Comparison of Alcohol Dehydrogenase Expression in 
*Drosophila melanogaster* and *D. simulans*

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Alcohol dehydrogenase (ADH) gene expression was analyzed in *Drosophila melanogaster* and its sibling species *D. simulans*. The levels of ADH activity, ADH-cross-reacting material (CRM), and ADH-mRNA were analyzed for several strains of each species, which derive from diverse geographic locations around the world. There is considerable quantitative variation in ADH activity, CRM level, and RNA level among strains within species at all developmental stages. However, the only consistent differences between the two species are in pupal RNA level and in late-adult activity and CRM level. Late-adult *melanogaster* flies that are homozygous for the Slow allozyme have approximately twice the level of ADH activity and CRM as do *simulans* flies. The regression of activity on CRM over strains is highly significant and essentially the same for each species, which means that most, if not all, of the activity difference between the species is due to a difference in concentration of the ADH protein. In contrast, there is no significant regression of CRM level on mRNA level in adults of either species; nor is there a significant difference in RNA level between species. Therefore, the difference in ADH protein concentration is not due to RNA template availability. Thus, the interspecific difference in ADH level in adults must be due either to a difference in the rate of translation of the two RNAs or to a difference in protein stability.

**Introduction**

*Drosophila melanogaster* and its sibling species *D. simulans* are essentially cosmopolitan in distribution and live in close association with human populations (Patterson and Stone 1952). Both species utilize fermenting fruits in which ethanol concentrations range up to several percent by volume (McKechnie and Morgan 1982; David and Van Herrewege 1983). In laboratory tests, *melanogaster* adults consistently show a higher level of tolerance both to ethanol and to other alcohols than do *simulans* adults (McKenzie and Parsons 1972; David et al. 1974; David and Bocquet 1976; Daggard 1981; Gibson and Wilks 1988). Tolerance in larvae has been measured for only a small number of strains, but in those cases *melanogaster* also shows a higher tolerance to ethanol than does *simulans* (McKenzie and Parsons 1972; Parsons et al. 1979; Parsons 1980). Several authors have suggested that the difference in alcohol tolerance detected in the laboratory may affect the distribution of the two species in nature. A consistent observation in studies of winery populations of *Drosophila* is that
melanogaster is very abundant inside wineries where ethanol concentrations are often very high, while simulans is very rare inside compared with immediately outside these same wineries (e.g., see McKenzie and Parsons 1972; McKenzie 1974; Marks et al. 1980; Gibson et al. 1981; Gibson and Wilks 1988). Two additional studies suggest that melanogaster larvae are found more frequently than simulans larvae in fermenting fruits with high alcohol contents (McKenzie and McKechnie 1979; Oakeshott et al. 1982). It is not clear whether environmental alcohol concentrations actually determine differences in the distributions of these two species on different substrates, but studies of many populations in different parts of the world clearly show that the adults have different tolerances to alcohol under laboratory conditions (see discussions in Gibson et al. 1981; Gibson and Wilks 1988).

The alcohol dehydrogenase enzyme (ADH; E.C.1.1.1.1) of D. melanogaster clearly plays an important role in alcohol detoxification and metabolism. Flies homozygous for an ADH gene (Adh) null allele are extremely sensitive to the toxic effects of environmental alcohols compared with flies possessing an active enzyme (David et al. 1976). More than 90% of the ethanol that is metabolized to lipid in larvae goes through a pathway that is dependent on ADH activity (Geer et al. 1985). Furthermore, genetic variation in ADH activity levels in melanogaster, particularly the difference between alleles, is frequently associated with variation in tolerance both to ethanol and other alcohols (see reviews in Gibson and Oakeshott 1982; Van Delden 1982).

Several investigators have reported that melanogaster adults have much higher ADH activity than do simulans adults (Pipkin and Hewitt 1972; McDonald and Avise 1976; Juan and Gonzalez-Duarte 1980; Daggard 1981; Oakeshott et al. 1982; Gelfand and McDonald 1983; Chambers et al. 1984; Dickinson et al. 1984; Gibson and Wilks 1988). From these studies it appears that AdhS melanogaster adults have about twice the simulans level of activity and that AdhF melanogaster adults have about four times the simulans level of activity. Many of the authors cited above have suggested that the higher alcohol tolerance of melanogaster adults is due to their higher ADH activity. Because of its potential adaptive significance and because interspecific gene transfer between melanogaster and simulans is feasible by P-element transformation (Spradling and Rubin 1982; Scavarda and Hartl 1984), we have initiated a study of the molecular basis of the difference in ADH expression between these species. The present report provides a description of the interspecific differences in ADH expression throughout development, at the levels of ADH activity, ADH-protein concentration, and ADH-mRNA concentration.

The melanogaster Adh produces two different transcripts, which are differentially expressed during development but produce identical proteins (Benyajati et al. 1983; Savakis et al. 1986). The sequence of the Adh from simulans (Bodmer and Ashburner 1984) indicates that it has the same transcriptional organization as melanogaster in terms of the positions of the distal and proximal promoters and of the three introns. Dickinson et al. (1984) showed that simulans produces primarily the proximal transcript in larvae and primarily the distal transcript in adults, a result which is consistent with the pattern shown by melanogaster. In the present report we provide a more detailed comparison of the relative abundances of the distal and proximal transcripts throughout development.

Gel-electrophoresis studies of populations from around the world have revealed no Adh allozyme polymorphism in simulans, whereas most melanogaster populations are polymorphic for two common allozymes, AdhF and AdhS (e.g., see Choudhary and Singh 1987). In comparison of ADH expression in melanogaster and simulans,
it is important to take into account the major systemic difference in ADH activity and in protein level due to the melanogaster allozyme polymorphism (see Laurie and Stam 1988). The DNA sequence data of Kreitman (1983), Bodmer and Ashburner (1984), and Cohn and Moore (1988) show that the Adh of simulans is more similar to melanogaster AdhS than to AdhF alleles. Among the 18 sites at which the consensus AdhS and AdhF sequences differ, there are 13 matches between simulans and AdhS. One of these matches includes the S/F amino acid substitution site so that the simulans ADH differs from the Slow melanogaster ADH by two amino acids and from the Fast melanogaster ADH by three. These data and the lower levels of polymorphism within AdhF sequences compared with AdhS sequences clearly suggest a recent origin of the high-activity AdhF allele within melanogaster (Kreitman 1983; Aquadro et al. 1986). Therefore, the most direct approach to studying interspecific differences in ADH expression is to compare simulans alleles and melanogaster Slow alleles. Thus, all of the melanogaster lines analyzed in the present report are AdhS.

Material and Methods

Fly Stocks

Seven melanogaster lines were made isoaallelic for the Adh region by extracting the entire second chromosome by using a balancer stock; these lines are Was (from Seattle), Jas (from Ishigaki, Japan), Afs (from Burundi, Africa), and Frs (from Bully, France), which were provided by Kreitman (1983), and KA27 (from Lawrence, Kans.), W109 (from Cochrane, Wisc.) and RI42 (from Providence, R.I.), which have been described by Laurie-Ahlberg et al. (1980). Eleven simulans lines were made isoaallelic for the Adh region by a procedure that makes use of the close linkage (1.5 map units in melanogaster; Lindsley and Grell 1968) between Adh and the black locus. A b* stock of simulans was obtained from E. H. Grell; this mutant is semidominant. A stock isoaallelic for the b region is made by crossing wild-type flies to b*/b* flies, backcrossing a single b*/ + F1 male to b*/b* females, intercrossing the b*/ + F2 progeny, and finally intercrossing the +/+ progeny. The wild-type flies for constructing the isoaallelic lines were obtained from 11 different isofemale lines: Tun-6 and Tun-16 (Tunisia), Fra-2 and Fra-20 (southern France), Afr S-5 and Afr S-10 (South Africa), and Con S-2 (Congo) were collected in 1983 and provided by R. Singh; Jap-1 and Jap-2 were collected in Munakata City, Japan, in 1986 and provided by T. Yamazaki; Aus-l was collected in Palmers Island, Australia, in 1986 and provided by J.S.F. Barker; and Ral l-2 was collected in Raleigh, N.C. in 1984.

Four melanogaster lines homozygous for the Slow ADH allozyme were established by pair-mating flies from four different isofemale lines. After producing progeny, each pair was analyzed by starch-gel electrophoresis (Laurie-Ahlberg and Weir 1979) to ensure that both members were Slow homozygotes. The four melanogaster pair-mated lines were derived from the following isofemale lines: V.France 16-2 (from Vienville, France) and B.W.Africa 27, collected in 1978 and provided by R. Singh; Japan Kochi 5, collected by T. Mukai in 1982; and Ral 124, collected in Raleigh, N.C., in 1982). Four pair-mated simulans lines were also established from the following isofemale lines: S.France 2 (from S.France) and B.Congo S2 (from Brazzaville, Congo), collected in 1983 and provided by R. Singh; Japan Muna-1, collected in 1986 by T. Yamazaki; and Ral S-5, collected in Raleigh, N.C. in 1984. Note that the four pair-mated simulans lines derive from four of the same isofemale lines from which isoaallelic stocks were also constructed.
The mutant stock, Adh\textsuperscript{fn23} pr cn, was obtained from W. Sofer. Hochi-R is a highly inbred Adh\textsuperscript{F} strain obtained from W. Doane.

Experimental Design

Experiment 1

One isoallelic line of each species was analyzed at each of 10 developmental stages. The lines were Was (melanogaster) and Ral 1-2 (simulans). For each line and developmental stage a total of four replicate samples were obtained. All samples were obtained as pairs, one from each line, and were reared simultaneously. All 40 pairs of samples are independent in the sense that each was obtained at a different time and from a different culture bottle. Each sample was divided into two parts. One part was homogenized and used for protein assays [ADH activity, cross-reacting material (CRM) level, and total protein level], and the other part was homogenized and used for RNase protection assays (quantitative level of ADH-mRNA relative to an internal standard, fn23, and visual assessment of the relative abundance of distal and proximal transcripts). Paired t-tests were performed to test the significance of the species difference at each stage. In addition, early- and late-adult data were analyzed together in an analysis-of-variance (ANOVA) model with sources: block (rearing time), sex, age, strain, and their interactions. In the devising of F-tests, all effects were considered fixed except block.

Experiment 2

Four isoallelic lines of melanogaster and 11 isoallelic lines of simulans were sampled as third-instar larvae and adult males. During each of two blocks of time, two replicate samples were obtained from each of the 15 lines. All larval and adult samples were analyzed for ADH activity, CRM level, and total protein level. In addition, RNase protection assays for total ADH-mRNA level were performed on the adult samples from the four melanogaster lines and four of the simulans lines (Cons-2, Jap-1, Fra-2, and Ral 1-2). These eight lines were analyzed to test for species differences. The ANOVA model contained the following main effects and all of their interactions: block, replicate within block, species, and line within species. In the devising of the F-tests, all effects were considered random except species. Eight of the simulans lines, two from each of four geographic locations (France, Japan, Tunisia, and South Africa), were analyzed to test for differences among geographic locations. The ANOVA model contained the following main effects and all of their interactions: block, replicate within block, location, and line within location. In the devising of the F-tests, all effects were considered random. Data from the two stages were analyzed separately.

Experiment 3

Four pair-mated lines from each species, as well as three isoallelic lines (Was, KA27, and Ral 1-2), were sampled as mid-third-instar larvae, mid-pupae, and late adults. The pair-mated lines represent one sample of each species from each of four continents (Europe, Africa, Japan, and North America). Collections of each stage were made independently of one another. During each of two blocks of time, two replicate samples were obtained from each of the 11 lines. Each sample was divided into two parts. One part was homogenized and used for protein assays (ADH activity, CRM level, and total protein level), and the other part was homogenized and used for the mRNase protection assay for total ADH-mRNA level. Data from each of the three stages were analyzed separately. The ANOVA model contained the following
main effects and all of their interactions: block, replicate within block, continent, and species. In the devising of the $F$-tests, all effects were considered random except species.

Developmental Staging

All flies were reared on standard cornmeal molasses medium at 25°C. A preliminary experiment was done to determine whether *melanogaster* and *simulans* develop at the same rate. The results are summarized in figure 1 in terms of range and median times for various developmental events. To investigate hatching time, embryos collected over a 1-h period were observed at hourly intervals beginning at 17 h postoviposition. From the curve of number hatched versus time, the median hatch time was estimated. Similar methods were used to estimate median times for the larval moults, pupariation, the appearance of red eye pigment, and eclosion. The results in figure 1 indicate that there are no clear differences in developmental timing between the species except for time of hatching. *Drosophila melanogaster* embryos hatched somewhat later than *D. simulans* embryos, so *melanogaster* embryos were aged slightly longer than *simulans* embryos, in order to sample the same developmental time.

In experiment 2, larvae were not precisely aged but were picked as actively feeding third instars directly from rearing vials. In experiments 1 and 3, staging was accomplished by collecting cohorts of individuals aged at standard intervals past developmental phenomena. The 10 developmental stages of experiment 1 are as follows: *melanogaster* embryos, 15–19 h postoviposition; *simulans* embryos, 14–18 h postoviposition; first instar, 0–4 h posthatch; second instar, 30–32 h posthatch; early third instar, 54–56 h posthatch; mid-third instar, 70–72 h posthatch; prepupae, 0–4 h post–white prepupa; early pupae, 20–24 h post–white prepupa; late pupae, 72–76 h post–white prepupa; early-adult males and females, 0–1 d posteclosion; and late-adult males and females, 7–10 d posteclosion. The three stages of experiment 3 are as follows: mid-third-instar larvae, 70–72 h posthatch; midpupae, 46–66 h post–white prepupa; and late-adult males, 7–10 d posteclosion.

Protein Assays

Samples were prepared for protein assays by grinding freshly collected flies in ice-cold potassium phosphate buffer (10 mM, pH 7.4, 1 mM ethylene diaminetetraacetate). Homogenates were centrifuged at 15,000 g for 10 min, and the supernatants were stored at −70°C.

For assaying ADH activity the spectrophotometric method described by Maroni (1978) was used with isopropanol as substrate. ADH units are expressed as nanomoles NAD+ reduced per minute per milligram total protein. Total protein was determined by the Folin phenol procedure (Lowry et al. 1951).

ADH-protein level was estimated as CRM by radial immunodiffusion (Mancini et al. 1965) using polyclonal rabbit antibodies. A dilution series of a standard fly extract (*Hochi-R, Adh*$^g$) was included on each immunodiffusion plate to ensure linearity over the range of sample values. ADH CRM units are given in terms of *Hochi-R* fly equivalents per milligram total protein. The radial immunodiffusion procedure was tested with purified ADH$^g$ from *melanogaster* and with purified ADH from *simulans* to verify that there is no difference between the two proteins in extent of antigen-antibody reaction. ADH purification steps were ammonium sulfate precipitation, gel filtration, anion-exchange chromatography, and ATP-affinity chromatography [according to the methods of Lee (1982) and Chambers et al. (1984a)].
FIG. 1.—Timing of developmental phenomena in *melanogaster* and *simulans* strains. Each vertical bar shows the median time at which the indicated phenomenon was observed. Horizontal lines indicate the range of times observed, except for egg hatch, where they indicate the range of times over which 90% of eggs hatched. Arrowheads indicate that the entire range was not observed for some samples. Dotted lines denote values for *simulans* samples, and solid lines denote values for *melanogaster* samples. Letters identify strains: a = Ral 1-2; b = Con S-2; c = Was; d = Afs; e = W109; f = R142.

RNase Protection Assays

The RNase protection assay to quantitate total ADH-mRNA makes use of the null mutant *Adh\(^{fn23}\)* as an internal control and have been described in detail by Laurie and Stam (1988). The *fn23* mutant has in exon 3 a 34-bp deletion which causes it
to make a defective protein, but it has nearly normal levels of ADH-mRNA (Benyajati et al. 1983a). An RNase protection assay distinguishes between RNAs produced by the deletion-mutant and wild-type alleles. The pBSBD probe extends from the exon 2 BamHI site to an exon 3 DdeI site that lies just 4 bp beyond the 3' end of the fn23 deletion. RNA from wild-type flies protects two regions of this probe: 89 bases of exon 2 and 263 bases of exon 3. When RNA from fn23 homozygotes is used, the protected region from exon 3 is only 225 bases. In the experiments reported here, we used fn23 as an internal control for quantitating total RNA by mixing a homogenate of homozygous adult fn23 males with a homogenate of wild-type individuals of some developmental stage of either simulans or melanogaster. RNA was then isolated from the mixed homogenate, hybridized to radiolabeled pBSBD probe, and digested with RNase, and the protected fragments were separated on an acrylamide gel. Each sample produced both the 263-base fragment derived from the wild type and the 225-base fragment derived from the mutant. The amount of material in each fragment was estimated by cutting bands out of the gel and scintillation counting. Each sample was run as a dilution series, and the amount of wild-type RNA relative to the amount of mutant control was estimated as the slope of the linear regression of number of counts in the wild-type band on number of counts in the mutant band. RNA transcription, hybridization, digestion, and acrylamide-gel analysis were performed by the method of Melton et al. (1984), with modifications described by Laurie and Stam (1988).

Another RNase protection assay utilizes a different probe, pBSHB, which distinguishes proximal and distal ADH messages. The procedure was performed essentially as described by Fischer and Maniatis (1986). The pBSHB riboprobe extends from the HindIII site within the adult intron to the BamHI site in exon 2 (nucleotides 652-1257; Kreitman 1983). This probe protects a 315-base fragment of the second exon from both proximal and distal transcripts, a 134-base fragment specific to the distal transcript, and a 169-base fragment specific to the proximal transcript. The probe is transcribed from the pBSHB plasmid, which was constructed by isolating the 605-base HindIII/BamHI fragment from the pSPHB plasmid [described as pSP6-MEL by Fischer and Maniatis (1986) and provided by J. Posakony] and inserting it into HindIII/BamHI-cut pBSM13 vector (Stratagene, Inc.). RNA transcription, hybridization, digestion, and acrylamide-gel analysis were performed according to a method described by Laurie and Stam (1988), with the following exceptions: The plasmid was linearized with HindIII and was transcribed with T7 polymerase (1.25 units/μl), and hybrid digestions included RNase A (4 μg/ml). These samples were not run in a dilution series, and the bands were not quantitated by counting.

RNA was prepared essentially according to a method described by Fischer and Maniatis (1985), except that no Proteinase K was used. In the following, homogenate refers to the supernatant obtained after first grinding individuals in buffer in a Dounce glass homogenizer and then centrifuging at 12,000 g for 5 min. For experiments 1 and 3, aliquots of a single homogenate of fn23 adults were mixed with each of the wild-type homogenates that were prepared simultaneously (see Experimental Design). For experiment 2, 50 fn23 flies were mixed with 50 wild-type flies prior to homogenization. In experiment 1, the wild-type homogenate was split into two parts; RNA isolated from one part was used for the RNase protection assay to distinguish distal and proximal transcripts, and the other part was mixed with fn23 homogenate and was used for the RNase protection assay to quantitate total message. In the preparation of fn23 and wild-type mixed homogenates for the quantitative assay, the number of fn23 adults per milliliter homogenate was fixed, and the number of wild-type indi-
Individuals of each stage per milliliter of homogenate was varied so that the wild-type and mutant bands would have similar numbers of counts. The slope estimated as the linear regression of counts in the wild-type band on counts in the mutant band was multiplied by the ratio of milligrams protein in the mutant homogenate to milligrams protein in the wild-type homogenate. This adjustment allows direct comparison of the slopes estimated for different developmental stages.

**Results**

**Experiment 1: Developmental Profile**

The developmental profiles of Was (*melanogaster*) and Ral 1-2 (*simulans*) are shown in figure 2. The Was profile for ADH activity is very similar to the *melanogaster* profiles reported previously by Ursprung et al. (1970) for an Oregon-R strain and by Maroni and Stamey (1983) for a Samarkand strain. Activity per milligram total protein rises abruptly during larval development, until the third-instar stage, during which it declines somewhat. Activity falls off rapidly during the pupal stage and begins to rise

![Developmental profile of ADH expression](image)

**Fig. 2.** Developmental profile of ADH expression in one *melanogaster* strain (Was; circles) and one *simulans* strain (Ral 1-2; black dots). Asterisks show the level of significance of the difference between strains for each stage (*P* < 0.05; **P** < 0.01). The earliest pair of data points are for late embryos. The adult points are the averages of male and female data.
again at eclosion. The Was strain shows a large and significant increase in adult activity between the time points 0–1 d and 7–10 d posteclosion. Figure 2 shows only the average value for adult males and females, but the changes in the two sexes are very similar. Both Ursprung et al. (1970) and Maroni and Stamey (1983) also observed a steep rise in activity after eclosion.

The Ral-1-2 strain shows an ADH activity profile very similar in shape to that of Was, except that it does not show the increase from early to late adult. The difference in activity between early and late adults is not significant. ANOVA of the adult activities for both strains shows that there is a highly significant \( P < 0.001 \) age \( \times \) strain interaction.

Even though the two strains show developmental profiles of similar shape between embryo and eclosion, there are significant quantitative differences at most of the stages (fig. 2). The direction of the difference in activity between the strains changes twice during development. Compared with Ral-1-2, Was is significantly higher in embryos, consistently lower throughout larval and pupal development, not significantly different at eclosion, and again considerably higher in late adults.

For the two strains the developmental profiles for CRM level are virtually identical to those for activity. CRM level increases significantly \( P < 0.01 \) from early to late adulthood in Was and decreases significantly \( P < 0.001 \) in Ral-1-2 adults. The RNA profiles are also similar, except that changes are more abrupt and appear to anticipate changes in both activity and CRM, as expected. Furthermore, the rises in both activity and CRM that occur in Was between early and late adulthood are not accompanied by a rise in mRNA. Instead, in both strains, RNA levels show a significant \( P < 0.01 \) decline between the early- and late-adult samples. Therefore, the late-adult strain differences in both ADH activity and CRM are not accounted for by a difference in RNA level. In fact, the adult-RNA-level difference goes in the opposite direction. In contrast, the embryonic, larval, and pupal strain differences in both activity and CRM are accounted for by significant RNA-level differences that go in the same direction.

Figure 3 shows that the relative abundance of the proximal and distal transcripts is essentially the same in both strains throughout development. The pattern of relative abundance is very similar to that described by Savakis et al. (1986) for a \textit{melanogaster} strain. During the embryonic stage the proximal transcript is most abundant, but a low level of distal transcript is also observed. Throughout larval development, until mid third instar, the distal transcript is virtually undetectable, and the proximal transcript is abundant. At the mid-third-instar stage the distal transcript reappears and throughout the pupal and adult stages is clearly the most abundant of the two transcripts. On the basis of the analysis of this one \textit{simulans} strain, there are no apparent differences between the species in the developmental timing of usage of the distal and proximal \textit{Adh} promoters.

Experiment 2: Variation among Isoallelic Lines

In experiment 2 four isoallelic lines of \textit{melanogaster} (all \textit{Adh}^5) and 11 isoallelic lines of \textit{simulans} were sampled as third-instar larvae and late adults. Figure 4 shows that there is essentially no overlap between the species, in either ADH activity or CRM level, at either developmental stage. The direction of the difference switches between larvae and adults, as observed previously for Was and Ral-1-2. In larvae, the \textit{simulans} lines have significantly higher activity \( P < 0.001 \) and CRM \( P < .05 \) levels than do the \textit{melanogaster} lines. In adults the \textit{melanogaster} lines are significantly higher for activity \( P < 0.001 \) and CRM \( P < 0.05 \), but in adult RNA level there
FIG. 3.—Comparison of relative abundances of distal and proximal transcripts during development in Was (*melanogaster*) and Ral 1-2 (*simulans*)
is no significant difference between species. The variation among lines within species is highly significant ($P < 0.01$ in adults; $P < 0.001$ in larvae) for activity at both stages, is marginally significant ($P < 0.06$) for CRM at both stages, and is highly significant ($P < 0.001$) for RNA in adults.

In this experiment the differences detected between the *melanogaster* and *simulans* lines are not necessarily true species differences, since the lines within each species have some genetic background in common, because of the extraction procedure that made them isoallelic. The differences between the two groups of lines might be due to modifier genes contributed by the balancer stock used to isogenize the second chro-
mosomes of the four *melanogaster* lines and by the $b^2$ stock used to isogenize the *Adh* region of the 11 *simulans* lines.

Experiment 3: Variation among Pair-mated Lines

Experiment 3 analyzes variation among four lines of each species that have independent genetic backgrounds. These lines were established from single pair matings of flies from isofemale lines to ensure that each *melanogaster* line is homozygous for the ADH Slow allozyme. The isofemale lines were collected on four different continents. In addition, the isoallelic lines Ral 1-2 (*simulans*), Was, and KA27 (*melanogaster*) were included for comparison with previous results and to determine whether, with respect to ADH expression, they can be considered representative of their species. Table 1 and figure 5 show the lines' means. The results concerning adults are very similar to those described above for the isoallelic lines. Adults from the *melanogaster* pair-mated lines have a significantly higher ADH activity ($P < 0.01$) and CRM level ($P < 0.01$) than do the *simulans* lines, but in RNA level there is no significant difference between species. In contrast, there is no significant difference between species, in either activity or CRM, at either the larval or pupal stage, and figure 5 shows considerable overlap between the species at these stages. Larvae also show no species difference in RNA level, but there is a highly significant difference between species in RNA level at the pupal stage ($P < 0.0001$). Thus we conclude that the larval ADH-level difference observed in experiment 2 is not a true species difference. The only differences that appear to be species specific are activity and CRM in late adults and RNA level in pupae.

In this experiment the species × geographic location interaction in the ANOVA represents variation among lines within a species. This term is significant for larval activity ($P < 0.0001$), larval CRM ($P < 0.0001$), larval RNA ($P < 0.01$), pupal activity ($P < 0.001$), pupal CRM ($P < 0.0001$), and adult RNA ($P < 0.05$). Therefore, most of the ADH expression variables show genetic variation within a species.

Table 1 shows that for each of the variables the isoallelic lines Ral 1-2 and KA27 have values similar to the mean for their respective species and that therefore they

### Table 1
Mean Activity, CRM, and RNA Levels from Experiment 3

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Alcohol Dehydrogenase Expression in Drosophila

LARVAE

PUPAE

ADULTS

FIG. 5.—Variation in ADH expression among pair-mated lines. Circles denote values for melanogaster lines, and black dots denote values for simulans lines. The three left-most plots depict the relationships between ADH activity and CRM level; the three right-most plots depict the relationships between CRM level and RNA level. The lines represent significant regressions estimated over all points, ignoring species except for CRM vs. RNA in larvae; in that case, the regression is for the melanogaster points only. For each regression line the slope is significantly different from zero, with $P = 0.0001$. $R^2$ values from the regression analyses are given on each plot.

can be considered representative. Adh clones have been obtained from these lines and are being used to investigate the molecular basis of the ADH expression differences between the species.

Geographic Variation

The eight isolellic simulans lines from experiment 2, for which there was replication within geographic location, show a significant location effect on both adult activity ($P < 0.05$) and CRM ($P < 0.001$). The lines from France are high in activity (315 units), and those from South Africa are low (217), while Japan (241) and Tunisia (237) are intermediate. Significant location effects on adult activity ($P < 0.05$) and CRM ($P < 0.01$) were also observed in the pair-mated lines of simulans and melanogaster analyzed in experiment 3. In this case France is again high (359 units), and...
West Africa is again low (232), while North America (307) and Japan (308) are intermediate.

Biochemical Basis of Activity Variation

Figures 4 and 5 show, for each of the stages analyzed in experiments 2 and 3, the plots of activity versus CRM level. In each case, there is, for each species analyzed separately, a significant regression of activity on CRM. An analysis of covariance was performed on the data set represented by each of these plots, to test for a between-species difference in the slope of the regression line. The slope difference was significant ($P = 0.046$) for only one of the five data sets: adults from the pair-mated lines. Since the slope difference in this case is very small and since the equivalent test for the isoallelic lines was not significant, we conclude that the relationship between activity and CRM is essentially the same for both species. Thus, the regression lines drawn in figures 4 and 5 are taken over all the data points, ignoring species. These plots show that most of the genetic variation in ADH activity, both within and between species, is accounted for by variation in the amount of ADH protein.

The observation that the activity difference between adults is due to a CRM-level difference is consistent with previous reports in the literature. Chambers et al. (1984b) and Heinstra et al. (1987) each analyzed one strain of each species and reported in adults a CRM-level difference that parallels the activity difference (in both cases the melanogaster strain was Adh$^S$). Dickinson et al. (1984) analyzed one Adh$^S$ strain and one simulans strain and also found that in adults a CRM-level difference parallels the activity difference, which in this case was about fourfold.

Figures 4 and 5 also show the relationship, for experiments 2 and 3, between CRM level and RNA level. In only one case, melanogaster larvae in experiment 3, is there a significant regression of CRM on RNA. Thus, it appears that very little of the ADH-protein concentration variation among lines is accounted for by variation in ADH-mRNA.

Discussion

On the basis of a detailed developmental profile of one strain from each species—i.e., strains Was (melanogaster) and Ral 1-2 (simulans)—the patterns of change in ADH activity, in CRM level, and in RNA level are very similar for the two species. The only major difference in shape of the developmental profiles is due to the fact that, in adults between 0–1 and 7–10 d posteclosion, Was shows a large increase in both ADH activity and CRM whereas Ral 1-2 does not. In the relative abundance of distal and proximal transcripts through development, there is no apparent difference between these two strains.

Among strains within species there is, at all developmental stages, considerable quantitative variation in ADH activity, CRM level, and RNA level, but the only statistically significant species-specific differences are in pupal RNA level and in adult activity and CRM level. In both ADH activity and CRM level, in larvae and pupae, there are no significant differences between the species, and there is at these stages considerable overlap among the lines from each species. In adults melanogaster Adh$^S$ strains have, on the average, about twice the ADH activity level of simulans strains, which is consistent with previous reports in the literature. As discussed in the Introduction, in laboratory tests melanogaster adults also consistently have a higher alcohol tolerance than do simulans adults. The ADH activity difference may cause or at least contribute to this alcohol-tolerance difference, although other genes could be at least
as important as those affecting ADH levels. A number of studies of variation within *melanogaster* have shown that ADH activity is not always correlated with alcohol tolerance (see Gibson and Oakeshott 1982; Gibson and Wilks 1988). If ADH activity is a prime factor in determining alcohol tolerance, then the lack of a species difference in larval and pupal stages is difficult to reconcile with the suggestion that the two species show a difference in distribution with respect to alcohol-containing resources, since larvae and pupae are more intimately associated with the substrate than are adults. Nevertheless, it is possible that adult exposure to ethanol vapor during feeding and oviposition provides a selective agent that has influenced these species’ distributions.

Over strains within species, at each developmental stage there is a significant regression of ADH activity on CRM level, and this regression is homogeneous between species. Furthermore, the regressions have very high $R^2$ values. These results indicate that most, if not all, of the activity variation within and between species is accounted for by variation in the concentration of ADH protein. In contrast, the regression of CRM level on RNA level is not significant, except in *melanogaster* larvae. This result indicates that the ADH concentration variation among lines that is observed in pupae and adults is not accounted for by variation in mRNA template concentration. Therefore, the variation in ADH concentration must be due either to variation in rate of translation of the mRNA or to variation in protein stability. Both translational efficiency and protein stability might be due either to genetic background differences between the species (*trans*-acting modifier genes) or to *cis*-acting factors located within the *Adh* sequences that specify the mature message. Comparison of the *melanogaster Adh*\(^5\) consensus sequence of Kreitman (1983) and the *simulans Adh* sequence of Cohn and Moore (1988) shows two amino acid differences, which could confer differential protein stability, and 19 other differences within the mature distal message, which could confer differential translational efficiency. We are presently conducting an interspecific gene transfer experiment, using P-element-mediated transformation, to determine the relative contributions of *cis*- and *trans*-acting factors to the *Adh* expression difference between adult of these two species.

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LITERATURE CITED


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