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In vitro studies on the metabolism of aflatoxin B_1 and aldrin in testes of genetically different strains of *Drosophila melanogaster*

Roland E. Foerster[1] and Friedrich E. Würgler

Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, Schorenstraße 16, CH-8603 Schwerzenbach, Switzerland

Abstract. As *Drosophila melanogaster* occupies an important position within the test battery for mutagens and carcinogens, it is of interest to study the xenobiotics metabolism of this insect. Likewise, the genetic control of these important enzyme systems falls within this interest.

Our attempt was to get new strains, which show changes in their xenobiotics metabolism. This was done by a mutagenization and selection procedure for the second chromosome. The 44 fertile homozygous inbred strains produced by this selection were first tested for DDT resistance. Some of them showed LT50 values which were remarkably higher than that of the original strain Berlin K.

Aflatoxin B_1 metabolism in two of the new strains (H349 and H362), Berlin K, and Hikone-R was compared, whilst aldrin epoxidase activity was compared in strains H349, H362, Berlin K, vestigial, and Karsnäs-R. The metabolism studies were carried out in vitro with testes tissue of the different strains. The metabolism in testes is of specific interest because this tissue is most often used in mutagenicity testing.

In the AFB_1 assays of the up to 12 observed metabolites three could be identified as AFB_{2a}, AFM_1, and AFR_0. Hikone-R produced mostly AFR_0 (3.43% of the initial AFB_1 concentration) and small amounts of AFM_1 (0.59% AF) and AFB_{2a} (0.36% AF). The strain Berlin K showed only a low production of AFB_{2a} (0.48% AF), while the strain H349 formed AFR_0 (6.02% AF) and AFM_1 (0.75% AF). The AFM_1 appeared in even higher amounts than with Hikone-R. On the other hand, H362 showed the lowest activity in AFB_1 metabolism. With this strain none of the determined metabolites could be detected in levels significantly higher than the control. The difference between H349 and the original strain Berlin K was highly significant. The production of AFR_0 and the binding of aflatoxin to macromolecules show a linear correlation. In both parameters measured, the strain H349 yielded the highest results. The determination of aldrin epoxidase activity gave the following results (in pmol dieldrin · mg^{-1} protein · min^{-1}): H349: 0.74; Karsnäs-R: 0.57; vestigial: 0.57; Berlin K: 0.32; H362: 0.27. Again the difference between H349 and Berlin K was statistically significant. The measured activities match values obtained with extrahepatic tissue of mammals.

It is concluded that the line H349 is a mutant in the xenobiotic metabolism. For the strains Hikone-R, Karsnäs-R, and H349 AFR_0 could be confirmed to be the main metabolite of AFB_1. The metabolism pattern was shown to differ strongly from strain to strain.

Key words: Aflatoxins – Aldrin – Drosophila – Xenobiotics metabolism – Testes

Offprint requests to: F. E. Würgler

Abbreviations: AFB_1, AFB_{2a} and AFM_1 = aflatoxins B_1, B_{2a}, and M_1; AFR_0 = aflatoxicol, DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane; EMS = ethyl methanesulfonate

1 *Present address:* Ciba-Geigy AG, CH-4002 Basel, Switzerland

Introduction

Drosophila melanogaster offers a variety of assay systems for the detection of chemical mutagens and carcinogens (Sobels 1972, 1974; Vogel and Sobels 1976; Würgler et al. 1984; Würgler 1980). Systems are available for the detection of point mutations, chromosome aberrations, nondisjunction and somatic mutations and recombination. The best validated of these assays is the sex-linked recessive lethal test, which detects mutagens inducing point mutations and small deletions. The mutations are observed based on their recessive lethal phenotype. This test is easy to perform and relatively fast and cheap.

It was found that over 83% of known carcinogens are detected as mutagens in the *Drosophila* sex-linked recessive lethal assay (Vogel et al. 1980; Lee et al. 1983).

An important advantage of *Drosophila* is its capacity to metabolize xenobiotics in a manner similar to mammals. Therefore, using *Drosophila* based assay systems, it is possible to detect pro-mutagens and pro-carcinogens (Vogel and Sobels 1976).

The xenobiotics metabolism of mammals is most active in the liver (Bentley and Oesch 1982). These enzyme systems have been studied extensively during the last decades. For insects, especially for *Drosophila*, most data available were obtained only recently. Initially the metabolism of insecticides was of primary interest (Casida 1969; Wilkinson and Brattsten 1972; Perry and Agosin 1974; Wilkinson 1976). These studies demonstrated the presence of xenobiotics metabolizing systems in insects, including flies. Microsomes obtained from whole insects or particular organs had metabolic activities similar to those of mammalian liver microsomal preparations. Most of the enzyme activity in insects is located in the gastrointestinal tract, the Malpighian tubules and the fat body (Casida 1969). The first indications that *Drosophila* activates pro-mutagens were obtained by Clark (1959, 1960, 1963) and Pasternak (1962, 1963, 1964). They discovered the strong

mutagenic activity of pyrrolizidine alkaloids and of nitrosamines. In the last few years the biochemistry of the metabolism of pro-mutagens was studied in *Drosophila* (Kulkarni et al. 1976; Baars 1980; Baars et al. 1977, 1979; Holzer et al. 1978; Holzer 1981). These studies indicate that *Drosophila* has potent enzyme systems, most of which are comparable to those of mammals. In modified Ames tests, crude microsomal fractions from either *Drosophila* larvae or adults activated pro-mutagens to bacterial mutagens (Nix et al. 1981; Baars 1980; Hällström et al. 1981).

In earlier studies with insecticides (Georghiou 1965) and in recent studies (Nigsch 1978; Holzer 1981; Hällström et al. 1982), the second chromosome of *Drosophila* was shown to carry genes important in the control of xenobiotics metabolism. Therefore, *Drosophila* offers the opportunity to study the genetic control of the enzyme system(s) active in xenobiotics metabolism. By isolating flies which most probably carried a second chromosome mutation, and by characterizing the testes of homozygous carriers with respect to their metabolic activities, we have investigated the genetic control of the metabolism of aldrin and aflatoxin B_1.

Materials and methods

a) Chemicals and materials. DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] was purchased from Fluka, Buchs, Switzerland; EMS (ethyl methanesulfonate) from K+K, ICN Pharmaceutical Inc., Life Sciences Group, Plainview, NY; dieldrin and aldrin as Pestenal (HEOD) and Pestenal (HHDN) from Riedel-DeHaen AG, Seelze, Hannover, FRG; all aflatoxin standards (AFB_1, AFB_{2a}, AFM_1, and AFR_0) from Senn Chemicals, Dielsdorf, Switzerland.

[^{14}C]-aflatoxin B_1 was produced biosynthetically by the method of Hsieh and Mateles (1970) with *Aspergillus flavus* ATC 15517 grown on medium containing [1-^{14}C]-sodium acetate (purchased from New England Nuclear, Boston, MA; specific activity 57.7 mCi/mmole). The radiospecific activity of the product was 30.4 mCi/mmole.

A modified Bio-Rad assay was used for all protein determinations (Bradford 1976; Foerster 1979).

b) Drosophila strains. The following strains were used: Berlin K (wild type; from E. Vogel, Dept. of Radiation Genetics and Chemical Mutagenesis, State University Leiden, The Netherlands), DTS-91 (a strain with a dominant temperature-sensitive mutation with the following genetic constitution: CyO/l(2)91^{DTS}; from the California Institute of Technology, Pasadena, CA), Karsnäs-R (selected for Hg-resistance, from the Dept. of Genetics, University of Umea, Sweden), and Hikone-R (selected for insecticide resistance including DDT; from the Dept. of Genetics, University of Umea, Sweden). All strains, except DTS-91, were grown on standard medium at 25° C. For DTS-91 Instant Medium (Carolina Biological Supply Company, Burlington, NC) was used.

c) Mutagenization and selection. Usually 30 Berlin K males were fed 5.0 or 2.5 mM EMS, respectively, in a 5% sucrose solution for 4 h using the method of Vogel and Lüers (1974). These males were inbred in groups of 10 with 15 females of the strain DTS-91. The resulting first selection generation was raised at 23° C. From the offspring only the non-Curly flies with the genetic constitution Berlin K*/DTS-91 were used (Berlin K* indicates the mutagenized second chromosome of Berlin K). These males were crossed individually to three females of the strain DTS-91. The progeny were raised at 29° C in order to eliminate all offspring carrying the DTS-91 chromosome. The only offspring were flies with the genetic configuration Berlin K*/CyO. From each of the second selection cross the offspring were inbred to get flies homozygous for the mutagenized second chromosome (Berlin K*/Berlin K*). This cross was again performed under standard conditions (25° C). Homozygous offspring of any such inbred cross is homozygous for the second chromosome (Berlin K*) and is potentially homozygous for EMS-induced mutations.

d) DDT tests. The DDT tests were used as a first screening for physiological changes in strains homozygous for a mutagenized second chromosome compared to the original strain Berlin K. We tested the DDT-resistance in the short term test developed by Bochning (1954) as modified by Nigsch (1978). For each test 1–2-day-old flies were used. They were placed in groups of 10 females and 10 males into petri dishes coated with DDT (0.2 mg per dish) or into control dishes without DDT. Flies which were lying on their backs were counted hourly for up to 8 h for both sexes separately. All tests were repeated at least twice. The data were analyzed with computer programs based on the multinomial distribution (Berchtold 1982).

e) Preparation of testes tissue. For each preparation at least 1,500 adult males were collected during the 2 days before the experiment and kept in bottles with standard medium and live yeast. All steps of the preparation were performed on ice with precooled media and material. Portions of 100–200 flies were immobilized by cooling and pressed through a cellector (Bellco Glass Inc., Vineland, NJ) with sieve nr. 2 (860 µ within of mesh) and the sieve rinsed with cold autoclaved Ringer's solution (Ephrussi and Beadle 1936). The suspension obtained by this step was collected in a 200-ml beaker. This step was repeated until all flies were prepared. The resulting suspension was sedimented at 1 *g* for 5 min and the volume reduced to 20 ml by aspirating the major part of the supernatant. This suspension was then filtered through sieve no. 3 (520 µ within of mesh) and the sieve rinsed with an excess of fresh Ringer's solution. This suspension was sedimented at 1 *g* for 20 min and the supernatant was again discarded. The pellet was transferred to a petri dish and the testes were collected with small pasteur pipettes under stereomicroscopes. The testes were identified by their characteristic shape. This procedure yielded 100–200 intact testes per preparation. The medium was then changed from Ringer's solution to balanced saline (Chan and Gehring 1971). Antibiotic-antimitotic solution (Gibco AG, Basel, Switzerland) was added to the sterile filtered balanced saline prior to use.

f) Aflatoxin assay. For all assays freshly isolated testes tissue was used. For each strain duplicate charges with intact and heat-inactivated tissue were studied. All charges were preincubated for 10 min at 35° C in a shaking water bath (75 stpm). The incubation was started by adding 20 µl [^{14}C]-aflatoxin B_1 (1.8×10^5 dpm, 2.67 mmole) dissolved in methanol and stopped after 60 min by transferring the tubes into ice water and adding 5 ml dichloromethane. Each charge was extracted three times with 5 ml dichloromethane. The organic fractions were pooled, and dried on sodium sulfate, evaporated in a N_2 stream and redissolved in 20 µl benzene. All four charges of one strain were then applied individually to the corners of a HPTLC plate (10 × 10 cm, Kieselgel 60 F_{254}, Merck, Darm-

stadt, FRG). These plates were run contrarotating first with system A ($CHCl_3$: acetone : H_2O = 90 : 10 : 0.3) and second in the other dimension with system B ($CHCl_3$: acetone : iso-amyl-alcohol = 80 : 10 : 10) in a HPTLC linear chamber (Camag, Muttenz, Switzerland). The plates were then incubated with X-ray film at −80° for three different periods: 20.3, 44.8, and 199.3 h. Each plate was marked asymmetrically by spots of 2 µl of a solution containing 2×10^6 dpm/ml [^{14}C]. This was necessary for the identification and for the quantitative densitometry of the films.

All hydrophilic phases were extracted with 5 ml pentane and the organic phase evaporated to remove remaining dichloromethane. The purified fractions were separated in a high- and low-molecular fraction by vaccuum dialysis. Aliquots of each fraction were counted in a scintillation counter.

g) Aldrin assays. Incubation conditions were the same as used for the aflatoxin assays. After the preincubation of 10 min the incubation was started by adding 10 µl of a solution of aldrin in methanol (final concentration 3.0 µM). After 20 min the incubations were stopped by adding 1 ml of ice-cold *n*-hexane. After extraction, 1 µl of the organic phase was analyzed by gas chromatography (HRGC 4160, Carlo Erba, Milan, Italy; Temperature program: 2 min 60° C, 50° C/min to 160° C, 5° C/min to 240° C, 30 min 240° C). Standard solutions of aldrin and dieldrin were analyzed after each experimental sample.

Results

a) Selections and DDT-resistance tests. Among nearly 600 selection crosses 44 inbred strains were fertile. The strains were tested for their DDT-resistance in parallel with the three laboratory strains Berlin-K, DTS-91, and vestigial. All values are given as lethal time 50 (LT50), which indicates that 50% of the flies were lying on their backs at this time. The distributions of the data observed are shown in Fig. 1. Four of the new strains showed a LT50 of more than 8.8 h for both sexes.

The most striking result was obtained with females of the line H349. Their LT50 was more than 27 h. The value of the corresponding males was 7.4 h. The DDT-resistance was in most of the cases similar to that of the Berlin K strain, with LT50 values around 6.9 h.

b) Aflatoxin metabolism. The data for the aflatoxin metabolism of four tested strains are shown in Table 1. Aflatoxin B_1 and its analyzed metabolites are shown in Fig. 2. All values are given in percent of the original AFB_1 concentration. The control values obtained with heat-inactivated tissue were 0.43% for AFM_1, 0.55% for AFB_{2a}, and 2.25% for AFR_0. These metabolites were most probably present as contaminations of the AFB_1 used, since AFR_0 has the same R_f value in the chromatography system (system A) which was used to purify the AFB_1, whilst AFB_{2a} can be formed non-enzymatically (Campbell and Hayes 1976). Contamination of the incubation mixtures with microorganisms capable of metabolizing AFB_1 is most improbable for the following reasons: (1) All solutions were sterilized prior to use. (2) An antibiotic-anti-

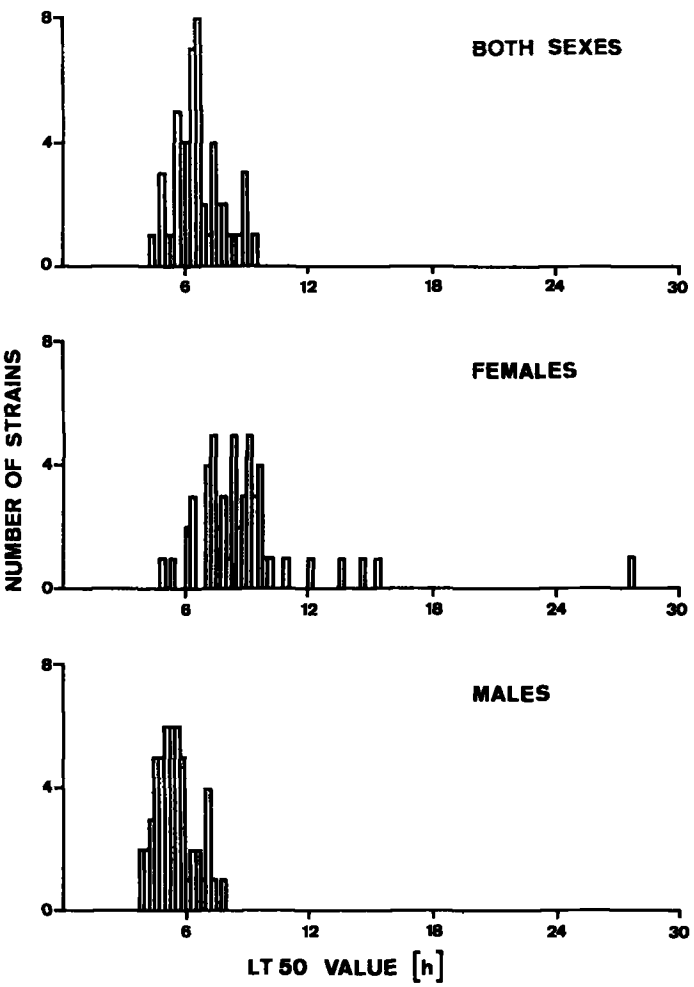

Fig. 1. Distributions of DDT resistance of all tested strains

Table 1. Conversion of aflatoxin B_1 to its metabolites AFB_{2a}, AFM_1, and AFR_0. All values are in percent of the original AFB_1 concentration. The control values resulted from incubations with heat inactivated tissue. The last line of the Table gives the binding of aflatoxins to macromolecules in % of aflatoxin B_1

Metabolite	Converted aflatoxin (%)				
	Control	Berlin K	Hikone-R	H349	H362
AFB_{2a}	0.55 ± 0.17	1.03 ± 0.66	0.91 ± 0.27	0.47 ± 0.01	0.54 ± 0.04
AFM_1	0.43 ± 0.12	0.44 ± 0.07	1.02 ± 0.16	1.18 ± 0.28	0.33 ± 0.01
AFR_0	2.25 ± 0.60	2.85 ± 0.53	5.68 ± 1.95	8.27 ± 1.98	2.60 ± 0.52
	Bound aflatoxins (%)				
	0.097 ± 0.002	0.264 ± 0.028	0.489 ± 0.042	0.76(± 0.036	0.176 ± 0.005

mycotic solution was included in the incubation medium. (3) The values of the three metabolites did not change during control incubation of up to 120 min. AFB_{2a} was formed only in small amounts by testes tissue of Berlin K and Hikone-R. For AFM_1 and AFR_0 the strains Hikone-R and H349 showed significantly higher values than control and Berlin K. Table 2 gives the metabolic activities (pmolAF · mg^{-1} protein · min^{-1}) of testes tissues of four strains for the metabolites of AFB_1. Several metabolites detected on the autoradiographs could not be identified. Polar metabolites like the 8,9-dihydrodiol of AFB_1 and/or aflatoxicol M_1 might be responsible for a spot near the front in both chromatographic systems. This spot was observed in incubations with testes from Berlin K, H349, and H362, but not in control incubations. The binding of AFB_1 to macromolecules is shown in Table 1. The highest binding was obtained with the strains Hikone-R and H349. Their values are significantly higher than the control value. In contrast, the binding of AFB_1 to macromolecules in incubations with Berlin K and H362 testes was not significant.

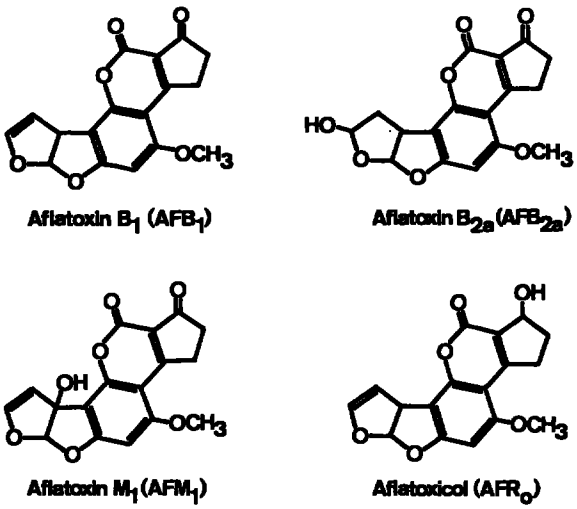

Fig. 2. Structures of aflatoxin B_1 and three of its metabolites

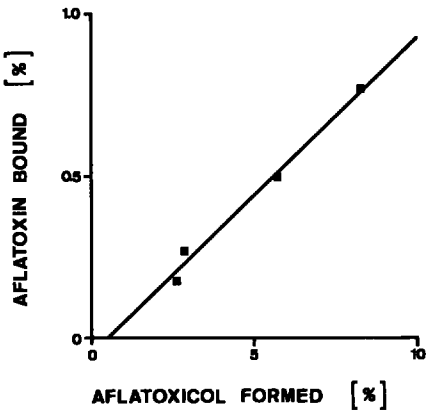

Fig. 3. Relationship between the binding of aflatoxin to macromolecules and the formation of aflatoxicol by the strains H362, Berlin K, Hikone-R and H349. The linear regression has a correlation coefficient of $r = 0.994$

Table 2. Activities of testes tissue of four different strains for the metabolites of AFB_1 in pmol AF · mg^{-1} protein · min^{-1}

Metabolite	Activities			
	Berlin K	Hikone-R	H349	H362
AFB_{2a}	21	18	–	–
AFM_1	–	29	23	–
AFR_0	26	170	187	9

c) Aldrin epoxidase. Table 3 shows the enzyme activities (pmol dieldrin · mg^{-1} protein · min^{-1}) of aldrin epoxidase of the testes of five strains. With the five strains studied the following sequence of activities was found: H349 > Karsnäs-R ≈ vestigial > Berlin K ≈ H362. Values for H349, vestigial, and Karsnäs-R were all significantly higher than that of Berlin K. On the other hand, the difference between Berlin K and H362 was not significant.

Discussion

The DDT-resistance tests showed that most of the LT50 values of the mutagenized strains were similar to that of the original Berlin K strain. Four strains (H314, H349, H350, and H364) show an increased resistance to DDT. The LT50 values of females are always higher than those of the males of the same strain. This may be explained by the ability of the females to store DDT in their fat body, which is much larger than that of the males. Therefore, in females a smaller fraction of the DDT dose entering the body becomes active in the nervous system than in males.

The selected strains H349 and H362 were used for the metabolism studies. H349 was selected as one of the most DDT-resistant strains, whereas H362 is one of the strains showing little difference to the original strain Berlin K. These two strains were compared to Berlin K and Hikone-R with respect to aflatoxin B_1 metabolism, and to Berlin K, vestigial and Karsnäs-R in terms of aldrin epoxidase activity.

On the autoradiographs of the aflatoxin assays up to 12 different spots were detectable; three of them could be identified as the metabolites AFB_{2a}, AFM_1, and AFR_0. None of the four strains produced AFB_{2a} in amounts statistically different from the control. Since this metabolite can also be formed non-enzymatically, we can deduce that it is not a metabolite of AFB_1 in *Drosophila* testes in vitro.

AFM_1, is, like AFB_1 itself, a very potent mutagen and carcinogen (Lutz et al. 1980). It also has the double bond in the 8,9-position, which can be oxidized to the 8,9-epoxide. There is good evidence that the 8,9-epoxide is the carcinogen of AFB_1 (Garner et al. 1972; Garner 1973; Swenson et al. 1975; Fahmy et al. 1978). The two strains Hikone-R and H349 produce

Table 3. Activities of aldrin epoxidase of testes tissue of five different strains. Activities in pmol · mg^{-1} protein · min^{-1}. All values are means of two determinations

Karsnäs-R	H349	Vestigial	Berlin K	H362
0.567 ± 0.144	0.744 ± 0.014	0.566 ± 0.008	0.320 ± 0.090	0.267 ± 0.115

AFM_1 in small amounts, whereas it could not be detected in testes of Berlin K and H362. The major aflatoxin metabolite formed in testes tissue in vitro seems to be AFR_0. In mammals this metabolite is formed by a cytoplasmic reductase. The reaction is reversible (Salhab and Edwards 1977). The conjugation of AFR_0 and its excretion is very slow compared to other AFB_1 metabolites. Therefore several authors assume that AFR_0 could act as a pool for AFB_1 (Salhab and Edwards 1977; Campbell and Hayes 1976). Nevertheless, AFR_0 itself is a strong mutagen (Wong and Hsieh 1976). The two strains Hikone-R and H349 again show the highest values of AFR_0 formation, whereas Berlin K and H362 form only small amounts. The binding to macromolecules gives strong indications of the susceptibility of a species or a strain to AFB_1 (Decad et al. 1979; Degen and Neumann 1981; Green et al. 1982; Lutz et al. 1980). In vitro studies with postmitochondrial fractions of liver preparations of rats and mice showed that, especially in male rats, the binding of AFB_1 to macromolecules was very high (Degen and Neumann 1981). The same was demonstrated in primary cultures of hepatocytes of rats and mice (Decad et al. 1978; Green et al. 1982). Lutz et al. (1980) showed that the in vivo binding of AFB_1 to DNA was clearly higher in rats than in mice. All these results correlate with the susceptibility of these species to AFB_1 carcinogenesis, which is higher in rats than in mice. The binding of AFB_1 to macromolecules of testes again showed significant differences between the four strains tested. The strain H349 showed the highest binding, followed by Hikone-R, whereas in the two other strains, Berlin K and H362, the binding was very low and not significantly different from the control value. The results for AFB_1 binding to macromolecules correlates with the AFR_0 formation. A plot shows the linear regression (Fig. 3). This fact can be interpreted in different ways. AFR_0 could act as a pool for AFB_1, increasing its binding in this way. On the other hand, it could itself bind to macromolecules. Nevertheless this would mean that strains with higher AFR_0 formation are more susceptible to AFB_1-induced toxicity.

The activities of aldrin epoxidase in *Drosophila* testes are far lower than those obtained with microsomes of other insects. Ray (1967) reported activities of about 230 pmol · mg^{-1} protein · min^{-1} with microsomes of *Musca vicina*, using the same incubation period as in our assay. Our results lie within a range of 0.2–0.8 pmol · mg^{-1} protein · min^{-1}. Similar differences could be shown for different tissues of mammals. Extrahepatic tissue of rats show activities in the range of 0.2–0.5 pmol · mg^{-1} protein · min^{-1} (Frei et al. 1982; Frei 1983; Kurihara et al. 1982). Therefore, the activities found in *Drosophila* testes are comparable to those of extrahepatic tissues of rats. Among the strains tested for aldrin epoxidase, H349 shows the highest activity, Karsnäs-R and vestigial are intermediate, and Berlin K and H362 are low.

In conclusion, there is strong evidence that the new strain H349 is a mutant with respect to xenobiotics metabolism. Aflatoxin metabolism and the aldrin epoxidase activity are both significantly different from the original strain Berlin K. The aldrin epoxidase activity is extremely high. In the AFB_1 assay H349 behaves like the multiply insecticide resistant strain Hikone-R. The two latter strains show significant differences in the formation of the AFB_1 metabolites AFM_1, AFR_0, and the binding of AFB_1 and metabolites to macromolecules compared to the strains Berlin K and H362.

AFR_0 was confirmed to be the major metabolite formed in vitro, which agrees well with earlier results from in vivo experiments in the Hikone-R strain (Holzer 1981). The strong correlation between binding of AFB_1 to macromolecules and the formation of AFR_0, as shown here, agrees well with the supposed role of AFR_0 as a pool for AFB_1 and therefore also for AFB_1-8,9-epoxide.

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