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GENETICS OF XENOBIOTIC METABOLISM IN *DROSOPHILA*

I. GENETIC AND ENVIRONMENTAL FACTORS AFFECTING GLUTATHIONE-*S*-TRANSFERASE IN LARVAE

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Abstract—The enzyme glutathione-*S*-transferase, which plays a crucial role in xenobiotic detoxification, was investigated in *Drosophila melanogaster*. Based upon examination of substrate specificities and pH optima, it was observed that the enzyme in *Drosophila* is considerably more restricted in its activities than in mammals. The effects of various xenobiotics on activities in third instar larvae were examined. While β-naphthoflavone and phenobarbital had no effect, pentamethyl benzene (PMB) administration resulted in a 50% increase in enzyme activity. Comparison of lines of known genetic composition indicates that the degree of response to PMB is modulated by genes on chromosome II, and that differences exist with respect to the patterns of response of activities towards the substrates 1-chloro-2,4-dinitrobenzene and ethacrynic acid. Results obtained suggest the existence of at least two loci on chromosome II that code for glutathione *S*-transferase isozymes.

Glutathione-*S*-transferases play a critical role in the Phase II conjugation of electrophilic intermediates [1], and are thus particularly important in the detoxification of alkylating compounds [2]. The enzyme has been most thoroughly studied in rat liver, in which at least six distinct cytosolic isozymes and one microsomal species have been purified and characterized [2–4]. While all of these isozymes demonstrate activity towards common substrates, each one can be distinguished on the basis of its relative activities towards different substrates. Thus, in this species, there exists a complex suite of isozymes, suggesting the evolution of a system designed to allow for the effective metabolism of a wide spectrum of substrates. Comparable complexity of isozymes has been reported in human [4, 5] and mouse preparations [6].

Glutathione-*S*-transferase activity is extremely high in insects and has been studied largely with respect to its role in the conferral of pesticide resistance [7–10]. In different species, glutathione-*S*-transferase activity is inducible by phenobarbital [11–13], as well as by various plant secondary products [14, 15]. Biochemical investigation of this enzyme in insects has been limited; however, most reports suggest the existence of no more than three isozymes [16–19]. Thus, the question remains as to whether glutathione-*S*-transferases in insects are as heterogenous as are those in mammals.

An approach that has not been exploited extensively with respect to this enzyme is the examination of the genetic control of its expression, largely due to the fact that the potential for genetic manipulation of studied organisms is limited. *Drosophila melanogaster* does not suffer from this limitation and has been demonstrated to possess considerable glutathione-*S*-transferase activity [18]. A recent report provides evidence for the existence of at least three distinct forms of the enzyme [19]. Furthemore, this organism is widely employed in genetic toxicity assays; thus, an understanding of the metabolic capabilities of the species is of critical importance in assessing its applicability as a model system. We therefore undertook to examine this enzyme in this species in a variety of manners. First, its activities towards several substrates were measured, in order to be able to compare the *Drosophila* enzyme to those of mammals. Second, lines of known genetic constitution were surveyed with respect to their inducibility by particular xenobiotics. The results obtained could then be interpreted both in terms of the control of this enzyme in *Drosophila* itself, as well as within the context of the question of the suitability of this species as a model for the study of detoxification mechanisms in higher organisms.

MATERIALS AND METHODS

Chemicals. Reduced glutathione, ethacrynic acid (EA), and Tween 80 were obtained from Sigma. 1-Chloro-2,4-dinitrobenzene (CDNB), 3,4-dichloronitrobenzene (DCNB), 1,2-epoxy-3-(*p*-nitrophenoxy)propane, *trans*-4-phenyl-3-butene-2-one, β-naphthoflavone (BNF), and pentamethyl benzene (PMB) were obtained from Aldrich.

Drosophila *stocks.* In initial characterization of glutathione-*S*-transferase, a heterogenous wild type stock, designated BMC, was employed. This stock was originally established from a collection of wild flies in Bloomington, IN, in 1976, and has been maintained in large population cultured since that

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time [20]. The isogenic lines utilized were constructed by Laurie-Ahlberg *et al.* [21], and are described therein. Briefly, each line is completely homozygous, but differs from every other one in the origin of one of the two major autosomes. Thus, any phenotypic difference that is evident between lines that differ only in the origin of one chromosome can be ascribed to the action of genes located on that particular chromosome.

Extract preparation and enzyme assays. Soluble extracts were prepared as follows. Ten to twenty larvae were homogenized in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, 1 mM EDTA, and 0.1 mM phenyl thiourea, using a motorized Potter–Elvehjem homogenizer. These preparations were then centrifuged at 12,800 *g* for 15 min, and the supernatant fraction was employed for enzyme assay. Microsomal pellets were obtained by centrifugation of this supernatant fraction at 105,000 *g* for 60 min.

Glutathione-*S*-transferase activity was assayed essentially as described by Habig *et al.* [2], with the following modifications. The pH of the reaction, as well as the concentration of the substrate, was adjusted to maximize activity while minimizing the degree of nonenzymatic conjugation (see Results). Assays were performed in semimicrocuvettes in an LKB model 4070 spectrophotometer, with the total cuvette volume being 0.4 ml. For every combination of substrate and pH, change in absorbance was monitored in the absence of enzyme, to allow correction of the data for nonenzymatic conjugation. Assays were performed for 2 min at 27°. Table 1 presents the substrate concentration and pH employed for standard assays with the different substrates. Protein concentrations were determined by the method of Bradford [22] with bovine serum albumin as standard.

Ammonium sulfate fractionation. Approximately 1 g of third instar larvae was collected and homogenized as described. Solid ammonium sulfate was added slowly, with stirring, to successive concentrations of 20, 40, 60, 75, and 90%. After each addition, precipitated proteins were collected by centrifugation at 10,000 *g*, resuspended in homogenization buffer, and assayed for glutathione-*S*-transferase activity as described.

Treatment of larvae. Administration of xenobiotics to third instar larvae was performed by a modification of the method of Hallstrom *et al.* [23]. Two- to four-day-old adults were allowed to oviposit on the surface of Brewer's yeast medium in 15 × 100 mm petri plates for 6–12 hr. These plates were then incubated at 25° for 60 hr, at which time they were warmed gently on a hot plate, until the young third instar larvae emerged on the surface of the medium. These larvae were then collected and transferred to half-pint milk bottles containing 2 ml of supplemented Penassay broth (see below) absorbed onto a piece of Whatman No. 1 filter paper.

Pentamethyl benzyne, β-naphthoflavone, and phenobarbital were administered in concentrations of 0.5%, 0.1% and 1.0% (w/v) respectively. Appropriate amounts of each compound were initially dissolved in 4 ml of Tween 80, after which Penassay broth [24] was added to a total volume of 25 ml. Two milliliters of this suspension was then placed in each bottle used for larval treatment. Control larvae were placed in bottles containing Tween 80 and Penassay broth alone. Larvae were incubated under these conditions for 48 hr at 18°, at which time they were prepared for enzyme assay as described. Under these conditions, there was no detectable larval mortality.

Table 1. Activities of glutathione-*S*-transferase in *Drosophila* towards various substrates

Substrate	Concn (mM)	Glutathione concn (mM)	pH	N*	Sp. Act.†
CDNB	1.0	1.0	7.1	4	1203 ± 16
EA	0.2	0.25	7.1	3	24.8 ± 3.2
DCNB	1.0	1.0	7.5	3	9.9 ± 2.1

* Number of preparations assayed.

† Mean ± S.E. Activities are in nmoles conjugate formed per min per mg protein at 27°.

RESULTS

It has been reported previously that high levels of enzymatic conjugation of CDNB to glutathione were demonstrated in preparations of *Drosophila* adults [18, 19]. Our first concern was to extend these observations to larvae, since this is a stage of active feeding and metabolism [25], and to determine the range of substrates upon which this enzyme could act. Accordingly, preparations of BMC larvae were assayed, using the substrates and conditions indicated in Table 1. The results presented indicate that, of the five substrates examined, only three (CDNB, EA, and DCNB) underwent measurable conjugation. Of these substrates, CDNB was clearly the most effective, in that the specific activity of the enzyme for it was over 100-fold higher than that for

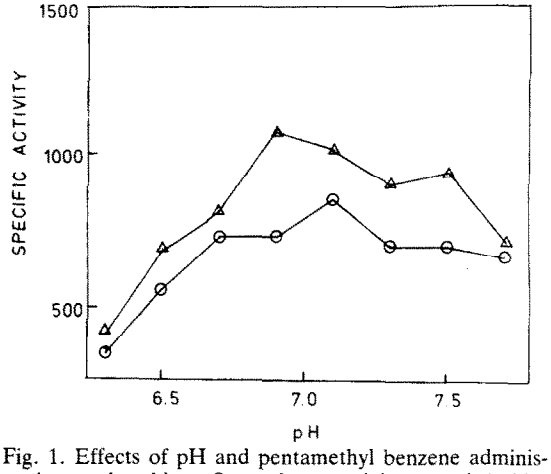

Fig. 1. Effects of pH and pentamethyl benzene administration on glutathione-*S*-transferase activity towards 1-chloro-2,4-dinitrobenzene. Key: (○—○) activity of third instar larvae placed on control medium; and (△—△) activity in preparations of larvae exposed to 0.5% pentamethyl benzene for 24 hr prior to assay. Each point represents the mean activity of four separate preparations. Specific activity is expressed as nmoles CDNB conjugated per min per mg protein at 27°.

DCNB. We then determined pH optima for each of these activities. Results, presented in Fig. 1, indicate that activity towards CDNB showed a pH optimum of about 7.1 to 7.5. At pH levels above this range, it was impossible to reliably assay activity towards CDNB, since the rate of nonenzymatic conjugation became excessively high. Similar examination of activity towards EA and DCNB gave similar results (data not shown).

The data reported thus far provided no direct evidence with respect to the question of the heterogeneity of this enzyme in *D. melanogaster*, although the range of substrates amenable to conjugation appears to be more limited than that of mammalian liver. A second source of adaptive flexibility in gene-enzyme systems is in their inducibility, as has been amply demonstrated in *Drosophila* [26–28]. Accordingly, we examined the inducibility of glutathione-*S*-transferases by likely xenobiotics. Larvae were treated with phenobarbital, BNF, and PMB, and the extracts obtained were assayed for activity towards CDNB. Neither phenobarbital nor BNF had any significant effect on enzyme activities at any pH (data not shown). Results from larvae treated with PMB are shown in Fig. 1. It is evident that, over the range of assay pH levels examined, dietary administration of this compound resulted in a significant increase in the specific activity of glutathione-*S*-transferase.

The existence of this response, combined with the availability of flies of well-defined genetic composition, allowed the further examination of the genetic basis of this response, especially, with regard to variation in response to PMB and coordinate control of different conjugation activities. Larvae from six isogenic lines, three with unique second chromosomes and three with unique third chromosomes, were treated as described and assayed for activity towards CDNB and EA at pH 7.1 (measurements of activity towards DCNB provided to be too variable for reliable analysis). Results of this experiment are illustrated in Fig. 2. Upon qualitative examination, it appeared, especially with respect to CDNB conjugation, that there were, in fact, strain-specific differences in control activities, as well as in the degree of response to PMB, among these lines. The differences are most notable among lines isogenic for different second chromosomes (Fig. 2A). This impression was confirmed by two-way, fixed-factor analyses of variance of the two activities [29]. The significant results can be summarized as follows:

(1) With respect to CDNB conjugation, among

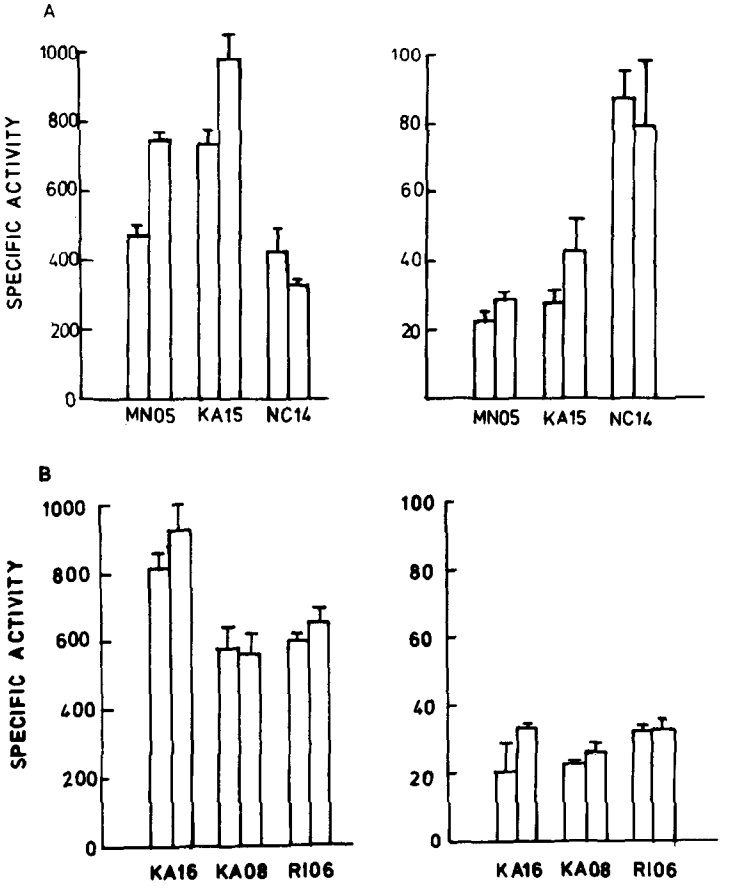

Fig. 2. Glutathione-*S*-transferase activities observed in larvae of isogenic lines. In each pair of bars, the left membrane represents activity of larvae reared on a control diet, while the right indicates activity of larvae exposed to 0.5% PMB for 24 hr. The left side of the figure describes activity towards CDNB, the right towards ethacrynic acid. Errors bar indicate the standard error of the mean of three (ethacrynic acid) or four (CDNB) independent measurements. Specific activities are given in nmoles of conjugate formed per min per mg protein. Figure 2A; second chromosome lines. Figure 2B; third chromosome lines.

lines differing for chromosome II, there existed significant line ($P < 0.001$) and diet ($P < 0.01$) effects, as well as a significant interaction between the two ($P < 0.001$). Among lines differing in their chromosome III (Fig. 2B) constitution, the only significant effect was observed among lines ($P < 0.001$). It is important to note that there was no significant effect of diet, indicating that none of these lines shows a measurable response to dietary PMB.

(2) Among the chromosome II lines, there were significant effects of line ($P < 0.001$) and pretreatment ($P < 0.05$) on levels of ethacrynic acid conjugation; however, no significant interaction between these factors was evident. No effect of either factor was evident among the chromosome III lines (Fig. 2B).

These results indicate that the major genetic factors affecting this enzyme are on chromosome II, and that significant variation exists that affects responses to PMB. Upon examination of Fig. 2, it is clear that the line–diet interaction observed among these lines in CDNB conjugation is due to the lack of significant response of NC14 to PMB. In contrast, the other lines, while differing in control activity, showed comparable degrees of response. In addition, there was no observable effect of treatment on EA conjugation in this line; however, the specific activity of this line in control preparations was approximately 3-fold higher than comparable preparations from the other chromosome II lines. Thus, this line is unique with respect to both inducibility and relative activity towards the two substrates.

These observations raised the question of how many structural genes are involved in coding for proteins with the enzymatic activities in question. In particular, while the overall correlation of EA and CDNB inducibility observed suggests that one protein is involved, the behaviour of NC14, particularly with respect to the difference from other lines in the relative amounts of the two activities, suggests the existence of two such proteins with different substrate specificities. If, indeed, two enzymes with different properties exist, it might be possible to physically separate them. Two experiments were performed. First, we collected a $105{,}000 \times g$ microsomal pellet from BMC larvae and assayed that pellet and the supernatant fraction for both activities. No activity was present in the pellet (data not shown); hence, we ruled out the possibility of there being both a soluble and a microsomal enzyme. Second, we performed ammonium sulfate fractionation of extracts of BMC larvae, as a first step towards purification of the enzyme. The results, presented in Fig. 3, demonstrate that indeed activity towards EA can be separated from that towards CDNB. While *ca.* 65% of the total activity towards CDNB was precipitated by 60–90% saturation with ammonium sulfate, only 27% of the total activity towards EA was found in those fractions. In contrast, 42% of the activity towards EA remained in the supernatant fraction following addition of ammonium sulfate to 90% saturation, while on 12% of that towards CDNB was found in this fraction. These data thus suggest the existence of two distinct proteins, the first of which exhibits a preference for CDNB as a substrate, and the second of which has higher activity towards CDNB. Further characterization of these fractions is in progress.

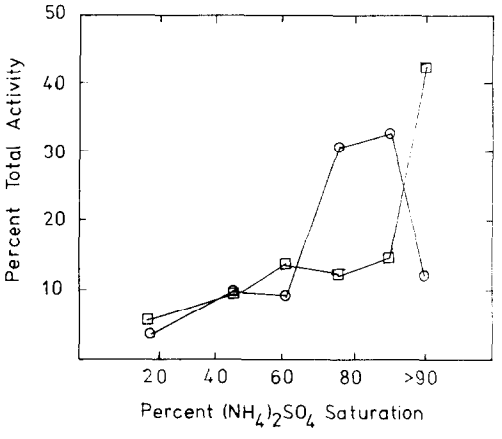

Fig. 3. Ammonium sulfate fractionation of glutathione-*S*-transferases. Key: (○--○) activity towards CDNB, and (□--□) activity towards EA. Activity indicated in the ">90%" fraction is that remaining in the supernatant after making to 90% saturation.

DISCUSSION

The data presented herein allow for certain inferences regarding the genetic control of glutathione-*S*-transferase in *D. melanogaster* and introduce new areas for speculation. Of primary concern is whether the enzyme or enzymes in this species provides the range of adaptive flexibility implied by the diversity of isozymes observed in mammals. Certain points are evident.

First, it is clear that, at least with respect to the substrates commonly employed for the assay of these proteins, the enzyme found in *Drosophila* is somewhat limited in its range of substrates. In particular, while we observed high activity towards some aromatic substrates, notably CDNB, activity towards other typical conjugation substrates was absent. Thus, the *Drosophila* enzyme appears to possess a range of substrate specificities more limited than those found in rat liver. Second, *Drosophila* does possess at least a limited adaptive potential with respect to the inducibility of the enzyme. In particular, PMB can act to elevate specific activities in larvae. Induction of glutathione-*S*-transferase has been reported in other insect species [11, 15] and, in some cases, differences in enzyme activity can be related to such processes as detoxification of plant secondary products [15] or insecticide metabolism [7]. In the case of *Drosophila*, no correlation is evident between enzyme activity and malathion conjugation [30], and our data, while demonstrating a potentially adaptive response, does not address the question of its physiological significance.

The major objective of this study was to attempt to exploit the genetic system of *Drosophila* as a means of making inference regarding the control of this enzyme. The data indicate that, although there are differences in the pattern of response of CDNB

and EA conjugating activities to exposure to PMB, these responses are, to a great extent, parallel. In particular, both chromosome II lines that showed significant responses when CDNB was employed as a substrate demonstrated comparable responses with respect to EA conjugation. Furthermore, the data indicate that there is little genetic variation on chromosome III that affects either conjugation reaction. Rather, we see that, in the lines examined, PMB appears to be ineffective as an inducer of either. This can best be explained as being due to the presence of factors on the chromosome II homolog carried by all these lines that prevent response.

The most interesting finding to arise out of the current study relates to the behavior of the enzyme in line NC14. It is unique in two ways. First, it was totally noninducible by PMB, as measured by either conjugation reaction. Second, activity towards EA, relative to that towards CDNB, was much higher than in any of the others. The first observation suggests that these two activities are controlled by a common genetic regulatory element, and that the variant carried by this line is nonresponsive to PMB. The second observation, however, suggests that a variant of an additional element must be present in this line, one that affects the relative magnitudes of the two activities, rather than their response to PMB. Two hypotheses can be put forward to explain these results.

(1) There are two glutathione-*S*-transferase isozymes, one of which exhibits higher relative activity towards CDNB and one towards EA. Structural genes for both are situated on chromosome II. Line NC14 either carries a structural allele of the EA-specific gene that produces a protein with high specific activity or is an overproducer of this form. Furthermore, this same line also carries a variant that prevents response to PMB.

(2) There is a single form of the enzyme, coded for by one locus on chromosome II. Line NC14 differs from other lines in that it carries an allele that produces a protein molecule with unusually high activity towards ethacrynic acid. In addition, the variant of some regulatory factor carried by this chromosome prevents response to PMB.

At present, it is impossible to fully distinguish between these possibilities. The fact that the two activities can be partially separated by ammonium sulfate fractionation supports hypothesis one and the existence of at least two distinct proteins. The most parsimonious explanation is that the two proteins are products of separate loci. The correlation between inducibility of activity towards CDNB and EA evident in Fig. 2 can best be explained as being due to the likelihood that the protein with high activity towards CDNB would also possess some activity towards EA, so that the degree of inducibility of this protein would, in fact, affect total EA conjugation activity observed.

Jansen *et al.* [19] have recently reported that at least three distinct forms of glutathione-*S*-transferase can be isolated from a 45–90% ammonium sulfate fraction from *Drosophila*. In the context of the present report, the activities they report would be combined within the single peak of CDNB activity evident in Fig. 3. Jansen *et al.* did not report data regarding activity in the >90% ammonium sulfate saturation supernatant fraction; neither did they examine activity towards ethacrynic acid. Thus, our data suggest the existence of an additional isozyme, one with relatively high activity towards EA. Further characterization of this heterogeneity awaits progress in the purification of these putative isozymes.

In summary, it is evident that, as is often the case, considerable genetic diversity has evolved in *D. melanogaster* that affects the function of this particular enzyme. Clearly, many questions remain; however, it is clear that the metabolic capabilities of flies are affected both by genotype and environmental factors. Therefore, such factors may be of considerable significance in modulating the observable mutagenicity and toxicity of particular xenobiotics.

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REFERENCES

1. W. Jakoby, *Adv. Enzymol.* **48**, 383 (1978).
2. W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. biol. Chem.* **249**, 7130 (1974).
3. W. H. Habig and W. B. Jakoby, *Meth. Enzym.* **77**, 398 (1981).
4. D. D. Dao, C. A. Partridge, A. Kurosky and A. C. Awasthi, *Biochem. J.* **221**, 33 (1984).
5. K. Kamisaka, W. H. Habig, J. M. Ketley, I. M. Arias and W. B. Jakoby, *Eur. J. Biochem.* **60**, 153 (1975).
6. C-Y. Lee, L. Johnson, R. H. Cox, J. D. McKinney and S-M. Lee, *J. biol. Chem.* **256**, 8110 (1981).
7. N. Motoyama and W. Dauterman, *Pestic. Biochem. Physiol.* **5**, 489 (1975).
8. N. Motoyama and W. Dauterman, *Rev. biochem. Toxic.* **2**, 49 (1980).
9. N. Motoyama, W. C. Dauterman and F. W. Plapp, Jr., *Pestic. Biochem. Physiol.* **7**, 443 (1977).
10. F. Oppenoorth, L. J. van der Pas and N. W. H. Houx, *Pestic. Biochem. Physiol.* **11**, 176 (1979).
11. J. A. Ottea and F. W. Plapp, Jr., *Pestic. Biochem. Physiol.* **15**, 10 (1981).
12. T. Hayaoka and W. C. Dauterman, *Pestic. Biochem. Physiol.* **17**, 113 (1982).
13. T. Hayaoka and W. C. Dauterman, *Pestic. Biochem. Physiol.* **19**, 344 (1983).
14. S. J. Yu, *Pestic. Biochem. Physiol.* **18**, 101 (1982).
15. S. J. Yu, *Pestic. Biochem. Physiol.* **19**, 330 (1983).
16. A. Clark and W. C. Dauterman, *Pestic. Biochem. Physiol* **17**, 307 (1982).
17. A. Clark, *Analyt. Biochem.* **123**, 123 (1982).
18. A. J. Baars, *Drug Metab. Rev.* **11**, 191 (1980).
19. M. Jansen, A. J. Baars and D. D. Breimer, *Biochem. Pharmac.* **33**, 3655 (1984).
20. B. J. Cochrane and R. C. Richmond, *Genetics* **91**, 491 (1980).
21. C. C. Laurie-Ahlberg, G. Maroni, G. C. Bewley, J. C. Lucchesi and B. S. Weir, *Proc. natn Acad. Sci. U.S.A.* **77**, 1073 (1980).
22. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
23. I. Hallstrom, A. Sundvall, U. Rannug, R. Grasfstrom and C. Ramel, *Chem. Biol. Interact.* **34**, 129 (1981).

24. Difco Laboratories, *Difco Manual*. Difco Laboratories, Detroit (1953).
25. T. Rizki, in *The Genetics and Biology of* Drosophila (Eds. M. Ashburner and T. Wright), Vol. 2b, p. 561. Academic Press, New York (1978).
26. C. C. Laurie-Ahlberg, J. H. Williamson, B. J. Cochrane, A. N. Wilton and F. I. Chasalow, *Genetics* **99**, 127 (1981).
27. S. Hori, S. Tanda, K. Fuzukawa and T. Hanaoka, *Jap. J. Genet.* **57**, 535 (1982).
28. B. J. Cochrane, J. C. Lucchesi and C. C. Laurie-Ahlberg, *Genetics* **105**, 601 (1983).
29. R. R. Sokal and F. J. Rohlf, *Biometry*. W. H. Freeman, San Francisco (1969).
30. B. C. Holwerda and R. A. Morton, *Pestic. Biochem. Physiol.* **20**, 151 (1983).