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Studies of Enzyme Polymorphisms in the Kamuela Population of *Drosophila mercatorum.* II. Evaluation of Glycolytic Intermediates

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A simple and effective cryogenic procedure for the extraction of glycolytic intermediates from whole Drosophila *has been developed. This procedure gives consistent results when a measure (μM/liter/OD_{260}) is adopted which corrects for differences in extraction efficiency. Using this measure and a homozygous strain of* D. mercatorum, *there are no significant differences among extracts for the levels of any of the 15 glycolytic intermediate or energy molecules considered. The profile of means is consistent across experimental designs and instrument types. Coefficients of variation are well below 50% for most variables. The methodology presented has the statistical power to detect a mean change of 10 to 50% using an experimental design which requires as few as 32 observations. The estimated energy charge for resting* Drosophila *from these studies is the expected value of 0.86.*

KEY WORDS: *Drosophila mercatorum;* glycolytic intermediates; energy charge.

INTRODUCTION

This is the second in a series of empirical studies undertaken to relate enzyme polymorphism to components of fitness in a natural population of *Drosophila.* In the first study (Clark *et al.,* 1981) we characterized the level and nature of the enzyme polymorphisms for the Kamuela, Hawaii, population of *Drosophila mercatorum.* In this study, we turn to the measurement of glycolytic

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intermediate phenotypes which are hypothesized to link inherited enzyme variations with components of reproductive fitness. This is the second step in a three-step "level-crossing" strategy designed to characterize the contribution of allelic differences to variability in intervening phenotypes which may contribute to fitness measures. We have chosen to apply this approach to glycolysis and to consider the glycolytic intermediate (GI) pools as intervening phenotypes, because the controlling glycolytic enzymes have typical levels of polymorphism (Clark *et al.,* 1981), the pathway has an important role in energy metabolism, the enzymes involved are specific and well known, and (as documented in this paper) methods for the measurement of GIs have proven adaptable to *Drosophila.*

A multivariate vector of glycolytic intermediate phenotypes provides additional information beyond that obtainable from separate studies of the individual variables. The response of glycolysis, as a system, to genetic and environmental perturbations is expressed as changes in both the levels of individual glycolytic pools and the relationships among these pools. A number of studies suggest that GI levels may be a physiological response to genetic and environmental stress treatments which are correlated with flight behavior. Sacktor and co-workers (Sacktor and Hurlbut, 1966; Sacktor and Wormser-Shavit, 1966) have reported altered levels of GIs in the blowfly, *Phormia regina,* during tethered flight. Preliminary work in our laboratory has shown elevated levels of certain intermediates in *D. mercatorum* in response to the stress induced by tethered flight, work, or cold. Evidence that allelic differences at glycolytic loci may have effects on flight behavior comes from studies of the screwworm fly, *Cochliomyia hominivorax,* by Bush and Neck (1976) and of *Drosophila* by O'Brian and Shimada (1974) and Curtsinger and Laurie-Ahlberg (1981). It is our working hypothesis that the effects of allelic differences at glycolytic loci are expressed as phenotypic differences at the physiological level that are correlated with flight behavior characteristics which contribute to reproductive fitness.

It is the purpose of the present study to develop the methodology for acquiring the measurements of the GIs in *Drosophila.* The ability to measure these phenotypes accurately will enable us to study a link in the chain of causation between genetic variability and variability in components of fitness.

MATERIALS AND METHODS

Our work to combine genetics and physiology has led us to prefer *Drosophila mercatorum* as an experimental organism. While there are several advantages to larger organisms such as *Phormia regina,* that is, single individuals are readily manipulated and may even suffice for an adequate sample for

glycolytic intermediate analyses, the extensive genetic information available for the *Drosophila* species gives them a distinct advantage. The species *D. mercatorum* is particularly useful because of its natural ability to reproduce both sexually and asexually. The asexual process (Carson, 1973; Templeton, 1979a) is a form of parthenogenesis in which the virgin females produce viable diploid eggs. The diploid state is restored to the egg by one of several mechanisms. Gamete duplication (postmeiotic duplication of the haploid gamete followed by fusion) is the most frequent. Each progeny arising from this mechanism is a female, homozygous at all loci, and a candidate to establish an all-female clone.

To produce a truly isogenic bisexual stock from a clone, a y chromosome is added by standard genetical methods. A cloned female is crossed to a male from a stock with a recessive visible marker on each chromosome. The F_1 male is backcrossed to a female of the original clone to produce a progeny which contains a fraction of males identical (except for the y chromosome) to the cloned females. Such males are identified by a test cross to the females of the marker stock. Males so identified are then crossed to a female of the original clone to produce a bisexual stock which is in all other respects identical to the clone. The absence of crossing-over in male *Drosophila* and the absolute homozygosity of the cloned females enable one to efficiently create such isogenic lines.

Congenic stocks for allelic variants from the Kamuela population are established on the isogenic background by crossing males of the isogenic bisexual stock to females of a variant line and then repeatedly backcrossing males of the isogenic stock to backcross daughters heterozygous at the locus of interest. These methods have enabled us to develop a series of clones and the isogenic and congenic stocks for the variants isolated from the Kamuela population.

The data reported in this paper are from samples of the isogenic bisexual stock K_{23}-o-bi and the congenic stocks K_{23}-o-bi (αGP-F) and K_{23}-o-bi (αGP-S), all derived from the clone K_{23}-o-im. The letter K denotes Kamuela, the collection site in Hawaii; the number 23 indicates the 23rd wild-caught female whose parthenogenetic daughter was the source of the clone K_{23}-o-im. The zero indicates the number of cross–backcross cycles (none) carried out to improve the asexual capacity of the stock (Templeton *et al.*, 1976; Templeton, 1979a). The abbreviations bi and im refer to bisexual and impaternate (all-female) stocks, respectively. The designations αGP-F and αGP-S indicate electrophoretic differences, fast and slow, at the locus coding for α-glycerophosphate dehydrogenase. The y chromosome in K_{23}-o-bi is derived indirectly from a single male of another Kamuela stock, K_1-o-bi, via a marked chromosome stock selected to assure the compatibility of the y chromosome with the rest of the genome.

Fly-Handling Procedure

The flies are raised in an incubator at 25°C on a 12 hr light/12 hr dark cycle. The medium, 75 ml in 6-oz bottles, contains water, cornmeal, molasses, agar, and brewer's yeast at the ratio 240:23:9:1.5:1 (w/v). The medium is supplemented with 0.1 g live yeast (dry) per bottle. Six milliliters of proprionic acid per 1200 ml of medium is used as a mold inhibitor. A standard culture is initiated with 15 pairs of flies, the parents are removed on the 19th day, and the progeny are collected on the 25th day. Progeny from 12 bottles are pooled and redistributed on fresh unyeasted bottles, 0.5 g to a bottle, and fasted for 24 hr prior to sacrifice.

Extraction

The flies which have been fasted for 24 hr are harvested by decanting directly into liquid nitrogen at −190°C. Individual flies boil the nitrogen for less than a second and sink immediately to the bottom of the Dewar flask. The bulk of the liquid nitrogen is decanted, leaving the fly sample in the flask in 2 to 3 ml of liquid nitrogen. The sample of flies is then decanted on top of a 3-vol pellet of frozen 3 M PCA in a tissue homogenizer tube maintained at −35°C to −50°C in a dry ice–acetone bath. A pestle at −20°C is attached to an automatic stirrer and the sample is subjected to 300 passes in three steps. The extract temperature is monitored with a YSI Model 42 SL telethermometer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) and held at between −8°C and −12°C. The sample is held for an additional 15 min in this temperature range to finish enzyme inactivation. At the end of this inactivation step, 3 vol of 1.0 mM EDTA in water is added (4°C) and stirred for 30 min. The sample is centrifuged at 5000*g* for 10 min to remove protein. It is then decanted, divided into aliquots, and frozen at −90°C until assayed. The samples are thawed on the morning of assay and the procedure given in Fig. 1 is followed. A subsample is removed for measurement of the OD_{260}. The remaining sample is neutralized and spun to clarity. ATP, ADP, AMP, and NAD are measured photometrically on this supernatant. A charcoal treatment is necessary prior to fluorometry to remove pigments from the extract that cause high interfering fluorescence and quenching. The temperatures and timing of the extraction procedure are derived from Lowry and Passonneau (1972).

Drosophila Assay Procedures for Intermediates

Excitation and fluorescence spectra are measured using a Farrand manual spectrofluorometer with a 16-cell turret and spectroprogrammer (Farrand Optical Co., Inc., Commercial Products Division, Valhalla, N.Y.) and a

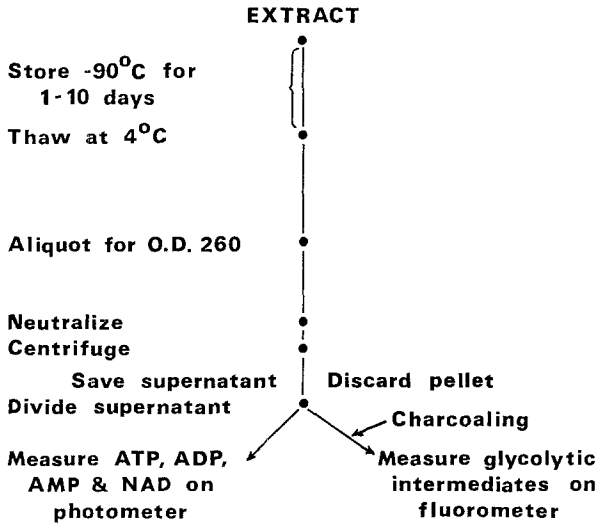


Fig. 1. The flow diagram for processing *Drosophila* extracts. The OD at 260 nm is measured on an aliquot of extract. The remaining extract is neutralized with calcium carbonate and centrifuged. Fractions of the supernatant are used for photometric and fluorometric analysis on successive days.

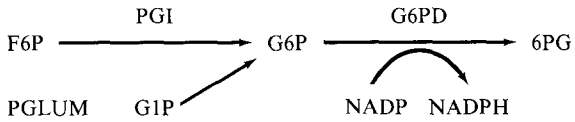
Brinkman Model 2541 potentiometric linear recorder (Goerz Electro, Brinkman Instruments, Westbury, N.Y.). The temperature is kept at a constant 25°C in the turret chamber by a circulating water bath (Thermomix Model 1480). The excitation light source is an 85-W mercury arc lamp (H 85A3/uv; GE Co., Nela Park, Cleveland, Ohio). Ten-millimeter slit widths are used, and the sensitivity range is set to 0.1. A Pyrex glass rod is used as a fluorescent standard for lamp intensity and wavelength calibration; it has an intensity of 30–40 units at range 0.1. The gain is set at position 20. The standard occupies position 0 in the turret and is read before each set of sample readings each time the turret completes a revolution. Any variation in lamp intensity can therefore be adjusted with the gain setting.

Glycolytic intermediates are measured fluorometrically by the conversion of substrate to product in a reaction coupled to the production or consumption of a fluorescent compound, typically NADH or NADPH, with a known stoichiometry. Standard curves of fluorescence versus NADH concentration in appropriate blanks (reaction mixture minus extract) are determined periodically to calibrate fluorescence. The inverse of the slope of the appropriate standard curve (μM NADH/liter/chart unit of fluorescence) times the net change in each sample tube (chart units of fluorescence) estimates the glycolytic intermediate concentration (μM intermediate/liter) since the stoichiometry is known. This value is adjusted for sample dilution and standard-

ized. Assays of AMP, ADP, ATP, and NAD are carried out on a Gilford 2400s recording spectrophotometer using quartz semimicro cuvettes (volume, 1.00 ml). All glassware used in fluorometric determinations is rinsed first in 50% nitric acid and then in deionized distilled water before use. All solutions with the exception of 1.0 M $MgCl_2$ and 0.9 M $Na_2HAsO_4 \cdot 7H_2O$ are prepared fresh on the day of assay. The assay procedures are derived from Minakami *et al.* (1965) as modified by Oelshlegel *et al.* (1972) unless otherwise noted.

G6P, F6P, and G1P—Fluorometric

This assay is based upon the reduction of cofactor NADP to NADPH by glucose 6-phosphate dehydrogenase and the conversion of G6P to 6-phosphogluconate. The increase in fluorescence due to NADPH is recorded. Subsequent reactions cycle their substrates through this same conversion reaction.

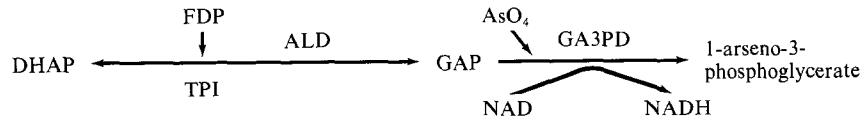


Scheme I

A 1-ml cuvette is loaded with 0.900 ml of PCA blank, 0.100 ml of PCA extract, and 0.005 ml of an NADP solution (50 mg/0.5 ml). After baseline readings have been taken, G6P is measured after the addition of 5 μl of glucose-6-phosphate dehydrogenase (Boehringer No. 127655, 5 mg/ml). Successively, F6P is assayed after the addition of 5 μl phosphoglucose isomerase (Sigma P 8391, 5000 units/1.3 ml), and G1P after the addition of 10 μl phosphoglucomutase (Boehringer Mannheim No. 108383, 10 mg/ml).

GAP, DHAP, and FDP—Fluorometric

This assay (Neissner and Beutler, 1973) measures the reduction of NAD to NADH in the glyceraldehyde-3-phosphate dehydrogenase-mediated conversion of GAP to 1,3-diphosphoglycerate. Subsequent cycling through this pathway allows us to measure also DHAP and FDP.



Scheme II

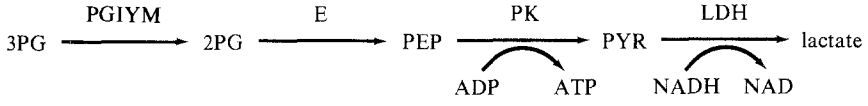
A 1-ml fluorometric cuvette is loaded with 0.8 ml PCA extract, 0.08 ml NAD solution (50 mg/ml), and 0.16 ml arsenate–mercaptoethanol solution (0.36 M sodium arsenate, 0.8 vol % mercaptoethanol). GAP is measured after the addition of 2 μl GA3PD (Boehringer Mannheim No. 105686 10mg/ml). Following GAP measurement, 12 μl additional GA3PD is added to speed the DHAP and FDP measurement reactions. DHAP and FDP are measured after the addition of the following enzymes, respectively: 5 μl triosephosphate isomerase (Sigma T7877, 10 mg/ml) and 10 μl aldolase (Sigma A 7145, reconstituted to 10 mg/ml).

αGP—Fluorometric

This assay of αGP is based upon the same reaction (conversion of GAP to 1,3-DPG with a concomitant reduction of NAD) as the above measurements of GAP, DHAP, and FDP. Because *Drosophila* have higher concentrations of αGP than GAP, DHAP, and FDP, it is necessary to use a much smaller aliquot of extract to avoid quenching. In this assay a 1-ml fluorometric cuvette is loaded with 0.700 ml PCA blank, 0.100 ml PCA extract, 0.080 ml NAD solution (50 mg/ml), 0.071 ml arsenate–mercaptoethanol solution (0.36 M sodium arsenate, 0.8 vol % mercaptoethanol), 10 μl GA3PD (Boehringer Mannheim No. 105686 10mg/ml), and 5 μl TPI (Sigma T7877, 10 mg/ml). After a baseline is established, 10 μl αGPD (Sigma G-6751 1000 units/0.67 ml) is added and the reaction recorded.

PYR, PEP, PG2, and PG3—Fluorometric

This series measures the decrease in fluorescence as NADH is oxidized to NAD in the lactate dehydrogenase-mediated conversion of pyruvate to lactate.

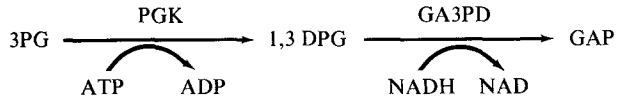


Scheme III

A fluorometric cuvette is loaded with 0.700 ml PCA blank, 0.300 ml PCA extract, 0.030 ml 1 M $MgCl_2$, 10 μl ADP (50 mg/ml), and 10 μl NADH (1 mg/ml in $NaHCO_3$–Na_2CO_3,*p*H 10.6, buffer). After a baseline is established, 2 μl of LDH (Sigma L 2500, 25000 units/2.5 ml) is added. The other intermediates are then measured by adding the following enzymes, respectively; 2 μl pyruvate kinase (Sigma P 1506, 5000 units/3.2 ml), 10 μl enolase (Sigma E-6126, reconstituted to 10 mg/ml), and 10 μl phosphoglycerate mutase (Boehringer Mannheim No. 108464, 5 mg/ml).

ATP—Photometric (from Minakami et al., *1965)*

This assay measures the oxidation of NADH in the coupled reactions mediated by PGK and GA3PD of the conversion 3PG to 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate. Absorbance at 340 nm is recorded.



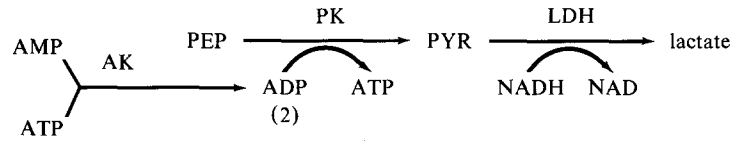
Scheme IV

A quartz semimicro cuvette is loaded with 0.900 ml PCA blank, 0.100 ml PCA extract, 5 μl 1.0 M $MgCl_2$, 0.100 ml 3PG (20 mg/ml), 10 μl NADH (10 mg/ml in $NaHCO_3$–Na_2CO_3, *p*H 10.6, buffer), 5 μl mercaptoethanol solution (1% in H_2O), and 5 μl GA3PD (Boehringer Manneim No. 105686 10mg/ml). Calibrated absorbance is set at 0.5, and baselines are established. Ten microliters of phosphoglycerate kinase is added (Boehringer Mannheim No. 108430 10mg/ml).

Note: Photometric measurement of ATP, NAD, ADP, and AMP must be carried out on *non*charcoaled extract. We found that the adenine and pyridine nucleotides are removed when charcoaling the extract. Also, the eye pigments do not interfere with absorbance readings.

ADP and AMP—Photometric

This series measures the oxidation of cofactor NADH in the lactate dehydrogenase-mediated conversion of pyruvate to lactate.



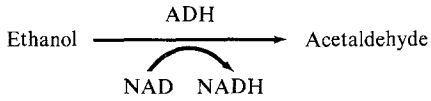
Scheme V

A semimicro quartz cuvette is loaded with 0.900 ml PCA blank, 0.100 PCA extract, 0.030 ml 1 M $MgCl_2$, 0.030 ml phospho*enol*pyruvate [(28 mg/ml) + ATP (28 mg/ml)], and 0.010 ml NADH (10 mg/ml in $NaHCO_3$–Na_2CO_3, *p*H 10.6, buffer). After a baseline is established, 2 μl of LDH (Sigma L2500, 25000 units/2.5 ml) is added. ADP and AMP are assayed by adding, respectively, 2 μl pyruvate kinate (Sigma P1506 5000 units/3.2 ml)

and 5 μl myokinase (Sigma M3003, 10 mg/ml). The calibrated absorbance setting for this series of assays is 0.2.

NAD—Photometric

This assay of NAD (Segal *et al.*, 1971) is based upon the reduction of NAD in the alcohol dehydrogenase-mediated conversion of ethanol to acetaldehyde. The *p*H of the extract is increased to 8.8 with K_2CO_3 (5 M) and the calibrated absorbance is set at 0.2. A quartz semimicro cuvette is loaded with 0.8 ml ethanol blank (30 mM tetrasodium pyrophosphate, 14.25% ethanol, *p*H 8.8) and 0.2 ml extract (*p*H 8.8). After a baseline is established, 10 μl of alcohol dehydrogenase (Sigma A3263, reconstituted to 3000 units/ml) is added and the reaction recorded.



Scheme VI

OD_{260}

Measurements of OD_{260} are done on a Gilford 2400S in matched sets of quartz semimicro cuvettes. Each cuvette is loaded with 0.900 ml PCA blank and 0.100 ml extract (noncharcoaled) and read against PCA blank at 260 nm.

Experimental Design

The data reported here were collected on unstressed flies cultured using the standard fly-handling procedures described above. The status "unstressed" denotes that the flies were at rest immediately prior to sacrifice. These data were taken from several different experiments designed (1) to estimate extraneous variability introduced by fly-handling extraction and assay, (2) to estimate the effects of genetic strain and environmental stress (only the unstressed control is reported here) on intermediate levels, or (3) to document the linear regression of intermediate levels on OD_{260} under the assay conditions described above.

In all cases the genetic variability was controlled by utilizing the isogenic stock K_{23}-o-bi or the closely related congenic stocks K_{23}-o-bi (αGP-F) and K_{23}-o-bi (αGP-S). The variability of the fly environment was minimized by the standard culture technique established by experiments which preceded the collection of the data reported here. Possible bottle effects were eliminated by first pooling and then dividing approximately 1800 flies from 12 bottles (each

strain) into samples of 250 to 300 flies. Each sample was maintained in a bottle containing fresh unyeasted medium for a 24-hr fast prior to extraction.

The design of experiments depended on the instrument available and the complexity of the experimental protocol. In the first set of experiments, design 1, four aliquots of each of four extracts of K_{23}-o-bi were assayed for each GI variable. We used this design to determine the relative contributions of extraction (extracts) and assay (aliquots) to the total variance of a glycolytic intermediate. In the second set of experiments, design 2, two aliquots of each of eight extracts were assayed for each variable. The object of this design was to contrast two genotypes (K_{23}-o-bi (αGP-F) and K_{23}-o-bi (αGP-S) in this study) each at two treatment levels. Each treatment strain combination involved two extracts, each with two aliquots. Two aliquots of each of eight extracts of K_{23}-o-bi were assayed for each variable in the study of the regression of OD_{260} on grams wet weight/liter and the regressions of the glycolytic intermediates on OD_{260}. In these regression experiments the extracts were prepared at four concentrations, with duplication at the extract level.

Both design 1 and design 2 require more assays than there are cuvette positions; consequently multiple runs are required. When pooling data from multiple runs we have taken care not to underestimate aliquot within extract variability. This underestimation occurs due to correlation of errors within a run and, if ignored, could lead to spurious significance in statistical tests. Due to different instrument limitations we have managed the distribution of samples among runs differently for the spectrophotometer and the spectrofluorometer. In the case of the spectrophotometer we have randomized the run errors across extracts which represent the treatment combinations. In the case of the spectrofluorometer all treatment combinations are assayed in the same run. In both cases, the aliquot within extract error is not underestimated due to the correlation of errors which could occur if both aliquots were measured in the same run.

RESULTS AND DISCUSSION

Choice of Measure

The glycolytic intermediates were measured as the micromolar concentration per liter of extract (μM/liter) and standardized by division with three alternate measures of the amount of fly extracted. The three measures were grams wet weight per liter (g wet wt/liter), grams of resolubilized PCA-precipitated protein per liter, and OD_{260}. The first two measures are likely to differ from the third, OD_{260}, if there are differences in extraction efficiencies among extracts. The first two reflect the amount of fly intended for extraction, the weighed amount, but do not vary with the extraction efficiency as OD_{260}

does. For this reason, μM/liter/OD_{260} is a potentially superior measure of GIs. It is also a convenient metric, in that an aliquot of extract may be used to measure the quantity of fly at a time subsequent to extraction. This is a particularly relevant advantage if a fly-handling procedure, such as weighing, is likely to alter GI pools.

The realization of the potential superiority of the measure μM/liter/OD_{260} depends on the demonstration that the OD_{260} and GIs are released in the same proportion over the range of extraction efficiencies encountered. The first characteristic is demonstrated in Fig. 2 by the linear regression of OD_{260} on g wet wt/liter. Approximately 97% of the variability in fly weight is predicted by the OD_{260}. The extractions of GIs and OD_{260} in fixed proportions are demonstrated by the linear regressions summarized in Table I. We have pooled the photometric data from nine experiments ($N = 92$) but present the fluorometric data separately for two experiments ($N = 16$ in each) to exemplify the repeatability of the results. In general, the linear regressions are significantly different from zero except for those intermediates at the lowest concentrations. There were no significant second- or third-order regression effects.

Design 1 resulted in significant differences among extracts within strains for glycolytic intermediates expressed as μM/liter of extract. These differences in GI measures remained when the data were expressed as μM/g PCA-precipitated protein or μM/g wet wt but were removed when expressed

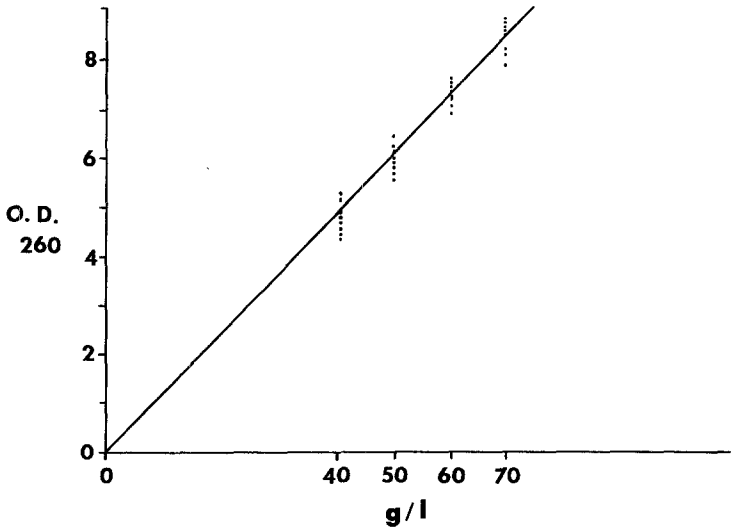


Fig. 2. The linear regression of OD at 260 nm on grams wet weight of flies per liter ($\beta = 0.121$). Thirty-two observations over four concentrations in our working range are graphed. The OD at 260 nm predicts 97% of the variability in grams per liter.

Table I. Linear Regression, μM/liter on OD_{260}

	Spectrophotometer				Spectrofluorometer									
	Sets 8–16 ($N = 92$)				Set 27 ($N = 16$)					Set 29 ($N = 16$)				
Variable	$\hat{\beta}$	$SE_{\hat{\beta}}$	R^2	Sig.	$\hat{\beta}$	$SE_{\hat{\beta}}$	R^2	Sig.	CV	$\hat{\beta}$	$SE_{\hat{\beta}}$	R^2	Sig.	CV
GIP	—	—	—	—	1.44	0.29	0.66	0.0001	15	1.82	0.27	0.77	0.0000	14
G6P	2.57	0.19	0.67	0.0000	4.22	0.57	0.79	0.0000	9	4.64	0.47	0.87	0.0000	7
F6P	—	—	—	—	1.02	0.40	0.32	0.0220	33	1.49	0.24	0.73	0.0000	12
FDP	0.68	0.16	0.17	0.0000	0.56	0.14	0.56	0.0013	25	0.34	0.09	0.52	0.0017	25
DHAP	1.00	0.17	0.27	0.0000	1.11	0.24	0.60	0.0004	19	0.61	0.21	0.36	0.0136	26
αGP	5.74	0.96	0.28	0.0000	10.36	3.37	0.70	0.0374	6	14.23	0.94	0.99	0.0007	1
GAP	—	—	—	—	0.15	0.07	0.45	0.0707	29	0.08	0.04	0.43	0.0795	27
3PG	—	—	—	—	1.35	0.33	0.77	0.0093	43	0.99	0.17	0.74	0.0001	15
2PG	—	—	—	—	0.15	0.07	0.27	0.0492	13	0.36	0.11	0.67	0.0251	14
PEP	—	—	—	—	—	—	—	—	—	0.51	0.18	0.59	0.0269	18
PYR	0.93	0.24	0.14	0.0002	—	—	—	—	99	1.76	0.54	0.51	0.0088	22
	Spectrophotometer													
	Sets 8–16 ($N = 92$)				Set 27 ($N = 16$)					Set 29 ($N = 16$)				
AMP	0.68	0.15	0.17	0.0000	—	—	—	—	—	1.30	0.43	0.61	0.0226	13
ADP	3.72	0.35	0.55	0.0000	6.86	1.68	0.54	0.0011	23	6.98	1.81	0.55	0.0023	28
ATP	23.02	1.81	0.64	0.0000	24.85	1.73	0.94	0.0000	7	29.05	2.60	0.95	0.0000	5
NAD	—	—	—	—	2.77	0.53	0.66	0.0001	13	2.12	0.44	0.66	0.0004	17

as μM/liter/OD_{260}. We interpret the μM/liter of extract differences as arising from differences in extraction efficiency which are standardized by the OD at 260 nm. In addition, a comparison of the linear regressions of μM GI/liter of extract on OD_{260} and g wet wt/liter reveals slightly higher R^2 values for the regression on OD_{260}. For reasons of the convenience, adjustment for differences in extraction efficiency, and improved regression, we find the OD_{260} to be a superior measure of fly quantity in our extraction procedure.

GI Means and CVs

The means and CVs for 146 measurements of each glycolytic intermediate on aliquots of 68 extracts of *D. mercatorum* are presented in Table II. While there are small differences in scale, the rank order of the means is consistent

Table II. A Summary of Pool Sizes and Coefficients of Variation for Resting Flies

	Design 1		Design 2			
	Spectrophotometer ($N = 16$; μM/liter/OD_{260})		Spectrophotometer ($N = 48$; μM/liter/OD_{260})		Spectrofluorometer ($N = 82$; μM/liter/OD_{260})	
Variable	$\bar{X}$	CV[a]	$\bar{X}$	CV[b]	$\bar{X}$	CV[b]
GIP	—	—	—	—	1.05	21
G6P	2.21	9	1.83	16	3.27	18
F6P	—	—	—	—	1.04	28
FDP	0.45	38	0.56	30	0.42	23
DHAP	0.81	24	0.64	38	0.75	18
αGP	7.50	16	6.19	23	11.06	10
GAP	—	—	—	—	0.11	10
3PG	—	—	—	—	0.88	23
2PG	—	—	—	—	0.21	74
PEP	—	—	—	—	0.24	36
PYR	0.83	40	0.60	46	1.26	31
	Spectrophotometer					
OD_{260}	8.69	7	6.86	19	9.40	8
NAD	—	—	—	—	2.53	14
AMP	0.79	22	0.42	45	0.72	61
ADP	3.70	12	3.79	12	5.65	20
ATP	24.23	7	23.51	13	20.95	14
ATP/ADP	6.59	8	6.30	19	4.07	24
ATP/AMP	31.40	20	65.13	143	44.76	79
Adenylate	28.71	6	27.69	11	27.98	15
Energy charge	0.91	1	0.92	1	0.86	6

[a] Attributable to series, extract, and aliquot within extract.
[b] Attributable to extract and aliquot within extract differences.

across experimental design and instrument types. Most of the intermediates are measured with sufficient precision by the methods we have developed to detect differences of 10 to 50% change in the mean GI concentration. A discussion of the statistical power to detect alterations in individual GI concentrations is given below. The intermediates, GAP, 2PG, and PEP, are present in whole fly extracts at low concentrations which yield a change in fluorescence in the range of the background. This contributes to relatively large coefficients of variation. The improvement of the estimation of these intermediates will depend upon a significant reduction in background fluorescence relative to the response in fluorescence due to the glycolytic intermediate.

Coefficients of variability are a function of the experimental design. The CVs in design 1 include variability due to extract, series, extract by series interaction, and aliquots. Variance components were estimated from the analysis of variance (Snedecor and Cochran, 1980). For the 8 intermediates reported under design 1, on the average, the extract effect determines 7% of the total phenotypic variance, with a range from 0 to 28%. The average aliquot effect on variance for the same intermediates is 69%, with a range from 28 to 100%. The combined effect of series and the extract by series interaction on the total variance is small by comparison and not statistically significant at the 0.05 level of probability. The CVs under design 1 indicate that GIs can be measured with acceptable precision on whole fly extracts of *D. mercatorum.*

The CVs for design 2 are a measure of the variability of duplicate extracts within runs and are an indication of our ability to make and measure extracts repeatably. A run is a session at the instrument in which a group of concurrent measurements is made on a set of extracts. Here again, the CVs are acceptable for most variables.

The *Drosophila* extracts contain significant amounts of biological substances which fluoresce over a range of excitation wavelengths overlapping the excitation wavelength for NADH. These fluorescing substances can be removed with a charcoal treatment but this also removes AMP, ADP, ATP, and NAD. Consequently these four substances are still measured on the spectrophotometer. The other intermediates are measured with marginally better CVs on the spectrofluorometer if the extract is charcoal treated.

In Table III we have compared the means and CVs (extracts) for our measures of glycolytic intermediates with those of Sacktor and co-workers (Sacktor and Hurlbut, 1966; Sacktor and Wormser-Shavit, 1966) made on *Phormia regina,* the blowfly. The blowfly resting means were estimated from the zero-time points of the figures of Sacktor and co-workers. For comparative purposes the *D. mercatorum* means are converted from μM/liter/OD_{260} to μM/g wet wt using the equality, 1 g wet wt/liter equals 0.121 OD units, estimated from the regression of OD_{260} on g wet wt/liter for the *D. mercatorum* data (Fig. 2). The *Drosophila* data are for whole flies, while the *Phormia*

Table III. Comparisons of Means and CV (Extracts), μM/g wet wt

	Drosophila mercatorum whole fly extracts ($N = 82$)		Phormia regina[a] thorax extracts ($N = 39$–157)	
	Spectrofluorometer		Spectrophotometer	
Variable	Mean	CV	Mean	CV
G6P	0.40	18	0.18	25
F6P	0.13	28	0.05	50
FDP	0.05	23	0.15	20
DHAP	0.09	18	0.14	20
αGP	1.34	10	1.50	15
GAP	0.01	41	0.03	50
3PG	0.11	23	0.10	20
2PG	0.03	74	0.04	45
PEP	0.09	36	0.05	30
PYR	0.15	31	0.20	32
	Spectrophotometer			
AMP	0.09	61	0.12	25
ADP	0.68	20	1.50	16
ATP	2.53	14	7.00	11
Energy charge[b]	0.86	6	0.90	

[a] From Sacktor and Hurlbut (1966) and Sacktor and Wormser-Shavit, (1966); at least 39 and a maximum of 157 extracts.
[b] Atkinson (1977).

data are for thoraxes. This difference in tissues extracted may account for the generally higher means in the *Phormia* data, particularly the ATP and ADP levels, since it is likely that the resting flight muscle is metabolically more active, on a per-gram basis, than the whole fly. The higher values of G6P and F6P in the *Drosophila* data may reflect a species difference in the pool sizes of these intermediates or a greater recovery of upper-pathway intermediates in *Drosophila* due to the more rapid temperature drop of this much smaller fly upon liquid nitrogen emersion. Given the many possible sources of difference, the *Phormia* and *Drosophila* data are remarkably similar.

The coefficients of variation, based on extract variability, for the *Drosophila mercatorum* data measured on the spectrofluorometer and the *Phormia regina* data (Sacktor and Hurlbut, 1966; Sacktor and Wormser-Shavit, 1966) measured on the spectrophotometer are also presented in Table III. With a few exceptions, these results are roughly equivalent and indicate that we are measuring most of the glycolytic intermediates with a precision that is comparable to that of published values.

In Table IV we have indicated the differences in treatment means which

Table IV. Percentage Change in Resting Mean Detectable for a Given Sample Size[a]

Variable	$\alpha = 0.05$			$\alpha = 0.01$		
	$N = 4$	$N = 8$	$N = 16$	$N = 4$	$N = 8$	$N = 16$
OD_{260}	11	7	5	17	10	7
GIP	28	18	12	46	28	18
G6P	24	16	11	40	24	16
F6P	39	25	16	63	37	25
FDP	32	20	14	51	31	20
DHAP	24	16	11	40	23	16
αGP	7	5	3	12	7	5
GAP	57	38	29	95	67	38
PG3	31	20	14	50	29	19
PG2	100	66	43	160	95	62
PEP	49	33	20	77	45	33
PYR	42	27	18	68	40	27
AMP	84	54	37	135	80	53
ADP	28	18	12	45	27	18
ATP	19	12	8	30	18	12
NAD	19	12	8	30	18	12

[a] N = number of extracts in each of two treatments; degrees of freedom = $2N - 2$.

we could detect with our methodology, for the indicated variables, sample sizes, and Type I experimental errors. These are calculated from the means and variance of the variables of the data set (Table II, fluorometric) measured as $\mu M/liter/OD_{260}$. It can be seen that for $\alpha = 0.05$ and N (each treatment) = 16, most variables can be measured well enough to detect 10 to 50% changes in the mean levels.

Figure 3 is a graphic of glycolytic intermediate means from the fluorometric data (design 2) expressed as $\mu M/liter/OD_{260} \pm 2$ SD calculated from the variance of extracts within runs (design 2). This represents the 95% confidence interval for extracts. The adenosine nucleotides, NAD, and OD_{260} are also included. The intermediates are arranged in their metabolic sequence within glycolysis. The profile of means and the estimated variability of observations about the means summarizes the scale and variability of the intermediates as measured in our laboratory.

CONCLUSIONS

We have developed an extraction procedure for glycolytic intermediates in *Drosophila mercatorum*. This procedure leads to consistent results when a measure is adopted which corrects for differences in extraction efficiencies. The unit $\mu M/liter/OD_{260}$ is such a measure. Using this measure, we found no

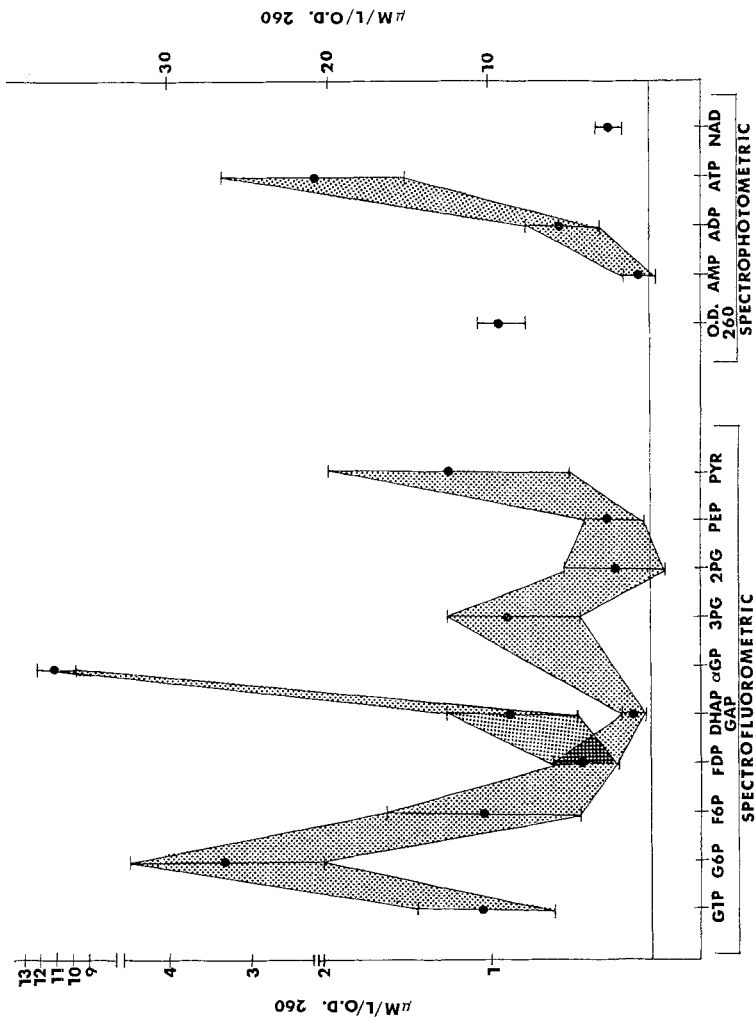


Fig. 3. Glycolytic intermediate mean ± 2 SD. The intermediates are arranged in metabolic sequence and connected to indicate the flow across pools. The shaded areas represent the 95% confidence intervals in our measurements.

significant differences among extracts for any of the measured metabolic intermediates using the extraction procedure we have developed.

We have compared the means and CVs of the intermediates assayed by the procedure we have developed for *Drosophila mercatorum* with those obtained for *Phormia regina* by Sacktor and co-workers (Sacktor and Hurlbut, 1966; Sacktor and Wormser-Shavit, 1966). These measures of ability to extract glycolytic intermediates are roughly equivalent in similar studies. We have estimated that our methodology has the capacity to detect a 10–50% change in mean level using an experimental design which is convenient.

In summary, the studies reported here document that methods are now available that have an accuracy sufficient to detect relatively small genetic and/or environmental treatment effects on glycolytic intermediate pool levels.

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