

AUTOSOMAL FACTORS WITH CORRELATED EFFECTS ON
THE ACTIVITIES OF THE GLUCOSE 6-PHOSPHATE AND
6-PHOSPHOGLUCONATE DEHYDROGENASES IN
*DROSOPHILA MELANOGASTER*¹

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ABSTRACT

Isogenic lines, in which chromosomes sampled from natural populations of *D. melanogaster* are substituted into a common genetic background, were used to detect and partially characterize autosomal factors that affect the activities of the two pentose phosphate pathway enzymes, glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). The chromosome 3 effects on G6PD and 6PGD are clearly correlated; the chromosome 2 effects, which are not so great, also appear to be correlated, but the evidence in this case is not so strong. Examination of activity variation of ten other enzymes revealed that G6PD and 6PGD are not the only pair of enzymes showing a high positive correlation, but it is among the highest in both sets of lines. In addition, there was some evidence that the factor(s) affecting G6PD and 6PGD may also affect two other metabolically related enzymes, transaldolase and phosphoglucose isomerase.—Rocket immunoelectrophoresis was used to estimate specific CRM levels for three of the enzymes studied: G6PD, 6PGD and ME. This experiment shows that a large part of the activity variation is accounted for by variation in CRM level (especially for chromosome 3 lines), but there remains a significant fraction of the genetic component of activity variation that is not explained by CRM level.—These results suggest that the autosomal factors are modifiers involved in regulation of the expression of the X-linked structural genes for G6PD and 6PGD, but a role in determining part of the enzymes' primary structure cannot be excluded with the present evidence.

SOME basic problems in evolutionary biology are to determine the amount and the nature of genetic variation in natural populations and to understand the roles that different types of variation have played in long-term evolutionary changes. In recent years, approaches to this problem have focused primarily on genetic variation in the structure of enzymes, which has proven very extensive by electrophoretic and other criteria (*cf.*, LEWONTIN 1974). In contrast, very little information is available about the amount or nature of variation in regu-

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latory sequences, which may constitute a much larger fraction of the eukaryotic genome than sequences coding for the primary structure of proteins. This gap in knowledge appears particularly significant in view of the possibility that polymorphism of regulatory elements may be a much more important source of variation for adaptive evolutionary change than structural variability (BRITTEN and DAVIDSON 1969; WILSON 1976).

Some of the reasons why population studies of regulatory elements have not been undertaken are, of course, that the mechanisms of regulation in eukaryotes are not well understood and the phrase "regulatory element" does not have a precise definition in molecular terms in the way that "structural element" does. Here we will use the term modifier gene, rather than regulatory element, to mean a locus that affects enzyme activity levels without affecting the primary structure of the polypeptide(s) at the time of translation (with no implication about molecular mechanisms).

We have initiated a study of genetic variation of enzyme activities in natural populations of *Drosophila melanogaster* in order to detect and characterize polymorphism of modifiers. To date we have discovered extensive genetic variation in the activities of ten different enzymes; 9 of them show evidence of variation of modifiers that are not linked to the structural locus of the enzyme (LAURIE-AHLBERG *et al.* 1980 and unpublished). This paper is a progress report on our efforts to characterize the autosomal factors that affect the activity levels of glucose 6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44), which catalyze the first and third steps, respectively, of the pentose phosphate pathway.

The *Drosophila melanogaster* G6PD and 6PGD enzymes have been the subject of a large number of genetic, biochemical and physiological studies, which have recently been reviewed (LUCCHESI, HUGHES and GEER 1979 and GEER *et al.* 1980). Two naturally occurring electrophoretic variants at each locus were used to assign the structural locus for 6PGD (*Pgd*) to the tip of the X chromosome (1-0.6, YOUNG, PORTER and CHILDS 1964 and 2D3-5, GERASIMOVA and ANANIEV 1972) and the structural locus for G6PD (*Zw*) to the proximal end of the X (1-63, YOUNG 1966 and 17B-18F, STEWART and MERRIAM 1974). Subsequently, several null and low activity variants of both enzymes have been induced on the X chromosome (see LUCCHESI, HUGHES and GEER 1979), some of which also alter electrophoretic mobility (GVOZDEV *et al.* 1976 and 1977). Only one of the two enzymes is affected by any one of these variants.

Both enzymes have been purified and characterized to some extent biochemically. 6PGD has been purified by WILLIAMSON, KROCHKO and GEER (1980), who reported a native molecular weight of 105,000 with subunits of 55,000 and 53,000. HORI and TANDA (1980) also reported a native molecular weight of 105,000 but found only one subunit of 58,000. The 6PGD thus appears to be a dimer, which is further supported by the observation of an intermediate hybrid band in females heterozygous for the two naturally occurring allozymes (YOUNG, PORTER and CHILDS 1964). The two naturally occurring allozymes of G6PD do not exhibit a hybrid band in heterozygotes. STEELE, YOUNG and

CHILDS (1968) originally showed that the B (slow) form has a native molecular weight of 317,000 and the A (fast) form, 147,000. They concluded that the polymorphism is due to the instability of subunit association, which has recently been confirmed by HORI and TANDA (1980). LEE, LANGLEY and BURKHART (1978) and HORI and TANDA (1980) reported a single subunit after SDS electrophoresis of purified enzyme; whereas, WILLIAMSON (in GEER *et al.* 1980) found two similar subunits of 61,000 and 66,000. The B variant is therefore apparently a tetramer and the A variant is a dimer.

The pentose phosphate pathway consists of an oxidative branch in which glucose 6-phosphate is converted to ribulose 5-phosphate with the reduction of NADP^+ in the reactions catalyzed by G6PD and 6PGD and a nonoxidative branch that allows for interconversion of glycolytic intermediates with pentose phosphates. The metabolic role of the pathway is generally considered to be the production of NADPH for lipid biosynthesis and pentose phosphate for nucleotide synthesis. The importance of G6PD and 6PGD in producing NADPH for lipid biosynthesis in *D. melanogaster* is well documented (see review by GEER *et al.* 1980), but their role is not essential since stocks that are null for both enzymes are viable and fertile. Stocks null for 6PGD only are, however, lethal or semilethal, apparently due to the toxic effects of 6-phosphogluconate accumulation (HUGHES and LUCCHESI 1977 and GVOZDEV *et al.* 1976 and 1977).

The mechanisms that regulate or cause variation in the activity levels of these enzymes are being investigated at several levels. With respect to the enzyme activities at a given stage in the life cycle, both environmental and genetic causes of variation have been identified, as well as causes that involve an interaction between environment and genotype. Two types of environmental variation can be distinguished: short-term fluctuations in the concentrations of metabolites that directly modulate activity levels and long-term influences of the diet that may cause variation in the rates of synthesis or degradation of the enzymes. For example, both G6PD and 6PGD are competitively inhibited by NADPH, from which GEER *et al.* (1980) concluded that the NADPH/ NADP^+ ratio probably regulates flux through the pentose shunt. Dietary modulation provides a much coarser type of control. GEER *et al.* (1976) and subsequently others reported large changes in the activities of G6PD and 6PGD in response to changes in the concentrations of dietary factors. Increases in sucrose, for example, increase the activities of both enzymes and also cause an increase in the rate of lipid synthesis. The increase of G6PD activity is accompanied by a change in the level of cross-reacting material, indicating a change in the rate of synthesis or degradation of the enzyme (GEER *et al.* 1980).

The genetic causes of activity variation can be divided into sex-specific and sex-nonspecific effects. Because the structural genes for G6PD and 6PGD appear to be X linked, they have been the subject of many studies of dosage compensation (see reviews by LUCCHESI 1977 and STEWART and MERRIAM 1980). Even though normal males have only one X chromosome and females have two, the activities of G6PD and 6PGD, as well as other X-linked enzymes, are equal

in the two sexes in similar tissues. Examples of sex-nonspecific effects are the lack of dosage compensation within each sex (LUCCHESI 1977) and the differences in activities between the A and B allozymes of 6PGD (BIJLSMA 1979; CAVENER and CLEGG 1981). An example of an environment \times genotype interaction effect is the genetic variation with respect to the inducing effect of dietary sucrose on G6PD and 6PGD activities (COCHRANE and LUCCHESI 1980). The work reported here deals primarily with the detection and characterization of genetic effects on G6PD and 6PGD activities in a standard laboratory culture environment. Particular attention is given to the possibility of coordinate genetic control because of the closely related functions of these two enzymes.

In an earlier report (LAURIE-AHLBERG *et al.* 1980), we described an experiment in which 50 chromosome 2 and 50 chromosome 3 isogenic substitution lines were screened for variation in the activities of seven enzymes, including G6PD and 6PGD. In that experiment, a significant genetic component of variation in G6PD activity was found in both sets of lines and in both sexes, but no significant genetic component of the 6PGD variation was found. However, the two activities were highly correlated: $r = 0.81$ for females and $r = 0.71$ for males for chromosome 2 lines, and $r = 0.64$ for females and $r = 0.80$ for males for chromosome 3 lines ($p < 0.001$ for all four). These observations suggested the possible existence of polymorphic autosomal factors that have correlated effects on the activities of G6PD and 6PGD. In order to investigate this possibility, the five lines with the highest G6PD activity and the five with the lowest activity (after weight-adjustment) were selected from each set of chromosome substitution lines and used for the experiments reported here (along with Ho-R, the genetic background line). Because the environmental component of enzyme activity variation is greater for adult females than males, only males were used in this study.

MATERIALS AND METHODS

General procedures

Stocks: Two sets of isogenic chromosome substitution lines were used in this study. The constitution of a line of each type is: $i_1/i_1; +_2/+_2; i_3/i_3$ (referred to as a chromosome 2 line) and $i_1/i_1; i_2/i_2; +_3/+_3$ (a chromosome 3 line), where i refers to a chromosome from a highly inbred line (Ho-R) and $+$ refers to a chromosome sampled from a natural population. The $+$, but not the i , chromosomes vary within a set of lines. Both sets of lines contain the same X chromosome (from Ho-R), which carries the "A" (fast) electromorph for both G6PD and 6PGD. The construction and electrophoretic analysis of these lines is described in LAURIE-AHLBERG *et al.* (1980).

Rearing conditions and sampling: All of the flies used for enzyme assays were raised at North Carolina State University on standard cornmeal-molasses medium. The standard procedure for obtaining samples from the isogenic lines was to place 50 pairs of parents in a half-pint bottle for 48 hr, rear the offspring at 25°, collect them within 18 hr of emergence, age the imagoes for X days (usually $X = 6$) in vials (15 per vial), weigh the live flies and freeze the samples at -70° .

Statistical analyses: The genetic correlations and their standard errors were computed with our own FORTRAN program. All other analyses were performed by using various procedures of the "SAS" statistical analysis system (HELWIG and COUNCIL 1979).

Chemical abbreviations: DTT: DL-dithiothreitol, EDTA: ethylenediamine tetracetic acid (disodium salt), SDS: sodium dodecyl sulfate, PMSF: phenylmethylsulfonyl fluoride, BSA: bovine serum albumin, DCIP: 2,6-dichlorophenol-indophenol, TAPS: Tris (hydroxymethyl) methylaminopropane sulfonic acid, PIPES: piperazine-N,N'-bis (2-ethane sulfonic acid).

Experiment I

Sample collection: In April, 1979, two separate experiments with the same design were used to investigate G6PD and 6PGD activity variation among 10 chromosome 2 lines plus Ho-R and among 10 chromosome 3 lines plus Ho-R. On each of two days ("blocks"), three bottles of parents for each of the 11 lines of chromosome type were set up. The flies from each set of 3 bottles were pooled to obtain three samples of 6-day-old males, which were weighed and frozen whole in sets of 10.

Extraction and assay: The frozen flies were homogenized and assayed at the University of North Carolina, Chapel Hill by the methods described by LUCCHESI and RAWLS (1973). Units of activity are μ moles NADP⁺ reduced per minute at 30°.

Experiment II

Sample collection and preparation: In January, 1980, on each of three days within each of two weeks, four bottles for each of 21 lines (10 chromosome 2, 10 chromosome 3 and Ho-R) were set up. Nineteen days after a group of bottles was set up, 25 each of 5-, 6-, 7- and 8-day-old males were pooled, weighed as a set of 100, homogenized in 1.25 ml of 0.01 M potassium phosphate buffer, pH 7.4, and then centrifuged for 30 min at 12,000 × g. The supernatant was split into 4 aliquots, which were diluted 1:1 with the homogenization buffer (buffer A), buffer A with 2.0 mM EDTA (buffer B), buffer A with 0.2 mM DTT (buffer C) or buffer A with 2.0 mM EDTA and 0.2 mM DTT (buffer D). These four types of samples were split into a total of twenty 95 μ l aliquots and frozen for enzyme assays, rocket immunoelectrophoresis and general protein determination.

Enzyme assays: The enzyme assays were performed at the Research Triangle Institute with a GeMSAEC centrifugal fast analyzer. This instrument, developed at the Oak Ridge National Laboratory, is an automated spectrophotometric system, allowing simultaneous measurement of 16 reaction rates (ANDERSON 1969). The samples for a given enzyme (total of 132) were all assayed on the same day at 30°. The 12 enzymes in Table 1 were assayed by the methods described below. The AOX reaction was monitored at 600 nm, all others were monitored at 340 nm. In all cases, substrate concentrations are saturating for crude extracts of Ho-R. For all assays, 10 μ l of sample were used in a total reaction volume of 128 μ l. Concentrations are for the total reaction mixture.

TABLE 1
Enzymes assayed in this study

Name	Abbreviation	E. C. #	Map position†
Alcohol dehydrogenase	ADH	1.1.1.1	2-50.1
Arginine kinase	AK	2.7.3.3	—
Aldolase	ALD	4.1.2.13	3R
Aldehyde oxidase	AOX	1.2.3.1	3-56.6
Fumarase	FUM	4.2.1.2	1-19.9
α -Glycerophosphate dehydrogenase	GPDH	1.1.1.8	2-20.5
Glucose 6-phosphate dehydrogenase	G6PD	1.1.1.49	1-63
NADP-dependent Isocitrate dehydrogenase	IDH	1.1.1.42	3-27.1
Malic enzyme	ME	1.1.1.40	3-53.1
Phosphoglucose isomerase	PGI	5.3.1.9	2-58.7
Transaldolase	TA	2.2.1.2	—
6-phosphogluconate dehydrogenase	6PGD	1.1.1.44	1-0.64

† O'BRIEN and MACINTYRE (1978) and VOELKER *et al.* (1978).

ADH: Sample buffer B. Reaction mixture: 90.0 mM ethanol, 1.4 mM NAD⁺, 0.9 mM EDTA in 0.04 M glycine-NaOH, pH 9.5.

AK: Sample buffer C. Reaction mixture: 4.7 mM arginine, 0.44 mM NADH, 0.4 mM ATP, 8.0 mM MgSO₄, 80 mM KCl, 1.1 mM phosphoenolpyruvate, 1.7 units/ml pyruvate kinase and 0.4 units/ml lactate dehydrogenase (Sigma Co. P-1381) in 0.02 M TAPS-PIPES, pH 7.2.

ALD: Sample buffer D. Reaction mixture: 0.5 mM fructose 1,6-diphosphate, 0.29 mM NADH, 2.6 units/ml α -glycerophosphate dehydrogenase and 24.7 units/ml triosephosphate isomerase (Sigma Co. G-1881) in 0.02 M TAPS-PIPES, pH 7.6.

AOX: Sample buffer A. Reaction mixture: 25.0 mM acetaldehyde, 0.13 mM phenazine methosulfate, 36.0 μ M DCIP_{ox}, 0.04 mM EDTA in 0.1 M Tris-HCl, pH 7.4 with 1.0 mg/ml BSA.

FUM: Sample buffer C. Reaction mixture: 6.0 mM fumarate, 0.6 mM NADP⁺, 0.8 mM MgCl₂, 0.5 units/ml malic enzyme (Sigma Co. M-5257) in 0.02 M TAPS-PIPES, pH 7.8.

GPDH: Sample buffer C. Reaction mixture: 13.4 mM α -glycerophosphate, 2.26 mM NAD⁺ in 0.04 M glycine-NaOH, pH 9.5.

G6PD: Sample buffer D. Reaction mixture: 3.44 mM glucose 6-phosphate, 0.2 mM NADP⁺, 18.8 mM MgCl₂, 0.78 mM DTT, 0.02 M TAPS-PIPES, pH 7.8.

IDH: Sample buffer C. Reaction mixture: 2.62 mM isocitrate, 0.36 mM NADP⁺, 0.78 mM MgSO₄, 1.17 mM DTT in 0.02 M TAPS-PIPES, pH 9.0.

ME: Sample buffer D. Reaction mixture: 47.0 mM malate, 0.68 mM NADP⁺, 2.0 mM MgCl₂ in 0.02 M TAPS-PIPES, pH 8.0.

PGI: Sample buffer B. Reaction mixture: 2.34 mM fructose 6-phosphate, 0.38 mM NADP⁺, 1.37 units/ml glucose 6-phosphate dehydrogenase (Sigma Co. G-5760) in 0.02 M TAPS-PIPES, pH 8.3.

TA: Sample buffer D. Reaction mixture: 21.6 mM fructose 6-phosphate, 1.0 mM erythrose 4-phosphate, 0.4 mM NADH, 0.2 units/ml α -glycerophosphate dehydrogenase and 0.78 units/ml triosephosphate isomerase (Sigma Co. G-1881) in 0.02 M TAPS-PIPES, pH 7.0.

6PGD: Sample buffer A. Reaction mixture: 0.3 mM 6-phosphogluconate, 0.3 mM NADP⁺, 3.12 mM MgSO₄ in 0.02 M TAPS-PIPES, pH 8.2.

Rocket immunoelectrophoresis: Levels of specific immunologically cross-reacting material (CRM) were determined at the University of Calgary by one-dimensional immunoelectrophoresis (LAURELL 1966) with subsequent staining for specific activity of the antigen-antibody complexes for G6PD, 6PGD or ME. Gels were prepared by heating 30 ml of 1.0% agarose suspension on 0.088 M Tris, 2.8 mM EDTA, 0.025 M acetate buffer, pH 8.6, to 90°. After cooling to 55° in a waterbath, antiserum was added (40 μ l for malic enzyme gels, 100 μ l for 6PGD gels and 120 μ l for G6PD gels). Five-microliter samples of crude homogenate (buffer A for 6PGD, buffer D for G6PD and ME) were placed in wells and the gels were electrophoresed for 21 hours at 100 V in BioRad Model 4200 chambers. Specific enzyme activities were visualized by staining the gels with specific substrate (1.5 mM L-malate, 7.0 mM 6-phosphogluconate or 6.0 mM glucose 6-phosphate), 35.0 mM MgCl₂, 0.35 mM NADP⁺, 0.6 mM phenazine methosulfate, 2.4 mM nitroblue tetrazolium in 0.06 M Tris-HCl, pH 7.9.

The rocket heights for all three enzymes are linear for concentrations in the range of 0.1 to 0.8 of an Oregon-R fly per 5 μ l sample. Each gel contained 6 control samples and 14 experimental samples. The controls were three replicates each of two concentrations of an Oregon-R mass homogenate, 0.25 and 0.50 fly per 5 μ l sample, and the experimentals were all at a concentration of 0.25 fly per 5 μ l sample. More than 90% of the experimental rocket heights were between the control values.

For each of the 6 sampling days of the experiment, there is a corresponding measurement day on which one sample per line was split in two and each run on a different immunoelectrophoresis gel. Analyses of variance of the control rocket heights showed a highly significant component of variation among gels within a day for G6PD and 6PGD, but no significant variation for ME. Therefore, the experimental rocket heights for G6PD and 6PGD were adjusted by the control rocket heights for each gel separately: $\hat{R} = (R-a)/b$, where \hat{R} is the

adjusted rocket height, and a and b are the intercept and slope, respectively, of the regression of control rocket height on the concentration of the control homogenate (50% or 100%). The \hat{R} values for G6PD and 6PGD, which are on an arbitrary scale, and the original R values for ME (in mm) were used in all subsequent analyses and are hereafter referred to as CRM level. For the statistical analyses reported here, the mean of the two CRM levels determined for a given line on one day was used.

Antisera: The production of antiserum specific to ME has been described by GEER, KROCHKO and WILLIAMSON (1979). Antisera to G6PD and 6PGD were produced by subcutaneous injection of 100 μg of pure enzyme carried in Freund's complete adjuvant into young female San Juan rabbits. A second injection of 50 μg of pure enzyme in Freund's incomplete adjuvant was administered to each rabbit 14 days after the first injection. Ten days after the second injection, samples of antiserum produced strong, single precipitin bands on Ouchterlony double-diffusion plates. Blood was collected by heart puncture, allowed to clot at room temperature for three hr and at 4° for 24 hr. The serum was collected by centrifugation at 4000 \times g for 10 min and frozen in 1.0 ml aliquots. Both G6PD and 6PGD were purified 400- to 600-fold by sequential purification on 2',5'-ADP Sepharose 4B and Blue Sepharose CL 6B (WILLIAMSON, KROCHKO and GEER 1980 and unpublished).

Protein determination: Protein concentrations were determined at the University of Calgary, by the dye-binding method of BRADFORD (1976), using BSA as the standard. Triplicate assays were performed on each sample for each day that immunoelectrophoresis gels were run.

Dissections: On each of the 6 sampling days, 20 males from each of 4 lines were dissected into 5 body parts: head, thorax, alimentary tract, the reproductive organs and the abdominal wall. The 4 lines were RIO9II (a chromosome 2 line), RI22III and KA27III (chromosome 3 lines) and Ho-R. The 20 body parts were homogenized in 500 μl of buffer D, centrifuged for 10 min at 12,000 \times g and the supernatant was frozen. The protein determinations were done by the method of LOWRY *et al.* (1951) using BSA as the standard. The enzyme assays were performed at N.C. State University by monitoring the reduction of NADP⁺ at 340 nm and 30°. For both assays, 100 μl of sample in a total reaction volume of 1.0 ml was used. The total reaction mixture for G6PD contained 1.62 mM glucose 6-phosphate, 0.16 mM NADP⁺, 16.7 mM MgCl₂ in 0.05 M Tris-HCl, pH 7.6. The total reaction mixture for 6PGD contained 0.30 mM 6-phosphogluconate, 0.14 mM NADP⁺, 1.51 mM MgSO₄ in 0.05 M Tris-HCl, pH 7.6.

Effects of extraction buffers on G6PD and 6PGD assays

Sample collection and preparation: On each of two consecutive days, 4 bottles for each of 5 lines were set up. Nineteen days after a group of bottles were set up, 40 each of 5-, 6-, 7- and 8-day-old males were pooled and then divided into 16 sets of 10 flies each; the same was done for females. Two replicate homogenates using 8 different buffers for each sex \times line combination were made on each of the two days of the experiment. The 8 buffers represent all combinations of 0 or 0.4 mM PMSF, 0 or 1.0 mM DTT and 0 or 1.0 mM NADP⁺ in 0.01 M potassium phosphate, pH 7.4 with 1.0 mM EDTA. Each set of 10 flies was homogenized in 0.25 ml of buffer, centrifuged for 20 minutes at 12,000 \times g and then split into two aliquots (one for G6PD, one for 6PGD) and frozen. The lines were RIO9II (a chromosome 2 line), RI22III, KA27III, RIO6III (chromosome 3 lines) and Ho-R.

Enzyme assays: The assays for each enzyme (G6PD, 6PGD) were performed on two different days (corresponding to the two sampling days) at N.C. State University. For both assays, 80 μl of sample were used in a total reaction volume of 1.08 ml. The concentrations of reactants are the same as reported above for the centrifugal fast analyzer assays.

Acrylamide gel electrophoresis

Vertical slab acrylamide gel electrophoresis was performed at the University of North Carolina, Chapel Hill with the buffer system of DAVIS (1964). The gel consisted of 10% acrylamide and 0.8% bis with a 5% stacking layer. The electrode buffer contained 26 μM NADP⁺. The homogenization buffer and staining solution are described by FAIZULLIN and GVOZDEV (1973). Ten males were homogenized in 100 μl of buffer (with 10% sucrose) and 5

μ l were loaded in each slot. When PMSF was used in the homogenization buffer, it was added in the form of a 4.0 mM solution in isopropanol to a concentration of 0.8 mM.

Specificity of the G6PD assay

In this study, G6PD activity was estimated by monitoring the reduction of NADP⁺ that accompanies the conversion of glucose 6-phosphate to 6-phosphogluconolactone. In crude extracts, the 6-phosphogluconolactone is probably converted to 6-phosphogluconate by lactonase (HUGHES and LUCCHESI 1978), which can allow reduction of NADP⁺ by 6PGD. The possible contribution of 6PGD to the assay of G6PD in crude extracts was investigated by three methods: (1) Four assays were performed in the G6PD reaction buffer containing NADP⁺: (a) the no substrate blank, (b) saturating concentrations of both glucose 6-phosphate and 6-phosphogluconate, (c) a saturating concentration of 6-phosphogluconate only and (d) a saturating concentration of glucose 6-phosphate only (the standard G6PD assay). It should be noted that neither 6-phosphogluconate nor ribulose 5-phosphate inhibit *Drosophila* G6PD (GEER *et al.* 1980). If 6PGD contributes to the apparent G6PD rate, then the sum of (c) and (d) will be greater than the sum of (a) and (b). The two sums were very similar both for high and low activity lines. (2) The amount of purified yeast 6PGD (Sigma Co. P-0632) that is saturating with respect to the reduction of NADP⁺ at 0.3 mM 6-phosphogluconate and under the G6PD assay conditions was first determined (1.0 units/ml). The addition of this amount of pure 6PGD to the standard G6PD assay had no effect on the reaction rate for either high or low activity lines. (3) Crude extract from a double-mutant strain (*Pgdⁿ Zwⁿ*), which contains no G6PD or 6PGD activity but does contain 6-phosphogluconolactonase activity (HUGHES and LUCCHESI 1978), was supplemented with either purified *Drosophila* G6PD only or with purified *Drosophila* G6PD and 6PGD. There was no difference between the two rates. We therefore conclude that any contribution of 6PGD to the apparent G6PD rate in crude extracts is negligible.

RESULTS

Experiment I

The following model was used for analyses of variance and estimation of covariance components:

$$Y_{ijkl} = \mu_i + \beta_{ij} + \tau_{ik} + (\beta\tau)_{ijk} + \epsilon_{ijkl},$$

where μ_i is the mean of the *i*th variable (*i* = 1, 2 for G6PD, 6PGD), β_{ij} is the effect of the *j*th block for the *i*th variable (*j* = 1, 2), τ_{ik} is the effect of the *k*th line (*k* = 1, . . . , 11), $(\beta\tau)_{ijk}$ is the interaction effect and ϵ_{ijkl} is the error effect (*l* = 1, 2, 3). For adjustment of raw activities (*Y*), which are measured in terms of units of activity per 10 flies, by the live weight of the flies, regression of \bar{Y}_{ijk} on \bar{WT}_{jk} was performed for each of the two blocks (*j* = 1, 2); the sums of squares and products were then pooled over blocks to obtain a single regression coefficient, *b_i*. Adjusted variables (\hat{Y}) were then obtained as follows:

$$\hat{Y}_{ijkl} = Y_{ijkl} - b_i(WT_{jkl} - \bar{WT} \dots).$$

The ranges of line means (in units $\times 10^3$ /fly) are 1.80–4.37 and 1.48–2.75 for G6PD and 6PGD, respectively for the chromosome 2 lines. Excluding one line with an extremely high weight (line R in the figures), the chromosome 3 ranges are 1.78–3.68 and 1.51–2.63 for G6PD and 6PGD, respectively. Weight adjustment has very little effect on the magnitude of these ranges.

TABLE 2

Summary of analyses of variance for experiment I†

Variable		Chromosome 2 lines				Chromosome 3 lines			
		Block	Line	Block × Line	K	Block	Line	Block × Line	K
G6PD	Raw	ns	***	ns	0.38	ns	***	ns	0.76
	Wt-adj	*	***	ns	0.44	***	***	ns	0.48
6PGD	Raw	ns	***	ns	0.49	ns	***	ns	0.57
	Wt-adj	ns	***	ns	0.47	**	***	ns	0.52
WT	Raw	ns	ns	***	0.39	*	*	***	0.43

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. ns $p > 0.05$.

† The significance levels of the F -tests and a variance component ratio $K = \hat{\sigma}_l^2 / (\hat{\sigma}_l^2 + \hat{\sigma}_{bxl}^2 + \hat{\sigma}_e^2)$ are given for raw and weight-adjusted variables.

The results of the analyses of variance are summarized in Table 2. Lines are a highly significant component of variance for both raw and weight-adjusted G6PD and 6PGD activities in both sets of lines. The correlations over line means between live weight and activity are $r = 0.29$ and $r = 0.37$ ($p > 0.05$ for both) for G6PD and 6PGD, respectively, for chromosome 2 lines and $r = 0.77$ ($p < 0.01$) and $r = 0.71$ ($p < 0.05$) for G6PD and 6PGD, respectively for chromosome 3 lines. The higher correlations for the chromosome 3 lines are largely due to the high-weight outlier, line R. The variance component ratio K , where $K = \hat{\sigma}_l^2 / (\hat{\sigma}_l^2 + \hat{\sigma}_{bxl}^2 + \hat{\sigma}_e^2)$, is the proportion of variance among the observations (based on 10-fly homogenates) within a block that is attributable to lines ($\hat{\sigma}_l^2$ is the estimated line component of variance, $\hat{\sigma}_{bxl}^2$ is the block × line component and $\hat{\sigma}_e^2$ is the error mean square). The relative magnitudes of K for raw and weight-adjusted activities indicate that weight adjustment was effective only in substantially reducing the line component for G6PD in chromosome 3 lines. However, even in this case, most of the effect was due to line R, and the K value for the weight-adjusted G6PD is still quite large (0.48).

Figure 1 shows the relationship between the line means for G6PD and 6PGD raw activities. The corresponding correlations are $r = 0.78$ ($p < 0.01$) for the chromosome 2 lines and $r = 0.96$ ($p < 0.001$) for the chromosome 3 lines. The correlations over weight-adjusted line means are very similar: $r = 0.76$ ($p < 0.01$) for chromosome 2 and $r = 0.90$ ($p < 0.001$) for chromosome 3 lines. The product-moment correlations over line means are not necessarily good estimates of the correlation between the line (genetic) effects on activity. Therefore, the genetic correlations (r^*) were computed as follows: Let $\hat{\sigma}_{xl,yl}$ be the estimated covariance of line effects on enzymes x and y and $\hat{\sigma}_{xl}^2, \hat{\sigma}_{yl}^2$ be the estimated variance components for lines. Then $r^* = \hat{\sigma}_{xl,yl} / (\hat{\sigma}_{xl} \hat{\sigma}_{yl})$. Because the quantity r^* is not necessarily bounded by -1 and $+1$ and may not even be defined for negative variance component estimates, no test of the hypothesis that the true value of r^* equals zero is available, although the standard errors were computed by the method of MODE and ROBINSON (1959). The genetic correlations for the weight-adjusted activities are $r^* = 0.83 \pm 0.13$ for chromosome 2 and $r^* = 0.98 \pm 0.05$ for chromosome 3 lines. These results provide clear evidence for

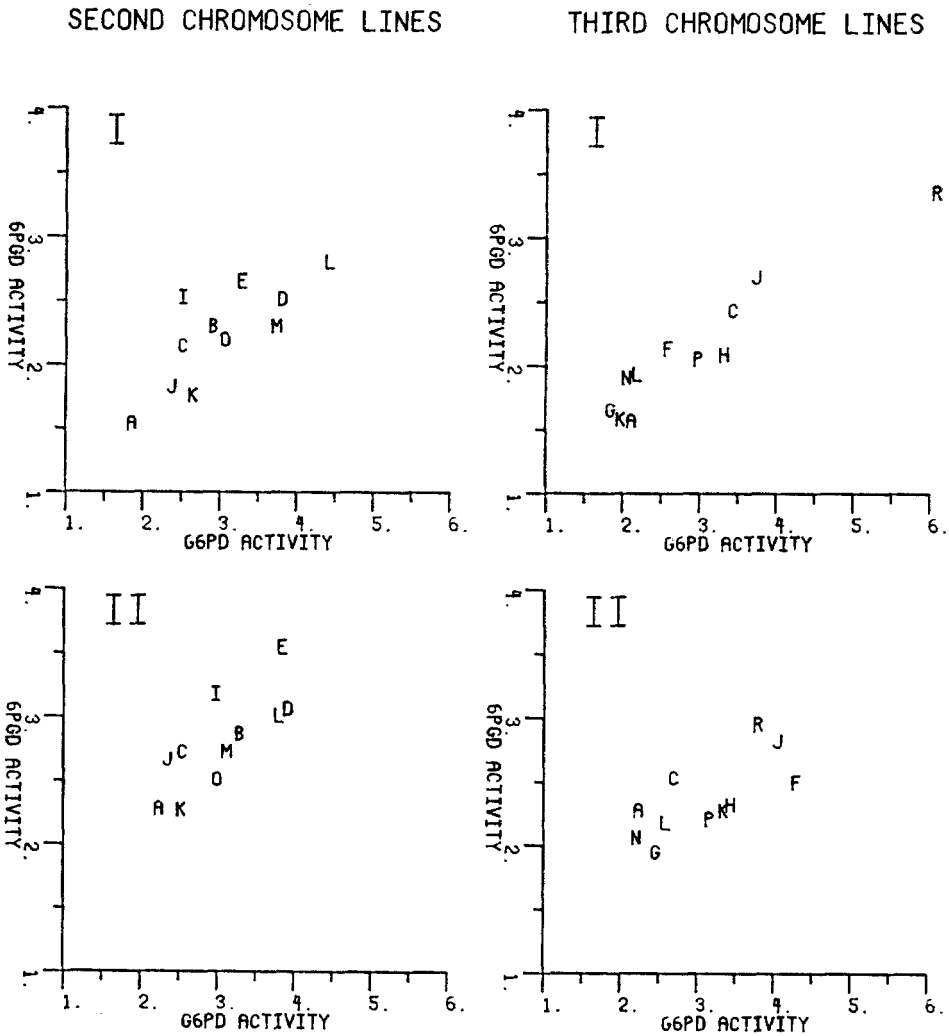


FIGURE 1.—Plots of the line means of 6PGD *versus* G6PD activity for Experiments I (upper half) and II (lower half) and for both sets of chromosome substitution lines (chromosome 2 on left, chromosome 3 on right). Each letter represents a line. Chromosome 2 and 3 lines are represented by the same letter if they were derived from the same isofemale line. The only line that is identical between chromosome 2 and 3 sets is the isogenic background stock, Ho-R, represented by the letter A. Both activities are in units $\times 10^3$ per fly.

autosomal factors with correlated effects on G6PD and 6PGD. Experiment II, which includes measurements of 12 different enzyme activities, was undertaken to investigate the specificity of this relationship.

Experiment II

The following model was used for analyses of variance and estimation of covariance components.

$$Y_{ijkl} = \mu_i + \beta_{ij} + \alpha_{ijk} + \tau_{il} + (\beta\tau)_{ijl} + \epsilon_{ijkl}$$

where μ_i is the mean of the i th variable ($i = 1, \dots, 12$), β_{ij} is the effect of the j th week of sampling ($j = 1, 2$), α_{ijk} is the effect of the k th day within the j th week ($k = 1, 2, 3$), τ_{il} is the effect of the l th line of a chromosome type ($l = 1, \dots, 11$), $(\beta\tau)_{ijl}$ is the week \times line interaction and ϵ_{ijkl} is the residual.

Two kinds of adjustments of the raw variables were made. Activities and CRM levels were adjusted for general protein or live weight, and activities were also adjusted for CRM level. Let Y be the dependent variable to be adjusted and X be the independent variable. The regression of Y on X over lines was performed for each of the 6 days of the experiment; the sums of squares and products were then pooled over days to obtain a single regression coefficient, b_i . Adjusted variables (\hat{Y}) were then obtained as follows:

$$\hat{Y}_{ijkl} = Y_{ijkl} - b_i(X_{ijkl} - \bar{X} \dots).$$

The ranges of line means for G6PD and 6PGD are similar to those in Experiment I, but are somewhat smaller: 2.18–3.85 for G6PD and 2.21–3.48 for 6PGD in chromosome 2 lines and 2.14–4.23 for G6PD and 1.89–2.89 for 6PGD in chromosome 3 lines (in units $\times 10^3$ per fly).

The results of linear regression using the line means ($N = 11$) of the 12 raw activities and the CRM levels on live weight or protein are summarized in Table 3. Many of the regression coefficients are not significantly different from zero, but more of the significant regressions are of activity or CRM on protein rather than on weight. These results suggest that adjustment by protein may be more effective than adjustment by weight.

The results of the analyses of variance of raw and protein-adjusted activities are summarized in Table 4. Lines are a significant component of variance for all of the raw variables. The variance component ratio $K = \hat{\sigma}_l^2 / (\hat{\sigma}_l^2 + \hat{\sigma}_{wsl}^2 + \hat{\sigma}_e^2)$ is the proportion of variance among the observations within a day that is due to differences among lines, (where $\hat{\sigma}_l^2$ is the estimated line component of variance, $\hat{\sigma}_{wsl}^2$ is the week \times line interaction component and $\hat{\sigma}_e^2$ is the error mean square). The values of K for raw variables range from 0.15 to 0.93. In most cases, the effect of weight or protein adjustment on the significance level of the line component or on the value of K is small. In some cases, however, lines become nonsignificant after adjustment. In particular, the chromosome 2 line component for G6PD and 6PGD loses significance after protein-adjustment (but not after weight adjustment). For the chromosome 3 lines, however, both G6PD and 6PGD have highly significant line components after either protein or weight adjustment, and the K values are quite large: 0.69 and 0.60 for protein-adjusted G6PD and 6PGD, respectively. These results suggest that the chromosome 2 line effects on G6PD and 6PGD may be due to nonspecific body size and tissue quantity variation. However, the week \times line interactions for both G6PD and 6PGD in the chromosome 2 lines are significant even after protein adjustment. This result indicates not only that there are some nonadditive genetic effects that cannot be removed by protein-adjustment, but it also means that the F -test for the main effect of lines is much less powerful than if the week \times line interaction were nonsignificant.

TABLE 3

Linear regression of activities and CRM level on weight or protein†

Variable	Chromosome 2				Chromosome 3			
	Weight		Protein		Weight		Protein	
	$H_0: \beta=0$	R^2	$H_0: \beta=0$	R^2	$H_0: \beta=0$	R^2	$H_0: \beta=0$	R^2
ADH	*	0.51	ns	0.24	ns	0.16	ns	0.26
AK	ns	0.09	*	0.40	ns	0.34	*	0.42
ALD	ns	0.28	**	0.62	ns	0.32	ns	0.18
AOX	ns	0.19	ns	0.01	ns	0.29	*	0.54
FUM	ns	0.09	ns	0.32	ns	0.11	ns	0.30
GPDH	ns	0.02	ns	0.06	**	0.69	ns	0.23
G6PD	ns	0.02	*	0.39	ns	0.17	ns	0.13
IDH	ns	0.28	ns	0.01	ns	0.02	ns	0.01
ME‡	**	0.54	**	0.57	ns	0.20	ns	0.00
PGI	*	0.38	***	0.78	ns	0.34	**	0.59
TA	ns	0.08	ns	0.01	ns	0.22	*	0.50
6PGD	ns	0.05	**	0.66	ns	0.24	ns	0.21
CRM-G6PD	ns	0.02	*	0.49	ns	0.02	*	0.39
CRM-6PGD	ns	0.02	ns	0.03	ns	0.09	ns	0.29
CRM-ME	*	0.40	**	0.71	ns	0.14	ns	0.00

† Regression over line means (10 or 11) from Experiment II. Significance levels of the regression coefficients (β) and the coefficients of determination (R^2) are given.

‡ The chromosome 3 ME null line is excluded.

The plots of the line means of the raw G6PD vs. 6PGD activities from Experiment II are shown in the lower half of Figure 1. The 2 or 3 lines at either extreme maintain their positions in both Experiments I and II. The correlations are also similar, although somewhat lower: $r = 0.77$ ($p < 0.01$) for chromosome 2 lines (compared with $r = 0.78$ in Experiment 1) and $r = 0.71$ ($p < 0.05$) for chromosome 3 lines (compared with $r = 0.90$ in Experiment I).

Table 5 shows the partial correlations over line means between pairs of enzymes with protein as the fixed variable ($r_{xy.p}$). For the chromosome 2 lines, only one of the partial correlations is significantly different from zero (ADH, AOX) and the numbers of positive and negative estimates are about equal (34 and 32, respectively). Although the G6PD, 6PGD partial correlation over chromosome 2 lines is not significantly different from zero, it is the fourth highest (out of 66), and the G6PD, TA correlation is third highest. For the chromosome 3 lines, there are 47 positive and 19 negative estimates and most of the negative estimates involve AOX. Three negative estimates are significantly different from zero, each involving AOX. The four significant positive estimates are between ADH and TA, G6PD and TA, G6PD and 6PGD, and TA and PGI. These results show that G6PD and 6PGD are not the only pair of enzymes showing a high positive correlation, but it is among the highest in both sets of lines, and most of the other high positive correlations involve enzymes that are also closely related in function. It should be noted that the correlations between G6PD and 6PGD and FUM (the only other enzyme in the set known to be X linked) are small.

TABLE 4

Summary of analyses of variance of experiment II†

Variable§	Chromosome 2					Chromosome 3				
	Week	Day in week	Line	Week × line	K	Week	Day in week	Line	Week × line	K
ADH(2) Raw	**	ns	***	***	0.93	ns	*	***	ns	0.64
Prot-adj	ns	**	***	*	0.85	ns	***	***	ns	0.61
AK Raw	ns	***	***	ns	0.49	*	***	***	ns	0.62
Prot-adj	*	***	**	ns	0.19	*	***	***	ns	0.54
ALD(3) Raw	ns	***	**	ns	0.48	ns	*	**	ns	0.28
Prot-adj	ns	***	ns	ns	0.13	ns	**	**	ns	0.23
AOX(3) Raw	ns	***	*	ns	0.26	ns	***	***	ns	0.68
Prot-adj	ns	***	*	ns	0.26	ns	***	***	ns	0.53
FUM(X) Raw	***	ns	***	ns	0.32	ns	**	***	ns	0.49
Prot-adj	ns	*	*	ns	0.22	ns	***	***	ns	0.41
GPDH(2) Raw	***	ns	***	*	0.82	ns	ns	*	*	0.52
Prot-adj	ns	*	***	**	0.82	ns	***	***	ns	0.51
G6PD(X) Raw	***	ns	*	*	0.40	**	ns	***	ns	0.66
Prot-adj	ns	ns	ns	*	0.25	ns	ns	***	ns	0.69
CRM-adj	**	ns	ns	*	0.10	ns	ns	***	ns	0.49
IDH(3) Raw	*	***	***	ns	0.88	ns	ns	***	ns	0.81
Prot-adj	*	**	***	ns	0.88	ns	ns	***	ns	0.84
ME‡(3) Raw	***	ns	*	ns	0.15	ns	ns	***	ns	0.90
Prot-adj	ns	**	ns	ns	<0	ns	ns	***	ns	0.91
CRM-adj	ns	***	ns	ns	<0	ns	ns	***	ns	0.39
PGI(2) Raw	*	**	***	‡	0.47	ns	***	***	ns	0.73
Prot-adj	ns	***	*	ns	0.09	ns	***	***	ns	0.56
TA Raw	ns	*	***	ns	0.51	ns	***	***	ns	0.70
Prot-adj	ns	ns	***	ns	0.54	ns	***	***	ns	0.63
6PGD(X) Raw	**	ns	**	**	0.53	ns	ns	***	ns	0.63
Prot-adj	ns	*	ns	*	0.20	ns	***	***	ns	0.60
CRM-adj	***	ns	**	*	0.50	ns	***	**	ns	0.40
WT Raw	***	ns	***	***	0.80	ns	**	***	ns	0.82
PROT Raw	*	***	***	ns	0.48	**	ns	ns	*	0.07

† The significance levels of the F -tests and a variance component ratio $K = \hat{\sigma}_i^2 / (\hat{\sigma}_i^2 + \hat{\sigma}_{wxl}^2 + \hat{\sigma}_e^2)$ are given for raw and adjusted variables.

‡ The third chromosome ME null line is excluded.

ns, $p > 0.05$. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.

§ The chromosomal location of the structural gene, if known, is given in parentheses.

As mentioned above, the correlations of line means are not necessarily good estimates of the correlations of line (genetic) effects. However, in this experiment, the two types of correlations are very similar. For example, the genetic correlations between protein-adjusted G6PD and 6PGD are $r^* = 0.55 \pm 0.41$ and $r^* = 0.71 \pm 0.17$ for chromosomes 2 and 3, respectively, compared with $r_{xy,p} = 0.58$ and $r_{xy,p} = 0.66$ for partial correlations over line means.

The analyses of variance of the CRM levels for G6PD, 6PGD and ME are summarized in Table 6. Lines are a significant component of variance for all the raw and weight-adjusted CRM levels in both sets of lines and for all the protein-adjusted CRM levels except for ME in the chromosome 2 lines. Note

TABLE 5
Partial correlations over line means with protein as the fixed variable†

	ADH	AK	ALD	AOX	FUM	GPDH	G6PD	IDH	ME	PGI	TA	6PGD
ADH	—											
AK	-0.31	—										
ALD	-0.14	0.32	—									
AOX	-0.38	0.63	-0.18	—								
FUM	-0.34	-0.24	-0.03	-0.48	—							
GPDH	-0.01	0.46	0.25	0.29	-0.41	—						
G6PD	0.01	0.58	0.52	0.35	0.46	-0.26	—					
IDH	0.46	0.07	-0.31	-0.12	0.16	0.05	-0.15	—				
ME	0.13	0.26	-0.22	0.07	-0.31	0.38	0.38	-0.28	—			
PGI	0.35	0.23	0.43	0.63	0.46	0.02	0.02	-0.28	0.25	—		
TA	0.16	0.42	0.20	0.61	0.42	0.60	0.51	-0.24	-0.49	0.12	—	
6PGD	0.65*	0.03	-0.37	0.31	0.15	0.78*	0.51	0.61	-0.49	0.75*	0.48	—
	0.54	0.34	0.13	0.07	0.13	0.66*	0.55	0.30	0.53	0.49	0.49	—

* $p < 0.05$.

† Chromosome 2 lines above diagonal; chromosome 3 lines below.

TABLE 6

Summary of analyses of variance of CRM level†

Variable	Chromosome 2					Chromosome 3				
	Week	Day (week)	Line	Week × line	K	Week	Day (week)	Line	Week × line	K
CRM-G6PD Raw	*	**	***	ns	0.35	ns	*	***	ns	0.48
Prot-adj	ns	***	*	ns	0.18	ns	***	***	ns	0.41
CRM-6PGD Raw	ns	***	*	ns	0.21	ns	***	***	ns	0.61
Prot-adj	ns	***	*	ns	0.21	ns	***	***	ns	0.54
CRM-ME Raw	ns	***	***	ns	0.36	ns	***	***	ns	0.77
Prot-adj	ns	***	ns	ns	0.10	ns	***	***	ns	0.78

ns, $p > 0.05$. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.† The significance levels of the F -tests and a variance component ratio $K = \hat{\sigma}_i^2 / (\hat{\sigma}_i^2 + \hat{\sigma}_{wxl}^2 + \hat{\sigma}_e^2)$ are given for raw and adjusted variables.

that the line components for chromosome 2 G6PD, 6PGD and ME protein-adjusted CRM levels are all significant, although this was not the case for the corresponding activities. Similarly, the chromosome 2 week × line interactions are significant for protein-adjusted G6PD and 6PGD activities, but not for the corresponding CRM levels.

The plots of the line means of activity *versus* CRM level are shown in Figure 2, and the corresponding linear regression analyses are summarized in Table 7. There is a significant regression of activity on CRM level for both G6PD and ME in both sets of lines and for 6PGD in the chromosome 3, but not the chromosome 2, lines. The multiple regression of activity on CRM level and protein shows that, in each case, CRM level accounts for variation in activity that is not accounted for by variation in protein (*i.e.*, the partial regression coefficients are significant). The partial correlations between activity and CRM level with protein fixed are all quite high, except for 6PGD in the chromosome 2 lines.

The results of the analyses of variance of the CRM-adjusted activities are shown in Table 4. CRM-adjustment, like protein-adjustment, causes lines to lose significance for G6PD and ME in the chromosome 2 set. Since there was no significant regression of 6PGD activity on CRM for the chromosome 2 lines, CRM adjustment did not appreciably change the line component. Even though the CRM level and activity were strongly associated for all three enzymes in the chromosome 3 lines (especially ME), lines remained highly significant for all three CRM-adjusted activities. These results for the chromosome 3 lines indicate that even though variation in CRM level can account for a large part of the variation in activity level, there is a genetic component to the activity variation that is not explained by CRM level, possibly some type of structural variation. The results for the chromosome 2 lines are not as clear-cut, but they provide indications of activity variation not accounted for by CRM level: the significant week × line interactions for G6PD and 6PGD activity, which are not significant for the corresponding CRM levels, and the lack of significant regression of 6PGD activity on CRM level.

SECOND CHROMOSOME LINES

THIRD CHROMOSOME LINES

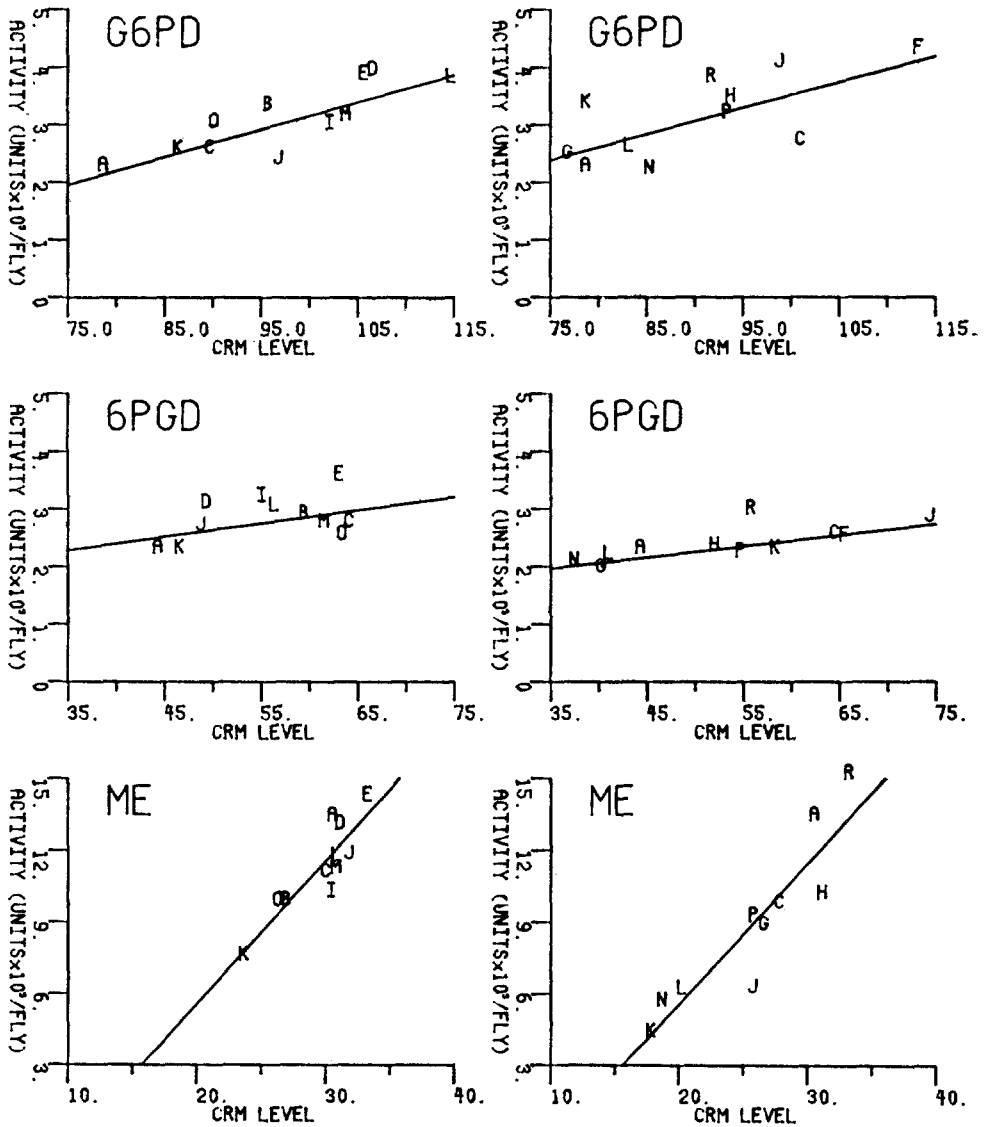


FIGURE 2.—Plots of enzyme activity *versus* CRM level for G6PD, 6PGD and ME with the corresponding regression lines. Each letter represents a line, as in Figure 1.

The correlations over line means between G6PD and 6PGD CRM levels are very similar to the correlations between the activities for the chromosome 3 lines: $r = 0.71$ for raw activities, $r = 0.73$ for raw CRM level, $r_{xy.p} = 0.68$ for the partial correlation between activities with protein fixed and $r_{xy.p} = 0.60$ for

TABLE 7

Linear regressions over line means of activity on CRM level and protein

	Activity on CRM level only†		Multiple regression of activity on CRM level and protein‡			
	$H_0: \beta=0$	R^2	CRM level $H_0: \beta_1=0$	Protein $H_0: \beta_2=0$	r_{AC-P}	R^2
<i>Chromosome 2</i>						
G6PD	**	0.69	*	ns	0.71*	0.69
6PGD	ns	0.19	ns	**	0.50	0.74
ME	***	0.78	*	ns	0.69*	0.78
<i>Chromosome 3</i>						
G6PD	*	0.49	*	ns	0.65*	0.50
6PGD	**	0.56	*	ns	0.67*	0.56
ME§	*	0.83	***	ns	0.91***	0.83

† Significance levels of the regression coefficient (β) and the coefficient of determination (R^2) are given.

‡ Significance levels of the partial regression coefficients (β_i), the partial correlation between activity and CRM level with protein fixed (r_{AC-P}) and the coefficient of multiple determination (R^2) are given.

§ The chromosome 3 ME null line is excluded.

ns, $p > 0.05$. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.

CRM levels with protein fixed. For the chromosome 2 lines, the correlations between CRM levels are less than between activities: $r = 0.64$ and $r_{xy,p} = 0.58$ for activities and $r = 0.31$ and $r_{xy,p} = 0.26$ for CRM levels.

One of the possible nonspecific causes of variation in enzyme activities is variation in the amount of a tissue type in which the enzyme is expressed. This situation could also explain parallel variation between activity and CRM levels, as well as correlations between functionally related enzymes. We therefore dissected adult males from two high (RIO9II and KA27III) and two low (Ho-R and RI22III) activity lines (based on Experiment I) into several body parts, which were then assayed for G6PD, 6PGD and general protein. The body parts are the abdominal wall (which contains most of the adult adipose tissue, as well as several other tissue types), the alimentary tract (midgut and hindgut), the head, the reproductive organs and the thorax (which consists mainly of flight muscle). The data for each high *vs.* low activity pair of lines were analyzed separately. Table 8 gives the difference between lines for each body part (with its standard error and a test of significance). The results of the *F*-tests of lines and the line \times body part interaction from the ANOVA of all body parts together are also given. These results show that the activity differences between lines are not simply due to variation in size or amount of a certain body part. For each pair of lines and for each enzyme, more than one body part shows a significant difference; whereas, none of the protein differences are significant.

Effect of PMSF on activity and electrophoretic mobility

HORI and TANDA (1980) recently reported a small change in the electrophoretic mobility of both A and B allozymes of G6PD that occurs during stor-

TABLE 8
Localization of activity differences to body parts of adult male†

Variable	Abdominal wall	Alimentary tract	Head	Reproductive organs	Thorax	Whole body	All body parts‡	
							Line	Line × Body part
G6PD	0.87 ± 0.17***	0.18 ± 0.07*	0.07 ± 0.03*	0.10 ± 0.03***	0.00 ± 0.04	1.55 ± 0.42**	*	***
6PGD	0.24 ± 0.09*	0.10 ± 0.02**	0.04 ± 0.02	0.02 ± 0.01	-0.01 ± 0.02	0.74 ± 0.17**	**	***
Prot	-1.6 ± 1.1	-0.1 ± 0.5	-0.4 ± 1.0	-1.8 ± 0.8	-3.4 ± 1.8	2.5 ± 3.9	ns	ns
<i>Difference between lines RI09II and Ho-R</i>								
G6PD	0.86 ± 0.20***	0.10 ± 0.08	-0.10 ± 0.04*	0.05 ± 0.02	0.03 ± 0.02	4.90 ± 3.17	**	***
6PGD	0.76 ± 0.12***	0.02 ± 0.03	-0.03 ± 0.03	0.01 ± 0.12	-0.04 ± 0.01**	4.68 ± 1.46*	ns	***
Prot	3.0 ± 2.0	-1.8 ± 0.8	-1.0 ± 1.1	-1.1 ± 2.1	-2.6 ± 1.2	1.0 ± 3.6	ns	**
<i>Difference between lines KA27III and Ho-R</i>								
G6PD	0.63 ± 0.18**	0.25 ± 0.07***	0.12 ± 0.03***	0.08 ± 0.02**	0.04 ± 0.03	4.53 ± 2.96	***	***
6PGD	0.44 ± 0.14**	0.10 ± 0.03**	0.07 ± 0.02**	0.01 ± 0.01	-0.01 ± 0.02	2.55 ± 1.46	***	***
Prot	2.0 ± 1.8	-0.6 ± 0.5	0.6 ± 1.2	-1.8 ± 1.9	-3.2 ± 1.8	1.1 ± 4.3	ns	ns
<i>Means over all 4 lines§</i>								
G6PD	1.23	0.29	0.35	0.23	0.20	2.67		
6PGD	0.79	0.19	0.21	0.13	0.10	2.41		
Prot	10.1	4.5	8.1	6.6	10.6	32.7		

† The mean difference ± s.e. is given with significance level indicated when $p < 0.05$. For G6PD, $N = 5$ for body parts and $N = 6$ for whole body; for 6PGD, $N = 6$ for body parts and whole body; for protein, $N = 6$ for body parts and $N = 18$ for whole body. Activities are units $\times 10^3$ /body part and protein is μg /body part.

‡ F-tests from ANOVAs of all 5 body parts for 2 lines.

ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

§ The body parts and whole-body assays were performed at different times and with somewhat different methods, so that the sum of body parts is not necessarily less than the whole body.

age of the crude extract at 4°. Each allozyme normally has one or two subbands that migrate slightly faster than the major band. After storage of crude extract at 4° for two days, HORI and TANDA found that virtually all of the activity shifted to the position of the subband. This change in mobility is apparently due to proteolytic activity in the crude extract since it is prevented by the addition of 0.4 mM PMSF, and it can be duplicated in a short time by the addition of trypsin.

HORI and TANDA's results suggested that proteolytic activity in our crude extracts might explain some of the activity variation. We duplicated their electrophoretic conditions and observed the same pattern of faintly staining subbands (Figure 3). Although the amount of activity present in the subbands varies somewhat (independent of the presence of 0.8 mM PMSF in the crude extract), there was no indication of consistent differences among lines (either chromosome 2 or 3) with respect to the distribution of activity among the major and subbands or with respect to the mobilities of the bands. We also investigated the effect of having PMSF in the extraction buffer on the activities of 5 lines—two typically low activity lines and three typically high activity lines. The experiment was set up with a factorial design, with two levels of each of three chemicals added to the extraction buffer: 0 or 0.4 mM PMSF, 0 or 1.0 mM DTT and 0 or 1.0 mM NADP⁺. In the analyses of variance of both G6PD and 6PGD, none of the three line \times chemical interactions were significant, and neither of the main effects for PMSF or DTT were significant, but NADP⁺ showed a small, significant enhancement of activity (about 2% for G6PD and 4% for 6PGD). We therefore have no evidence that proteolytic activity in the crude extract contributes to variation among lines, although a PMSF-insensitive protease could, of course, be involved.

DISCUSSION

The results presented here clearly demonstrate genetic variation in natural populations of *D. melanogaster* for autosomal factors that affect the activities of

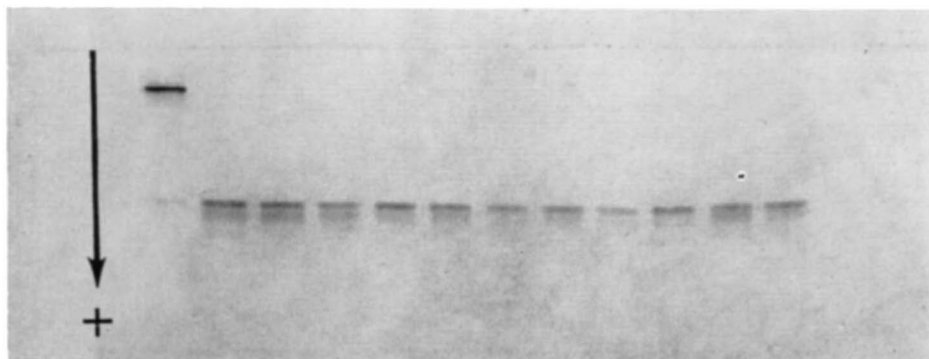


FIGURE 3.—Polyacrylamide gel stained for G6PD activity. Lane 1 (left) is a homozygous Zw^B (slow) line for comparison with the 11 chromosome 3 isogenic lines (Zw^A , fast) in lanes 2-12.

G6PD and 6PGD. The existence of such factors for G6PD has been suggested by others (STEEL, YOUNG and CHILDS 1969; RAWLS and LUCCHESI 1974; BIJLSMA 1980; HORI and TANDA 1981), but no systematic efforts were made to isolate and characterize particular variants, with one exception. BELOTE and LUCCHESI (1980) recently induced and characterized male-specific lethal mutants on chromosome 2 that affect the rate of X-chromosome transcription, as well as the activities of three X-linked enzymes (G6PD, 6PGD, FUM) in homozygous male larvae, but not in females. These mutants are therefore implicated in the process of dosage compensation. Although we did not measure activity levels in females, it is unlikely that the autosomal factors investigated in this study are involved in dosage compensation because the correlations between either G6PD or 6PGD and FUM are very small.

Because it is generally accepted that the structural genes that code for the subunits of G6PD and 6PGD are located on the X chromosome, the autosomal factors could be considered modifiers that are somehow involved in the regulation of gene expression. However, we must consider the evidence for each enzyme that there is just one structural gene located on the X chromosome that codes for its primary structure. The evidence, most of which has already been referenced in the introduction, is very similar for both enzymes: (1) The common, naturally occurring allozymes map to the X chromosome. (2) Several null and low activity alleles have been induced on the X chromosome, some of which also alter electrophoretic mobility. (3) There is dosage compensation between the sexes, but within each sex there is dependence on the dosage of the chromosomal region to which the allozyme variants have been localized. (4) The biochemical evidence is somewhat ambiguous: some workers report a single band after SDS electrophoresis of purified enzyme, indicating identical subunits; whereas, WILLIAMSON and coworkers report two bands for both G6PD and 6PGD (which could be due to proteolytic breakdown during purification or to an *in vivo* post-translational process). (5) GIESEL (1976) has speculated that *Zw* is not the structural locus for G6PD, but rather regulates the expression of two autosomal loci, one coding for the A and one for the B form. The basis for this suggestion is the apparent segregation of variants by both A and B forms, but the evidence provided is far from convincing, and no independent support has come from the many laboratories that have worked extensively with this enzyme. HORI and TANDA (1980) suggested that GIESEL's hypothesis is due to a misinterpretation of the zymogram since the number and relative mobility of GIESEL's "alleles" coincide with the proteolytic breakdown products they observe. In conclusion, there is certainly no compelling evidence on which to base a rejection of the hypothesis that *Zw* and *Pgd* are the sole structural genes for G6PD and 6PGD, respectively, but there definitely are plausible alternatives. Therefore, we cannot rule out the possibility that the autosomal factor(s) code for some part of the primary structure of the enzyme.

The chromosome 3 factors clearly have correlated effects on G6PD and 6PGD and the chromosome 2 factors seem to as well, but the evidence in the latter case is not as strong. Experiment II was designed primarily to investigate the specificity of this relationship. The selection of enzymes was made to include

one of the nonoxidative pentose shunt enzymes (TA); some related glycolytic enzymes (ALD, PGI); GPDH, which probably plays a role in lipid as well as in carbohydrate metabolism; the NADP-dependent enzymes ME and IDH, which, like G6PD and 6PGD, are believed to be important in providing NADPH for lipid synthesis and which are co-induced or co-repressed with G6PD and 6PGD by dietary factors (see GEER *et al.* 1976); as well as a few other enzymes with no obvious or intimate metabolic connection with the pentose shunt (ADH, AK, AOX, FUM). The results show that G6PD and 6PGD are not the only pair of enzymes showing a high positive correlation, but it is among the highest in both sets of lines. Moreover, many of the other high correlations are also between enzymes with closely related functions. For example, the TA, G6PD correlation is high in both sets of lines (0.60 in chromosome 2 and 0.78 in chromosome 3 lines), as is the TA, PGI correlation in the chromosome 3 lines (0.75). It is possible that the chromosome 3 factor(s) with correlated effect(s) on G6PD and 6PGD also affect TA and PGI. It is certainly conceivable that the enzymes in intimately interconnected pathways, such as glycolysis and the pentose shunt, are regulated in such a way as to maintain some constancy in relative amounts, and the genetic factors we have identified may be involved in such a process. This is only a speculation at present, but we are currently investigating it further by attempting to map the activity factors for all four enzymes (PGI, TA, G6PD, 6PGD). Whether or not they all map to the same location will be very informative with respect to this hypothesis.

A large part of the activity variation among chromosome 3 lines for G6PD, 6PGD and ME is accounted for by variation in CRM level, but there is a significant fraction of the genetic component of activity variation that is not explained by CRM level. This result suggests the existence of structural variability that influences catalytic efficiency, but it is also possible that our CRM level measurements are not sufficiently accurate to make the proper CRM adjustment of activity. Because the structural gene for ME is located on chromosome 3, some variation in enzyme structure is not unexpected. In the case of G6PD and 6PGD, some type of post-translational modification, such as that reported for XDH (FINNERTY and JOHNSON 1979), could be involved. An intensive search for direct evidence of structural variation of G6PD and 6PGD among these lines is in progress.

Two small experiments reported here argue against nonspecific causes of the correlated genetic effects on G6PD and 6PGD activities. The dissection of high and low activity lines indicate that the activity differences are not simply due to a gross change in the amount of one particular tissue, although more work of this type is clearly needed to characterize the extent of systemic *versus* tissue-specific effects. The lack of an effect of the protease inhibitor, PMSF, on activity or electrophoretic mobility indicates that proteolytic breakdown in crude extracts is not responsible for activity variation among the lines.

A basic requirement of any model of regulation of eukaryotic gene expression is some mechanism to account for the coordinate control of functionally related enzymes. Therefore, the detection of variants with correlated effects on en-

zymes of the same or related pathways is potentially very useful for investigating the mechanisms of regulation, as well as the importance of regulatory variation in evolutionary change. We conclude that continued characterization of the autosomal factors reported here is likely to provide unusual opportunities to investigate these problems.

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