After a second centrifugation, the clear supernatant was decanted and frozen at −35°C. The frozen extract was allowed to thaw slowly, causing the precipitation of any residual $KClO_4$. This final preparation was centrifuged for 10 min at 4°C, but the resulting supernatant was not decanted. The extract was chilled at 4°C in an ice water bath during the enzymatic analyses, not more than 1 h later.

Aspects of the experimental design might have influenced the accuracy and precision of the experiment. A primary concern during the sampling of tail tissue was the handling effects on tissue metabolite concentrations. Only a few tail movements can cause significant changes in metabolite concentrations; particularly labile are ATP and phosphocreatine [8,9]. Although metabolic activity might have been affected by the netting procedure, all fish were quickly netted in a similar manner to minimize bias by this factor. Due to the difficulty of dissection and the requirement for speed, the entire tail section was analyzed. Included in the tail section were the following tissues, by mean percentage (standard error of mean, $N = 5$) of total wet weight: skin and scales, 9.7 (0.9)%; vertebral column, 8.9 (1.3)%; caudal, dorsal, and anal fins, 7.4 (0.7)%; muscle, 67 (2.2)%; unaccounted tissues, 6.8 (3.6)%.

Metabolite analysis

The procedures of the enzymatic analysis of the metabolites were modified from Bergmeyer [10]. The details of the analysis for each metabolite are summarized in the Appendix. The concentrations of the following metabolites were measured: glucose-6-phosphate, G6P (GPI substrate), fructose-6-phosphate, F6P (GPI product), pyruvate, lactate, citrate, isocitrate, α-ketoglutarate, succinate, malate (MDH substrate), oxaloacetate (MDH product). The method of calculation is that of Bergmeyer [10], using calibration factors determined by the response to standards of a purity stated by the manufacturer. Reactions were monitored as the change in absorbance at 339 nm caused by the oxidation or reduction of the nucleotide cofactor, using a Beckman (Fullerton, CA) spectrophotometer model DU-70 (light path length 10 mm, cuvette temperature maintained at 27°C by water jacket). The median difference between duplicate measurements of the metabolites was 3.2% ($N = 97$, 25th and 75th percentiles, 1.3 and 6.5%, respectively). Recovery of metabolites by the extraction technique was tested by spiking frozen tissue with a known amount of each metabolite. Mean recovery was 99.6% ($N = 13$, 25th and 75th percentiles, 84.1 and 112%, respectively).

After their genotypes were identified by electrophoresis, 76 control fish and 72 mercury-treated fish were selected for analysis. However, not all of the metabolites could be measured in all fish due to the small tissue sample size. Metabolites were given priority for analysis from most to least important as follows: G6P, F6P, malate, oxaloacetate, lactate, isocitrate, α-ketoglutarate, pyruvate, citrate, and succinate.

Electrophoresis

Horizontal starch gel electrophoresis, using the methods and numbering system of Diamond et al. [3], identified genotype at the *Gpi-2* and *Mdh-1* loci. Anodal mobility of allozymes was determined relative to the most common allozyme at each locus. For example, the designation $Gpi\text{-}2^{100/100}$ indicates a fish homozygous for the most common *Gpi-2* allele. $Gpi\text{-}2^{100/38}$ indicates a heterozygous individual [3].

RESULTS

Water quality

Water quality (Table 1) in the control and exposure tanks was uniform throughout the experiment. The time-weighted average concentration of Hg was 0.857 mg/L (range 0.665–1.098 mg/L Hg) whereas in the control tanks the concentration was lower than the detection limit of 0.1 μg/L. Chloride concentration in the exposure tanks was slightly higher than that in the control tanks, due to the addition of $HgCl_2$ to the exposure tanks. Ionic balance calculations revealed that the measured ion concentrations adequately determined the major ionic constituents of the water used in the experiment. The median percentage of deviation

concentration ($F = 2.77$, $p = 0.021$, $d.f. = 5$). No other mercury treatment and genotype interactions were significant ($\alpha = 0.025$).

Mercury treatment

G6P and lactate concentrations, when considered without regard to genotype, were lower in mercury-treated fish (Table 3). Malate and oxaloacetate concentrations, on the other hand, increased in response to the mercury treatment.

Genotype and mercury treatment

Gpi-2. There were genotype-specific responses in metabolite concentrations to mercury treatment at the *Gpi-2* locus. Control concentrations of G6P and F6P were not significantly different between genotypes. Mercury treatment produced marked differences between genotypes in the concentrations of G6P, F6P, lactate, and malate (Table 4). In general, mercury treatment caused a depression in glycolytic metabolites, especially lactate. However, contrary to this general trend, glycolytic metabolite concentrations were high in *Gpi-2*$^{38/38}$ fish, nearly double the concentrations of G6P, F6P, and lactate found in any other genotype. *Gpi-2*$^{38/38}$ fish followed the trend of increased malate in response to mercury treatment, but they also had the highest observed mean level of malate.

Mdh-1. There were no differences between malate or oxaloacetate among the three *Mdh-1* genotypes in either the mercury treatment or the control group. As stated earlier, mercury treatment produced an increase in malate; only *Mdh-1*$^{118/118}$ fish had a great enough difference in malate concentration to be statistically significant (Table 5). Concentrations in the other two genotypes were also higher in the mercury treatment than those in the control, but not significantly so. There was no genotype-specific response of any metabolite to mercury treatment at the *Mdh-1* locus.

Table 3. Metabolite concentrations (nmol/g wet wt.) in tail tissues of mosquitofish in response to mercury treatment, mean (SE)

Metabolite	N_m	Mercury	N_c	Control	t[a]	p
G6P	72	162 (9)	76	233 (25)	2.29	0.024
F6P	72	33 (2)	76	39 (5)	1.16	0.250
Pyruvate	52	43 (5)	65	48 (5)	0.72	0.476
Lactate	72	691 (39)	76	951 (57)	3.76	0.001
Citrate	51	626 (28)	65	654 (28)	0.71	0.480
Isocitrate	72	20 (3)	76	13 (2)	1.80	0.074
α-Ketoglutarate	52	25 (3)	65	34 (5)	1.52	0.133
Succinate	20	133 (25)	16	99 (18)	1.07	0.290
Malate	72	85 (4)	76	64 (6)	2.87	0.005
Oxaloacetate	71	32 (3)	76	25 (2)	1.99	0.048

N_m = sample size mercury treatment.
N_c = sample size control.
t = Student's t statistic.
$\alpha = 0.05$.
[a]Satherwaite's approximation of degrees of freedom applied for unequal variances.

DISCUSSION

The physiological significance of allozyme variation has been studied in relation to thermal adaptation in fishes [12–14] and blue mussel [15], juvenile survival of red deer [16], oxygen consumption of a salamander [17], hyperosmotic stress in a copepod [18], and inhibition by metabolic intermediates [19]. In this study, the role of allozymes in the response of mosquitofish to inorganic mercury poisoning was determined by the measurement of glycolysis and Krebs cycle metabolite concentrations, including GPI and MDH substrates. Although several studies have demonstrated correlations between resistance to heavy metal pollution and specific allozyme genotype [1,3,20], little work has been done to identify physiological mechanisms of allozyme-specific sensitivity.

It has been suggested that direct differential inhibition could explain allozyme-specific sensitivity [2,3] to pollutants. With regard to inorganic mercury poisoning of mosquitofish, the present results do not support this hypothesis. There was no genotype-specific response to the mercury treatment at the *Mdh-1* locus. At the *Gpi-2* locus, the genotype-specific response of *Gpi-2*$^{38/38}$ fish (increased concentrations of lactate and G6P) suggested enhanced, rather than inhibited, flux at the GPI step and through glycolysis.

It is unlikely that the enhanced glycolytic activity itself could be responsible for the mercury sensitivity of *Gpi-2*$^{38/38}$ fish. The concentrations of G6P, F6P, and lactate in mercury-treated *Gpi-2*$^{38/38}$ fish were similar to control concentrations. More important, however, the concentrations of G6P, F6P, and lactate in the mercury-treated *Gpi-2*$^{38/38}$ fish were about double the concentrations of other mercury-treated *Gpi-2* genotypes. This indicated that *Gpi-2*$^{38/38}$ fish responded to mercury stress differently from the other *Gpi-2* genotypes that Diamond et al. [3] found to be more tolerant of the mercury poisoning. The enhanced glycolytic activity and increased Krebs cycle activity, indicated by increased malate, suggested that *Gpi-2*$^{38/38}$ mosquitofish might have responded to mercury stress with a hyperactive metabolism when fish of other genotypes were conserving energy

Table 4. Metabolite concentrations (nmol/g wet wt.) in tail tissues of mosquitofish, according to genotype at the *Gpi*-2 locus, in response to mercury treatment, mean (SE)[a]

Metabolite		*Gpi-2* genotype 100/100		100/66		100/38		66/66		66/38		38/38	
G6P	C	175	(33)	174	(23)	177	(41)	367	(148)	293	(59)	190	(48)
	Hg	179^{B}	(22)	180^{B}	(15)	154^{B}	(26)	139^{*B}	(11)	124^{B}	(13)	281^{A}	(96)
F6P	C	33	(8)	30	(5)	29	(8)	60	(31)	53	(11)	33	(9)
	Hg	35^{AB}	(6)	37^{AB}	(4)	30^{B}	(6)	27^{*B}	(3)	27^{B}	(3)	56^{A}	(26)
Pyruvate[b,c]	C	31	(6)	52	(7)	40	(11)	52	(22)	57	(18)	68	(18)
	Hg	53	(12)	53	(15)	54	(13)	25	(10)	32	(6)	48	(35)
Lactate	C	968	(134)	895	(86)	862	(169)	913	(119)	1042	(159)	1056	(180)
	Hg	780^{B}	(90)	654^{*B}	(74)	703^{B}	(103)	609^{*B}	(51)	560^{*B}	(58)	1217^{A}	(295)
Citrate[b,d]	C	695	(56)	687	(54)	659	(100)	538	(72)	684	(57)	572	(92)
	Hg	608	(66)	632	(56)	631	(44)	657	(93)	595	(63)	783	(153)
Isocitrate	C	16	(5)	15	(7)	9	(3)	16	(7)	9	(3)	11	(8)
	Hg	28	(7)	27	(12)	11	(5)	23	(6)	11	(3)	3	(2)
α-Ketoglutarate[b,c]	C	22	(6)	38	(9)	19	(6)	56	(23)	35	(15)	43	(11)
	Hg	32	(6)	29	(8)	21	(7)	21	(7)	25	(5)	17	(16)
Succinate[e,f]	C	126	(31)	78	(59)	99	(46)	72	–	94	–	69	(31)
	Hg	158	(43)	33	(17)	180	(41)	97	(36)	166	(126)	190	–
Malate	C	58	(16)	61	(11)	46	(13)	85	(17)	78	(13)	61	(18)
	Hg	78^{B}	(7)	82^{B}	(10)	100^{*B}	(11)	87^{B}	(6)	76^{B}	(7)	125^{A}	(23)
Oxaloacetate[g]	C	23^{AB}	(5)	24^{AB}	(5)	23^{AB}	(4)	40^{A}	(9)	24^{AB}	(5)	12^{B}	(6)
	Hg	39*	(4)	40	(10)	27	(7)	29	(6)	23	(8)	34	(16)

Sample sizes: control (C)—18, 16, 12, 9, 15, 6; mercury (Hg)—19, 14, 10, 11, 15, 3, respectively, unless otherwise stated.

*Different from control, Student's *t* test using Satherwaite's *d.f.* approximation for unequal variances when appropriate, $\alpha = 0.05$.

[a]Means with same letter are not different, comparison within each metabolite and treatment, Student-Newman-Keuls test, $\alpha = 0.05$. No letter indicates no difference.

[b]Control sample sizes 13, 15, 10, 8, 13, 6, respectively

[c]Mercury sample sizes 9, 11, 8, 7, 15, 2, respectively.

[d]Mercury sample sizes 9, 11, 7, 7, 15, 2, respectively.

[e]Control sample sizes 6, 3, 3, 1, 1, 2, respectively.

[f]Mercury sample sizes 6, 3, 3, 4, 3, 1, respectively.

[g]Mercury sample sizes 19, 14, 9, 11, 15, 3, respectively.

Table 5. Malate and oxaloacetate concentrations (nmol/g wet wt.) in the tail tissues of mosquitofish, according to genotype at the *Mdh-1* locus, in response to mercury treatment, mean (SE)

Metabolite		*Mdh-1* genotype 118/118	118/100	100/100
Malate	C	62 (8)	73 (15)	62 (10)
	Hg	91[a] (5)	88 (9)	80 (6)
Oxaloacetate[b]	C	26 (4)	23 (4)	25 (4)
	Hg	30 (5)	28 (4)	36 (5)

Sample sizes: control (C)—33, 21, 21; mercury (Hg)—19, 19, 34, respectively, unless otherwise stated.

[a]Different from control, Student's *t* test using Satherwaite's *d.f.* approximation for unequal variances when appropriate, $\alpha = 0.05$.

[b]Mercury sample sizes 18, 19, 34, respectively.

through depressed glycolysis or were exhibiting a smaller increase in Krebs cycle activity. As suggested by the findings of Newman et al. [4], the reduced time to death exhibited by *Gpi-2*$^{38/38}$ mosquitofish in both mercury and arsenate exposures indicates a nonspecific sensitivity to metal poisoning for which the *Gpi-2*$^{38/38}$ genotype may act as a genetic marker.

The genotype-independent response of mosquitofish to mercury poisoning consisted of generally depressed glycolytic activity (decreased G6P and lactate concentrations) and enhanced Krebs cycle activity (increased malate and oxaloacetate concentrations). These results agree with those of a previous study on mosquitofish in which mercury exposure induced decreased concentrations of G6P and F6P (lactate was not measured) and increased

concentrations of pyruvate, α-ketoglutarate, succinate, and malate (V.J. Kramer, M.C. Newman, and G.R. Ultsch, unpublished data). Lindahl and Hell [21] observed a 15 to 19% increase in oxygen uptake by liver slices and liver mitochondria taken from *Leuciscus rutilis* exposed to 1 mg/L phenylmercuric hydroxide. Glycolytic activity in the liver slices (measured as the production of lactate) decreased 20 to 25% in treated fish. Diminished swimming activity was casually observed in mercury-treated mosquitofish. This response could have resulted from decreased glycolytic activity in the tail muscle [22]. Increased Krebs cycle activity might have been a mechanism for supplying energy (from nonglycolytic sources) required to maintain homeostasis in response to the mercury stress.

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APPENDIX – METABOLITE ASSAY CONDITIONS

		Volume (μl)	Reaction time (min)
Assay A. Citrate, α-ketoglutarate, pyruvate			
Buffer	0.5 M glycylglycine, pH 7.8, 0.6 mM $ZnCl_2$	1,075	
Cofactor	20 mM NADH	25	
Sample	Neutralized extract, pH 8–9	300	5
Enzyme	Citrate lyase, 40 U/ml 2 M ammonium sulfate (AMS)	10	
	Malate dehydrogenase, 1.6 kU/ml 3.2 M AMS	10	10–30
	Glutamate dehydrogenase, 360 U/ml 2 M AMS	10	10
	Lactate dehydrogenase, 1.4 kU/ml 3.2 M AMS	10	10
Total		1,420–1,440	35–55
Assay B. Malate			
Buffer	0.17 M Tris, pH 9.5	1,050	
Cofactor	100 mM NAD	50	
Sample	Neutralized extract, pH 8–9	300	5
Enzyme	Malate dehydrogenase, 1.6 kU/ml 3.2 M AMS	5	5
Total		1,405	10
Assay C. G6P, F6P, isocitrate			
Buffer	0.4 M triethanolamine HCl, pH 7.6, 5 mM $MgCl_2$	1,050	
Cofactor	48 mM NADP	50	
Sample	Neutralized extract, pH 8–9	300	5
Enzyme	Glucose-6-phosphate dehydrogenase, 35 U/ml 3.2 M AMS	10	10
	Glucose phosphate isomerase, 315 U/ml 3.2 M AMS	10	10
	Isocitrate dehydrogenase, 20 U/ml 18 mM Tris citrate, 10% glycerol, pH 7.0	10	5
Total		1,410–1,430	30
Assay D. Succinate			
Buffer	0.3 M glycylglycine, pH 8.4, 41 mM $MgSO_4$	1,000	
Cofactors	20 mM NADH	50	
	15 mM inosine-5′-triphosphate, 11 mM coenzyme A, 21 mM phosphoenolpyruvate	100	
Sample	Neutralized extract, pH 8–9	300	
Water		1,450	
Enzyme	Pyruvate kinase, 600 U/ml 3.2 M AMS		
	Lactate dehydrogenase, 550 U/ml 3.2 M AMS	50	10
	Succinate thiokinase, 50 U/ml 3.2 M AMS	15	20–30
Total		2,965	30–40
Assay E. Oxaloacetate			
Buffer	0.3 M Tris, pH 7.8, 20 mM EDTA	1,700	
Cofactor	0.8 mM NADH	1,000	
Sample	Neutralized extract, pH 8–9	300	5
Enzyme	Malate dehydrogenase, 230 U/ml 50% glycerol	5	10
Total		3,005	15