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DOSAGE COMPENSATION IN DROSOPHILA: NADP-ENZYME ACTIVITIES AND CROSS-REACTING MATERIAL

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ABSTRACT

The relationships between gene dosage, enzyme activities and CRM levels have been determined for G6PD and 6PGD. Enzyme activities and CRM levels were directly proportional and increased in genotypes carrying duplications of the respective structural genes. When a duplication consisting of the distal 45% of the X chromosome was used to duplicate Pgd^+, 6PGD activity and CRM increased and G6PD activity decreased. When the proximal 55% of the X chromosome was duplicated, G6PD activity and CRM increased whereas 6PGD activity and CRM levels decreased. These observations support the model of dosage compensation of X-linked genes that invokes an autosomal activator in limited concentrations for which X-linked loci compete. The distal 45% of the X chromosome, when duplicated, caused a significant increase in NADP-malic enzyme activity and CRM levels, as if a structural gene for NADP-ME is sex-linked.

IN Drosophila and in mammals the phenomenon of dosage compensation ensures that levels of expression of sex-linked genes are equal in males having one X chromosome and females with two X chromosomes. In mammals dosage compensation is achieved by inactivation of one X chromosome in somatic cells of females (LYON 1972). No such mechanism of X chromosome inactivation occurs in Drosophila; instead, both X chromosomes are expressed in somatic cells of females (KAZAZIAN, YOUNG and CHILDS 1965). However, the activities of sex-linked genes are modulated such that they are equal in the two sexes. A variety of experimental approaches has led to the conclusion that in Drosophila dosage compensation of sex-linked genes is exerted at the level of transcription. The extensive evidence supporting this conclusion has been reviewed by LUCCHESI (1974; 1977).

The original description of dosage compensation was that of MULLER, LEAGUE and OFFERMANN (1931) using the apricot eye color system in Drosophila. The white-apricot (w^a, 1–1.5) allele is hypomorphic (i.e., leaky), allowing small amounts of pigment to be deposited in the adult eye. Males having one copy of this sex-linked allele have levels of pigment not different from the levels in females with two copies of the w^a allele. Thus one copy of the w^a allele in males causes deposition of as much pigment as do two copies of the w^a allele in

females. Eye pigmentation is, of course, a terminal phenotype and may in some way be unique, rendering these considerations of compensation suspect. To test the hypothesis of dosage compensation at a level more closely related to primary gene action, SEECOF, KAPLAN and FUTCH (1969) determined levels of enzymatic activity for glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in males and females with different numbers of copies of the structural genes coding for these enzymes (Pgd^+ for 6PGD, 1–0.64; Zw^+ for G6PD 1–63.) Normal (two-dose) females had the same levels of activity for these two enzymes as normal (one-dose) males. In aneuploid genotypes activities of both enzymes varied directly with the number of copies of their respective structural gene.

In an attempt to probe more closely the primary gene action of sex-linked loci, MUKHERJEE and BEERMANN (1965), MARONI and PLAUT (1973), and MARONI, KAPLAN and PLAUT (1974) employed autoradiographic techniques to study dosage compensation in Drosophila. They observed that rates of incorporation of ^{3}H-uridine into RNA by polytenic X chromosomes were equal in the two sexes. Thus dosage compensation in Drosophila has been observed at the level of eye color, enzyme activity and transcription.

Rates of X chromosome transcriptional activity intermediate to the rates of normal males and females have recently been demonstrated by MARONI and LUCCHESI (1980). These altered rates occur in individuals possessing a complete X chromosome plus relatively large fragments of X chromosomes. From these observations, one would predict that in individuals with two and one-half X chromosomes gene activities should vary by 20% in either direction depending on whether a gene was in the duplicated region or in the nonduplicated region of the X chromosome. In this paper we confirm this prediction and extend the observations of gene dosage effects to the level of specific proteins.

MATERIALS AND METHODS

Experimental animals: *C (1) RM, y v bb/0* females were collected within 12 hr of eclosion and aged 2 to 4 days on yeasted culture medium before mating with males carrying translocations involving the X and Y chromosomes (STEWART 1973; STEWART and MERRIAM 1974; 1975; Figures 1 and 2). One component of each *T(X;Y)* carried the dominant allele for body color (y^+) whereas the other component carried the dominant marker Bar-Stone (B^S). Complete descriptions of these genetic markers can be found in LINDSLEY and GRELL (1968). Crosses were made on cornmeal-yeast-sugar medium (LEWIS 1960), six to ten pairs per quarter-pint (120-ml) bottle, and incubated at 25° and 40% relative humidity. After 4 days the parents were transferred to fresh culture bottles for a second 4-day brood.

Progeny females less than 24 hr of age were homogenized in Tris-acetate buffer containing NADP (0.1 M Tris, 0.25 M acetate, 0.5 mM 2-mercaptoethanol, 0.1 mM NADP, pH 8.6). The homogenates were centrifuged at 8000 × *g* for 10 min and the resultant supernatant was used for activity and protein determinations and for rocket immunoelectrophoresis.

Enzyme assays: G6PD activity was measured using an assay solution containing 0.1 M Tris-HCl, pH 8.5, 1.2 mM glucose-6-phosphate, 0.17 mM $NADP^+$, 0.01 mM $MgSO_4$. 6PGD assay buffer consisted of 0.1 M Tris-HCl, pH 8.2, 1.4 mM 6-phosphogluconate, 0.14 mM $NADP^+$, and 0.008 mM $MgSO_4$. NADP-malic enzyme (NADP-ME) assay buffer consisted of 0.1 M Tris-HCl, pH 7.9, 10 mM L-malate, 0.34 mM $NADP^+$, 5 mM $MnCl_2$. All activities were determined by monitoring the reduction of $NADP^+$, at 340 nm and 30° and were converted to a micromolar concentration of $NADP^+$ reduced per min per mg of protein.

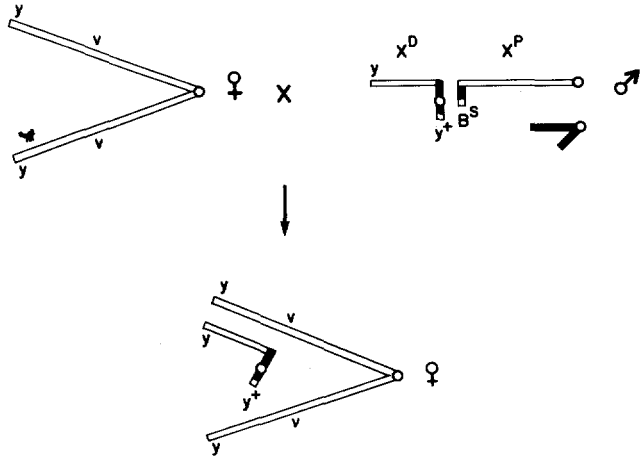

FIGURE 1.—Diagram of crosses used to generate females carrying duplications of the X chromosome. Open bars represent X chromosomes or segments of X chromosomes, solid bars represent Y chromosomes or segments of Y chromosomes and open circles represent centromeres. The genetic symbols are explained in the text.

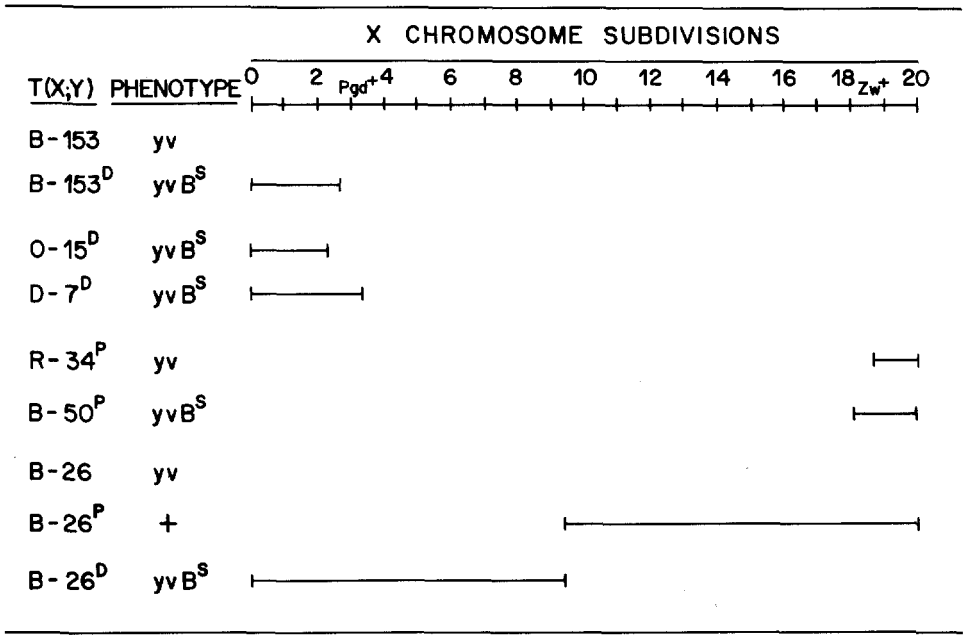

FIGURE 2.—Relative sizes of duplicated regions of the X chromosome in the nine experimental genotypes. The X chromosome is represented by numbered subdivisions of its polytene chromosome map. The loci of the structural genes Pgd^+ (6PGD) and Zw^+ (G6PD) are indicated. Pgd^+ is located in region 2D3-5 Zw^+ is located in region 18D. Thus $D\text{-}7^D$ and $B\text{-}26^D$ carry the Pgd^+ locus while $B\text{-}50^P$ and $B\text{-}26^P$ carry Zw^+. Females carrying one of these distal fragments carry three copies of Pgd^+ whereas females carrying one of these proximal fragments carry three copies of Zw^+.

Rocket immunoelectrophoresis: Levels of specific immunologically cross-reacting material (CRM) were determined by one-dimensional immunoelectrophoresis (LAURELL 1966; MEIDINGER and WILLIAMSON 1978). Five-microliter samples of crude homogenates were placed in 2.5-mm wells in agarose gels (10 × 20 cm) on glass plates. Gels contained 1% agarose in 0.088 M Tris, 0.25 M acetate, 2.8 mM EDTA, 0.1 mM NADP, pH 8.6, and specific antiserum (120 μl of anti-G6PD, 75 μl of anti-6PGD, or 40 μl of anti-NADP-ME in 30 ml of gel solution). All samples were run at 100 volts for 21 hr at 4°–7°. G6PD and 6PGD gels were stained to develop specific enzyme activity (rockets) in the appropriate enzyme assay buffer containing 0.35 mM $NADP^+$, 0.6 mM phenazine methosulfate (PMS), 2.4 mM Nitro Blue tetrazolium (NBT) and either 1.4 mM glucose-6-phosphate or 1.5 mM 6-phosphogluconate at 25°. NADP-ME gels were stained in 0.1 M Tris-HCl buffer containing similar concentrations of NADP, PMS and NBT, 10 mM $MgCl_2$ and 1.5 mM L-malate.

Levels of specific enzyme-CRM were determined by comparing rocket heights and protein concentrations to a series of dilutions of homogenates of the standard genotype [*C(1)RM, y v bb/Y*]. Eight wells on each plate were used to generate a standard curve of rocket height per milligram of protein for that gel.

Protein determinations: Protein concentrations were determined by using the dye-binding technique described by BRADFORD (1976) using bovine serum albumin as the standard.

Statistical methods: All statistical comparisons employ nonparametric methods described in SOKAL and ROHLF (1969) and statistical tables from ROHLF and SOKAL (1969). The KRUSKAL-WALLIS test was used to detect heterogeneity among genotypes when compared for CRM or enzymatic activity. WILCOXON'S signed-ranks test was used to compare each genotype to the *C(1)RM, y v bb/Y* female controls or to other appropriate controls, as indicated. Comparisons of pairs of genotypes employed WILCOXON'S two-sample test.

RESULTS

The objectives of our experiments were 1) to determine if levels of enzyme activity and levels of specific CRM vary coordinately in genotypes carrying duplications of the structural genes for G6PD (Zw^+) or 6PGD (Pgd^+) and 2) to determine if duplications of approximately half the X chromosome carrying one of these genes were associated with a concomitant decrease in enzyme activity and CRM levels determined by the other gene.

Using a series of X-Y translocations generated by BARBARA R. STEWART (1973) we produced *C(1)RM, y v bb* females carrying either distal or proximal fragments of the X chromosome (Figures 1 and 2). The distal fragment of *T(X;Y) B-153* segregates more or less randomly in the male parent and yields siblings some of which carry the X chromosome fragment and some that do not. Thus the *C(1)RM, y v bb/Y(B-153)* females (no fragment) were used as the standard against which all other genotypes were compared. Crosses involving *T(X;Y) B-26* generate three classes of females—those without a fragment *C(1)RM, y v bb/Y(B-26)* those carrying the proximal 55% of the X chromosome *C(1)RM, y v bb/* $B^SY(B\text{-}26^P)$ and those carrying the distal 45% of the X chromosome *C(1)RM, y v* bb/Yy^+ $(B\text{-}26^D)$. In detailed comparisons of these genotypes the values determined for *C(1)RM, y v bb/Y (B-26)* females were used as the standard against which $B\text{-}26^P$ and $B\text{-}26^D$ females were compared.

Although we have successfully used the technique of quantitative immunoelectrophoresis to detect as little as 20 ng of NADP-ME (GEER, KROCHKO and WILLIAMSON 1979), we were initially concerned that we would not be able to use this technique to measure levels of 6PGD in various genotypes. Our concern was generated by the report of KOGAN, ROZOVSKII and GVOZDEV (1977) that the 6PGD antigen-antibody complex retains no enzymatic activity in both kinetic

assays and in gels. Fortunately, we were able to circumvent this problem by the addition of NADP to grinding, gel and running buffers and by leaving the gel in the staining solution for up to 2 hr at 25°. Although there was no comparable problem with G6PD and NADP-ME activities in gels, we routinely added NADP to all buffers. For each of the three enzymes tested we observed a direct linear relationship between specific activities and CRM levels (Figure 3).

G6PD activity was significantly increased in *C(1)RM, y v bb/B-50^P* ($P < 0.01$) and *C(1)RM, y v bb/B-26^D* females ($P < 0.05$, Figure 4). In both genotypes levels of G6PD-CRM were also significantly increased ($P < 0.01$). In all other genotypes, except *C(1)RM, y v bb/B-26^D*, G6PD activities and CRM levels were not different from the control levels. In *C(1)RM, y v bb/B-26^D* females G6PD activity was 82% that of the control ($P < 0.01$).

Levels of 6PGD activity and CRM were elevated in *C(1)RM, y v bb/D-7^D* and *C(1)RM y v bb/B-26^D* females ($P < 0.01$; Figure 5). All other genotypes had levels of activity and CRM comparable to those of the control, except *C(1)RM, y v bb/B-26^P*. In this genotype 6PGD activity was 58% that of the control value ($P < 0.01$) whereas CRM levels were 75% of the control valued ($P < 0.01$). *C(1)RM, y v bb/B-50^P* females also had reduced levels of 6PGD-CRM (90%; $P < 0.01$).

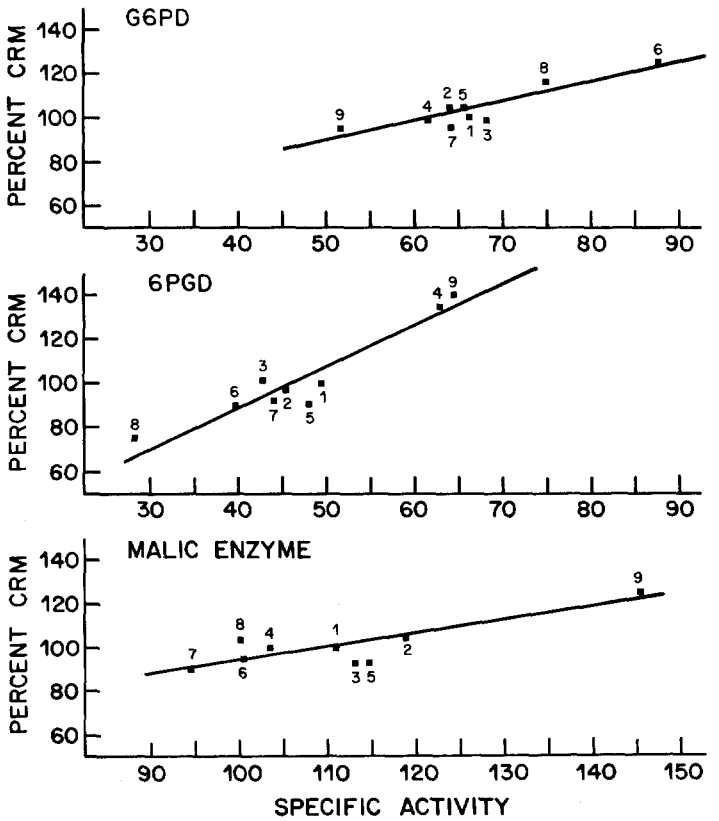

FIGURE 3.—Correlations of enzyme activities and CRM levels. Numbers refer to genotypes listed in Figure 2, from top to bottom.

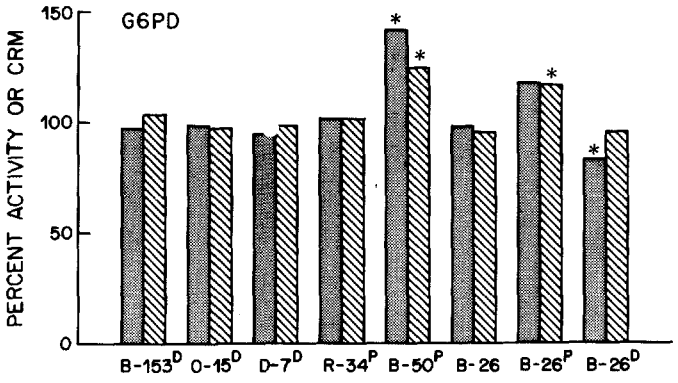

FIGURE 4.—G6PD activity (stippled bars) and CRM (cross-hatched bars) relative to control values. Values marked with * are significantly different from the control values.

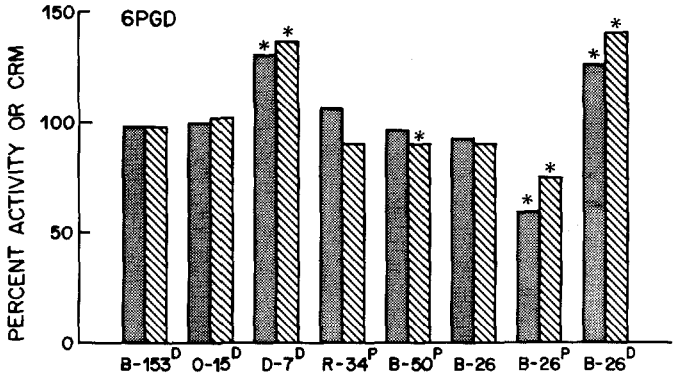

FIGURE 5.—6PGD activity (stippled bars) and CRM (cross-hatched bars) relative to control values. Values marked with * are significantly different from the control values.

NADP-ME activity and CRM levels were measured as an autosomally controlled, NADP-dependent enzyme and thus were expected to be comparable in all genotypes. This expectation held in every genotype expect *C(1)RM, y v bb/ B-26^D* (Figure 6). These females had elevated levels of NADP-ME activity and CRM ($P < 0.01$) suggesting that the X chromosome contains a region between *3C (D-7)* and *9C (B-26)* that, when duplicated, behaves as a structural gene for NADP-ME. This is somewhat surprising since *Men*, a gene on the third chromosome, is considered to be the structural gene for NADP-ME (VOELKER *et al.* 1981; WILLIAMSON 1982). Recent tests have further localized this increase in NADP-ME activity to region 8D10-12—9A1-2 of the X chromosome (M. M. BENTLEY and J. H. WILLIAMSON, unpublished results).

DISCUSSION

Two models of gene action have been proposed to explain dosage compensation of sex-linked genes in Drosophila. A "negative-control" model, first

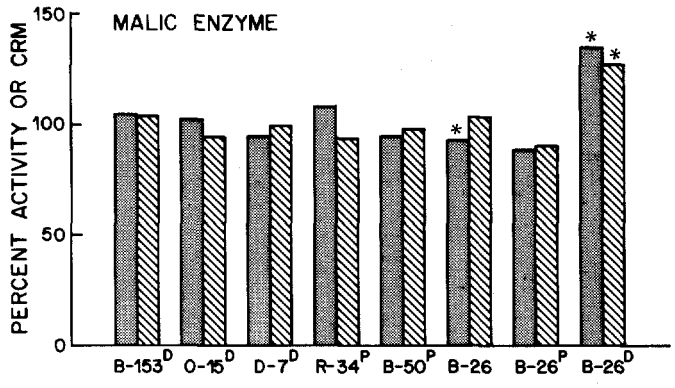

FIGURE 6.—NADP-ME activity (stippled bars) and CRM (cross-hatched bars) relative to control values. Values marked with * are significantly different from the control values.

proposed by MULLER (1950), invoked "compensator" genes, themselves sex-linked but noncompensated, that act to depress the activities of X-linked structural genes in females. Conversely, a model of "positive control" holds that an autosomally-coded factor, in limited supply and for which X chromosomes compete, activates X-linked loci (MARONI and PLAUT 1973). In males these loci interact with twice as many units of the autosomal factor as do individual X-linked loci in females thereby equalizing gene activity in the two sexes.

The technique of segmental aneuploidy was used by STEWART and MERRIAM (1975) to test the model of X-linked compensators; no evidence for such genetic control was observed. Negative evidence does not, of course, disprove the existence of X-linked compensators but does suggest that compensatory genes with major effects on activity of X-linked genes are improbable. The hypothesis of "positive control" by limited autosomal products was tested by MARONI and LUCCHESI (1980) employing X chromosome duplications of increasing lengths and techniques of autoradiography. They concluded that the sum of all transcription occurring on the X chromosome elements is constant in all of the genotypes tested. Furthermore, they concluded that within a genotype the rate of transcription was uniform over all individual X chromsomal elements. These observations are consistent with the model of a limited autosomal activator for which all segments of X chromosomes compete equally.

The model of autosomally-mediated dosage compensation of sex-linked loci predicts that duplication of half an X chromosome should lead to increased activity of genes within the duplicated region of the X chromosome (STEWART and MERRIAM 1975). The magnitude of increased activity should be approximately 20%. Conversely, activities of genes in the unduplicated region of the X chromosome would decrease by approximately 20%. Our observations on enzyme activity and CRM levels, derived from progeny of crosses of *C(1)RM, y v bb/O* females and *T(X; Y) B-26* males, are consistent with these predictions (Figure 7). Duplication of the proximal 55% of the X chromosome ($B\text{-}26^P$) containing Zw^+, the structural gene for G6PD, increased G6PD activity and CRM by 22% and 23%, respectively. In this same genotype, 6PGD activity was

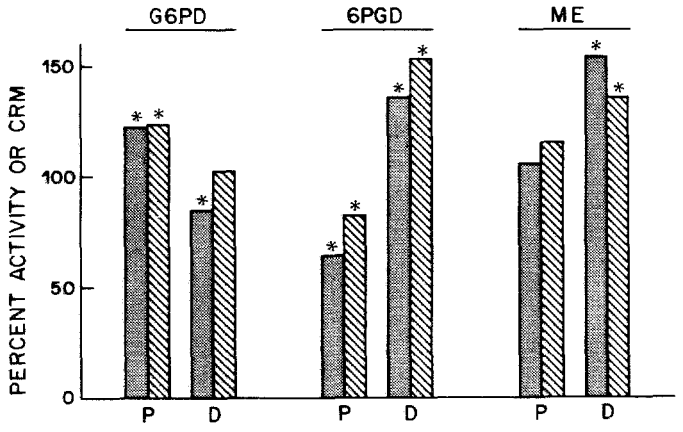

FIGURE 7.—G6PD, 6PGD and NADP-ME activities (stippled bars) and CRM levels (cross-hatched bars) in females carrying a duplication of the proximal 55% (P), or the distal 45% (D) of the X chromosome compared with their *C(1)RM, y v/Y* sisters. Values marked with * are significantly different from the control values.

reduced by 16% although 6GPD-CRM levels were not different from control levels. When the distal 45% of the X chromosome, containing Pgd^+, the structural gene for 6PGD, was duplicated, 6PGD activity and CRM were increased 36% and 54%, respectively. In the same genotype G6PD activity and CRM were reduced 35% and 18%, respectively. These observations must not be consequences of duplication of the structural genes, *per se*. This conclusion is derived from comparisons of G6PD and 6PGD activities in females carrying smaller duplications including Pgd^+ ($D\text{-}7^d$) or Zw^+ ($B\text{-}50^P$). In the first genotype 6PGD activity and CRM are increased 30% and 36%, respectively, whereas G6PD activity and CRM were not different from the control levels. Similarly, in *C(1)RM, y v/B-50*^{P} females G6PD activity and CRM were increased (41% and 24%, respectively) whereas 6PGD activity and CRM were not different from the control values.

The competition model of dosage compensation holds that an autosomally regulated factor, in limited quantities and for which X chromosomes compete, activates X-linked genes. Since females have two X chromsomes and males have only one X chromosome, and since quantities of the autosomal compensator are equal in the two sexes, a sex-linked gene in females is associated with only half as many compensator molecules as is the same gene in males. Therefore an X-linked gene in males functions at two times the rate as the same gene in females. On these assumptions and the additional assumption that regions of X chromosomes compete equally for the autosomal compensator, in females carrying a duplication for half an X chromosome (2.5 X's), genes in the nonduplicated regions would be associated with only 80% as much autosomal compensator as in normal females. Thus these genes should be associated with 80% of their normal activity. Table 1 summarizes the observed and expected 6PGD and G6PD activities in females carrying the X chromosome duplications used in these studies. The predicted values were based on proportionate

TABLE 1

A comparison of observed activities and CRM levels to levels predicted by the competition model of dosage compensation of X-linked genes

		6PGD			G6PD		
			Observed			Observed	
Translocation	Phenotype	Predicted	Activity	CRM	Predicted	Activity	CRM
B-153	y v	100	100	100	100	100	100
$B\text{-}153^{D}$	y v B^{s}	93.5	97	97	93.5	97	104
$O\text{-}15^{D}$	y v B^{S}	94.3	91	101	94.3	102	98
$D\text{-}7^{D}$	y v B^{S}	138.3	130	136	92.2	94	99
$B\text{-}34^{P}$	y v	96.6	106	90	96.5	101	101
$B\text{-}50^{P}$	y v B^{S}	95.2	96	90	142.8	141	125
B-26	y v	100	88	95	100	97	91
$B\text{-}26^{P}$	+	78.4	58	75	117.6	118	117
$B\text{-}26^{D}$	y v B^{S}	122.4	125	140	81.6	82	96

amounts of X-chromosomal material in each genotype. The correlations of observed activities and CRM levels were quite high (6PGD activity—0.91; 6PGD CRM—0.94; G6PD activity—0.98; G6PD CRM—0.88).

In an experiment similar to those described in this paper, STEWART and MERRIAM (1975) detected comparable differences in activities of G6PD and 6PGD in females hyperploid for large regions of the X chromosome. Because the differences were not statistically significant, these authors favored an alternative explanation over the competition model to account for dosage compensation.

NADP-ME activities and CRM levels were monitored in all comparisons as an autosomally coded, NADP-dependent enzyme. The rationale of our experimental design was that NADP-ME activity and CRM would be the same in all genotypes and thus would serve as an internal control for our experimental procedures. These expectations were, indeed, realized in that NADP-ME activities and CRM levels varied proportionately ($r = 0.98$; Figure 3). The unique observation with this enzyme was the increased activity and CRM levels observed in $C(1)RM$, y v $bb/B\text{-}26^{D}$ females (Figure 6). The increased levels of NADP-ME activity and CRM are assignable to region 8D10-12–9A1-2 of the X chromosome (M. M. BENTLEY and J. H. WILLIAMSON, unpublished results).

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