POPULATION GENETICS OF DROSOPHILA AMYLASE. I. GENETIC CONTROL OF TISSUE-SPECIFIC EXPRESSION IN *D. PSEUDOOBSCURA*[1]

JEFFREY R. POWELL AND JENIFER M. LICHTENFELS

Department of Biology, Yale University, New Haven, Connecticut 06520

Manuscript received October 2, 1978
Revised copy received January 22, 1979

ABSTRACT

Drosophila pseudoobscura is polymorphic for tissue-specific expression of α-amylase in adult midguts. This enzyme is encoded by a single locus, *Amy*, on the third chromosome. In this paper we show: (1) Up to about 12 days post-eclosion, the midgut activity patterns remain stable; after 12 days areas not showing activity previously begin to show activity. Thus, the genes controlling the expression of *Amy* are temporally acting. (2) Diet affects the quantitative, but *not* the qualitative, expression of *Amy*. (3) The expression of *Amy* in adult midguts is under genetic control. Selection for different frequencies of patterns is possible; realized heritabilities are 0.20 to 0.50. Partial linkage with third chromosome inversions has been demonstrated; the genes or elements controlling *Amy* expression are not, howevr, confined to the third chromosome. (4) The genetic elements controlling tissue-specific expression of amylase do not coordinately control the expression of five other "digestive-type" enzymes that were studied.—This polymophism appears to be analogous to that studied by ABRAHAM and DOANE (1978) in *D. melanogaster*, wherein they have mapped regulatory genes.

THAT adaptive evolution, both on the level of local population adaptation and speciation, occurs by genetic changes is hardly doubted by any biologist today. However, the exact nature of the genetic basis of adaptive evolutionary change seems to become less clear as our knowledge of genetics increases. For example, despite over ten years of extensive work (reviewed in LEWONTIN 1974; NEVO 1978; KIMURA 1977; AYALA 1976), the adaptive significance of protein polymorphisms and amino acid substitutions remains unclear. Strong arguments can be made that nearly all of the protein variation within populations is adaptively neutral and amino acid substitutions generally occur by random events. If so, then we must look for another type (or types) of genetic variation that can account for Darwinian evolution. One proposal is that changes in gene regulation are more important than changes in structural genes themselves (WILSON 1976). That is, variation in when, where and how much of a protein is made is more important than variation in the amino acid sequence of the protein itself.

The *sine qua non* of population genetics research is polymorphism. Therefore,

[1] Supported by National Science Foundation Grant DEB76-19971.

if we are to reach a full understanding of the role that variation in gene regulation plays in evolution, we need to define and study naturally occurring polymorphisms. Recently, ABRAHAM and DOANE (1978) have documented a polymorphism in *Drosophila melanogaster* that, by almost all definitions, would be considered variation in gene regulation. The polymorphism is for tissue-specific expression of the enzyme α-amylase (E.C. 3.2.1.1, α-1,4-glucan-4-glucanohydrolase) in the midgut of adult flies. A Mendelian factor has been identified that controls the presence or absence (or at least much reduced) of α-amylase activity in the posterior midgut of young adult flies. A second such factor has tentatively been identified that controls activity in the anterior midgut (DOANE 1977).

Because *D. pseudoobscura* is especially suitable for population genetic studies (DOBZHANSKY and POWELL 1975), we undertook a survey of this species in a search for a similar polymorphism. As in *D. melanogaster*, we have found variation in the tissue-specific expression of α-amylase in natural populations of *D. pseudoobscura*. This first paper of a series will report the results of our attempts to characterize various aspects of the variation in gene expression and to determine its genetic basis. The accompanying paper (POWELL 1979) reports geographic patterns in natural populations of this polymorphism and compares it with variation at the structural gene locus and inversion polymorphisms.

MATERIALS AND METHODS

Strains of *D. pseudoobscura* from four localities have been used in these studies to elucidate the genetic basis of the variation in α-amylase expression. Strains labelled FLAG were derived from a large cage population begun in 1973 by 23 isofemale lines originally collected by W. W. ANDERSEN in June, 1973. Strains labelled AM are from Amecameca near Mexico City; twelve isofemale lines collected in 1975 have been studied. ZIR strains are from Zirahuen (near Patzcuaro, Mexico); 32 isofemale lines were studied. HAMP is a hybrid population (described in POWELL 1978) originating from a mixture of flies from four widely separated localities. Lines homozygous for a single 2nd or 3rd chromosome were derived using standard crossing schemes with marker stocks *Delta/Bare* (Inv) and *orange Lobe* (SC)/*orange* (ST) respectively. Second chromsomes homozygotes were from Furnace Creek in Death Valley and were kindly supplied by STEPHEN H. BRYANT. Unless explicitly stated, all flies were reared on standard cornmeal-molasses-agar medium at 19°. To test for the effects of diet on the patterns, we used two other media described by DOANE (1969). One was a sucrose medium consisting of 88.5% water, 1.5% agar, 5.0% inactivated yeast, 5.0% sucrose. The other was a starch medium consisting of 90.5% water, 1.5% agar, 5.0% inactivated yeast, 3.0% soluble starch. Because these media are particularly susceptable to microorganism infections, a somewhat higher than usual concentration of proprionic acid (0.6%) was used as a mold inhibitor.

Midgut amylase activity were ascertained according to the method of ABRAHAM and DOANE (1978). Midguts were dissected from three- to six-day-old adults (except when explicitly stated otherwise) in Shen's solution. Flies were placed on fresh medium for 12 to 48 hr prior to dissection. After three rinses in clean saline, the midgut was placed on a slide that had a thin film of Mayr's albumin on it. The slide was air dried about five minutes; this assured lysis of cells and release of intracellular enzymes. A drop of starch-agar suspension kept just above the point of solidification (50 to 55°) was layered over the gut. This suspension consisted of 0.25% Connaught hydrolysed starch and 1.5% Bacto-agar in 0.05 M Tris-HCl buffer, pH 7.4, 0.003 M $CaCl_2$. After ten min of incubation at room temperature ($\simeq$ 22°) the slide was fixed in Carnoy's solution, rehydrated through two concentrations of ethanol (50% and 30%), stained in I_2-KI

solution, rinsed in distilled water, and air dried. α-Amylase activity is indicated by clear areas surrounded by dark-blue stained starch. All slides were scored within a few hours of preparation, though they remain quite stable for at least several days.

The structural gene for α-amylase production is designated *Amy*. Electrophoretically detectable variation at this locus was determined by polyacrylamide gel disc electrophoresis, as described by DOANE (1969).

Stains for other enzymes were as follows: Esterase: α-naphthyl acetate or β-naphthyl acetate in a 0.1 M phosphate buffer, pH 6.5, with fast garnet GBC salt added (6 mg/10 ml). Acid phosphatase: 10 mg α-naphthyl acid phosphate and 10 mg Fast Blue BB salt in 10 ml 0.125 M acetate buffer, pH 5.0. Alkaline phosphatase: 10 mg α-naphthyl acid phosphate, 10 mg Fast Blue BB salt, 6 mg $MgCl_2$, 6 mg $MnCl_2$ in 10 mg 0.05 M Tris-HCl buffer, pH 8.6. Peptidase: 7 mg L-leucyl-β-naphthylamide HCl, 4 mg Black K Salt in 5 ml 0.2 M maleic anhydride solution and 5 ml 0.2 M NaOH; pH of resulting solution is 5.0.

RESULTS

Patterns and nomenclature: Figure 1 is a photograph of two common amylase activity patterns from *D. pseudoobscura*. Following the nomenclature of DOANE (1978), we recognize three regions in the anterior midgut (AMG) and two in the posterior midgut (PMG); no activity has ever been observed in the middle midgut (MMG). Phenotypes will be designated as follows: *e.g.*, AMG-123, PMG

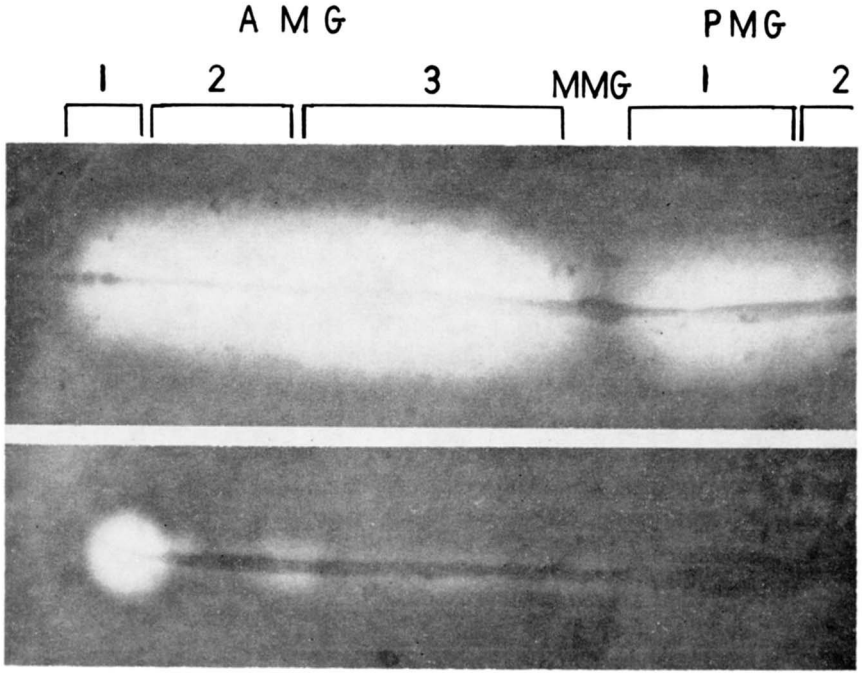

FIGURE 1.—Photographs of two patterns of α-amylase activity in adult midguts of *D. pseudoobscura*. AMG is anterior midgut, MMG middle midgut, and PMG posterior midgut. Upper midgut is from a female and region PGM2 extends further; this pattern is AMG 123 PMG 10. The shorter midgut in lower photograph is a male with pattern AMG 100 PMG 00. Designation of areas follows approximately the nomenclature of DOANE (1978), although relative lengths of areas seem to be somewhat different compared to *D. melanogaster*.

10, where a 1,2, or 3 indicate presence of activity in a particular area and a "O" absence of activity. It may be possible to further subdivide regions, but we have not attempted to do so until more information becomes available.

We have not detected any significant differences between male and female patterns. Therefore, in the data that follow, the sexes are combined.

Age effects: In Table 1, we report the results of testing for age effects on frequencies of midgut activity patterns. Several things can be seen in these data. First, with very rare exceptions, we always observed amylase activity in the AMG-1 region (the cardia) and seldom observed activity in PMG-2 region. Secondly, age does affect the tissue-specific expression of amylase. Early in adult life, the expression is often limited to specific areas; as the fly ages, more areas show activity until essentially the whole midgut (with the exception of the MMG and PMG-2) has activity. Exactly when the transition occurs is temperature dependent. At lower temperatures, it occurs later than at high temperatures. (This temperature range, 10 to 25°, represents the approximate limits at which *D. pseudoobscura* can breed.) In any case, no matter what the temperature, until about 12 days post-eclosion, the polymorphism remains. We have tested other populations and strains of *D. pseudoobscura*, and the results for ZIR are true for the species as a whole, as far as we know.

Thus, the genes involved in the control of midgut amylase activity are temporally acting. This is in agreement with ABRAHAM and DOANE's (1978) findings with *D. melanogaster*. Except when explicitly stated otherwise for all subsequent work, we have used only three to six day old adults.

Media effects: In *D. melanogaster* and *D. hydei*, the type of medium on which a fly has been reared affects the level of amylase activity (DOANE 1969). Generally flies reared on medium with a high concentration of starch produce more amylase activity than do flies reared on a starch-free medium.

TABLE 1

Effect of adult age on frequencies (in percent) of midgut amylase activity patterns in D. pseudoobscura

Pattern		10°						19°					25°			
AMG	PMG	4 days	8 days	12 days	16 days	20 days	24 days	4 days	8 days	12 days	16 days	20 days	4 days	8 days	12 days	16 days
123	10	49	46	51	50	77	100	43	48	57	74	99	46	44	60	98
123	10	2	1	0	0	0	0	5	3	1	0	0	4	3	1	0
120	10	20	22	21	27	19	0	24	21	28	20	1	28	29	29	1
120	00	6	6	5	1	0	0	6	5	3	0	0	4	3	1	1
103	10	11	9	10	12	3	0	9	8	7	2	0	12	9	6	0
103	00	1	3	1	0	0	0	0	1	0	0	0	0	2	0	0
100	10	9	11	11	10	0	0	11	13	4	4	0	6	10	3	0
100	00	3	2	2	0	0	0	2	1	0	0	0	1	0	0	0
n		109	101	110	103	100	97	100	101	100	100	98	110	100	100	99

Several isofemale lines from Zirahuen were combined and were allowed three generations of interbreeding in a large population cage. Newly emerged adults from this population were then aged on standard medium at different temperatures and about 100 midgut assays were made every four days; *n* is number assayed.

TABLE 2

Media effects on midgut amylase activity patterns in D. pseudoobscura

Pattern AMG	Pattern PMG	Standard	Starch	Sucrose
123	12	4	8	3
123	10	98	93	47
123	00	3	10	2
120	10	68	55	35
120	00	5	4	2
103	10	22	25	12
103	00	2	10	6
100	10	13	12	8
100	00	8	8	3
	Standard *vs.* Starch:	$\chi^2 = 6.86$	$p = 0.55$	8 d.f.
	Standard *vs.* Sucrose:	$\chi^2 = 3.43$	$p = 0.90$	8 d.f.
	Starch *vs.* Sucrose:	$\chi^2 = 12.27$	$p = 0.14$	8 d.f.
	Overall 3×9	$\chi^2 = 14.96$	$p = 0.53$	16 d.f.

Flies are from a large population cage derived from the Flagstaff, Arizona, natural population. The numbers of each pattern are shown. Contingency χ^2 tests were nonsignificant for all tests. (One AMG 003 PMG 10 phenotype was found in the starch-reared group; it is excluded from the χ^2 calculations.)

We reared the FLAG population of *D. pseudoobscura* on standard medium, starch medium, and sucrose medium. Table 2 shows the results of surveys of adult midgut activity patterns from each group. While there were clear quantitative differences in strength of amylase staining (starch > standard > sucrose), the qualitative patterns remained the same.

Thus we conclude that the qualitative patterns of midgut amylase activity are independent of the environment, at least with respect to the three media used here. From Table 1, we can also conclude that up to about 12 days of age, the patterns are independent of temperature.

Selection experiments: We carried out several selection experiments on both HAMP and FLAG in an attempt to derive lines that would breed true for a single midgut activity pattern. We selected for presence or absence of activity in the PMG or in the AMG separately. Selection was done by allowing single pairs to mate and dissecting both parents after five to six days (at 25°) when the female had deposited eggs. Figure 2 shows the response to selection of several lines from the two populations. The number of lines selected varied from four to 15 per population per character. For example 11 lines from FLAG were selected for increase in PMG 10 frequency and 15 for decrease in PMG 10 frequency. Because of the possibility that a high concentration of starch (such as in standard medium) may inadvertently select against flies with limited amylase activity, we also carried out selection on four HAMP lines maintained on sucrose medium. The response to selection for no (or little) PMG amylase activity on sucrose medium (dotted line in Figure 2A) was marginally more successful as compared to standard medium (dashed line Figure 2A). Selection for less activity

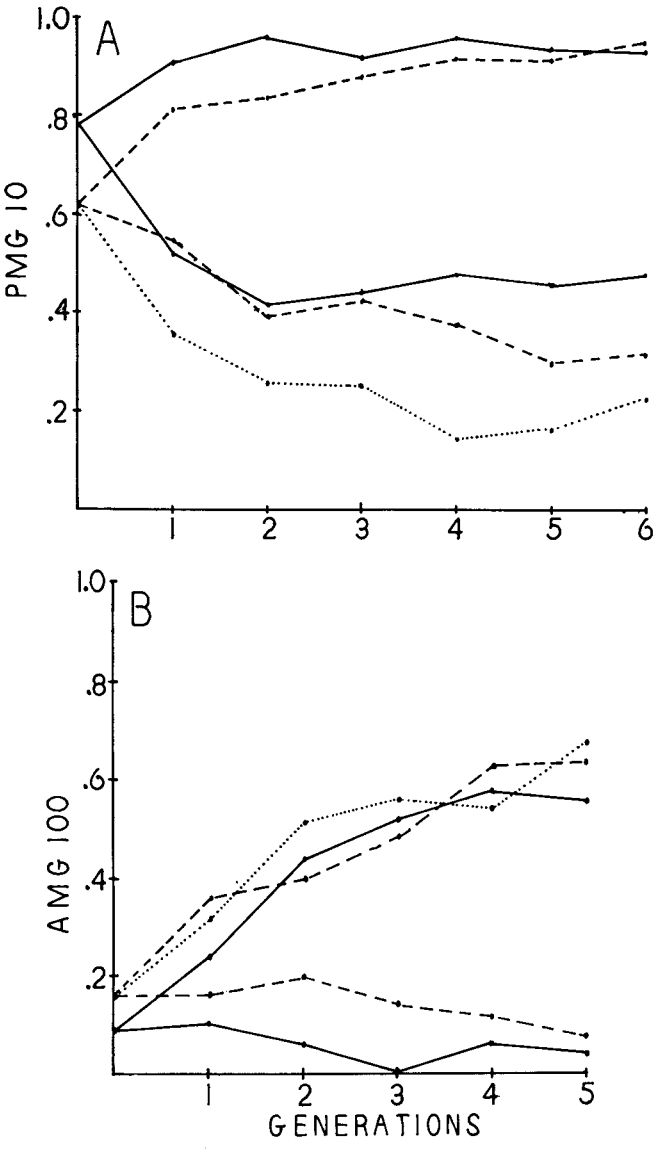

FIGURE 2.—Results of selection experiments. Each point on the graphs represents a mean of four to 15 lines selected in each direction. The solid line is for the FLAG population; dashed line is HAMP population; dotted line is HAMP-SUC (HAMP on sucrose medium rather than standard medium). Graph A is selection for or against posterior midgut activity, the alternative phenotypes being PMG-10 and PMG-00. Graph B is selection for and against anterior midgut activity the phenotypes being AMG 100 *versus* AMG 120, AMG 103, or AMG 123; that is, presence of activity in either region 2 or 3 or both was considered as one alternative.

in the AMG on sucrose *versus* standard media was not different (dotted *vs.* dashed line in Figure 2B).

Table 3 presents the realized heritabilities from the data in Figure 2. Realized heritability is the ratio of selection gain to selection differential; it represents the proportion of variance of a character due to additive genetic variance (*cf.*, FALCONER 1960). Because of fluctuations in selection response, after the first two generations some lines give a negative heritability. The mean heritability for the PMG patterns for the first two generations is 0.34 ± 0.05 and 0.20 ± 0.03 for the AMG patterns. [Other methods of calculating heritability for an "all-or-none" trait exist (*e.g.*, FALCONER 1965; ROBERTSON and LERNER 1949). However, these methods require assumptions about the underlying genetic basis of a trait, assumptions that may greatly influence any heritability estimates (KIDD and CAVALLI-SFORZA 1973). Realized heritability is probably more informative and interpretable.]

It should also be noted that a trait with a realized heritability of less than one may be completely under genetic control. This quantity does not take into consideration variance due to interactions among genes nor variance due to dominance (for which there is evidence).

Selection has been carried out on some lines for as many as 12 generations. However, little further gain than shown in Figure 2 was achieved. We have yet to completely fix any line for a single pattern.

Dominance and segregation: We have made many crosses to learn more about the genetic basis of the different patterns in *D. pseudoobscura*. ABRAHAM and DOANE (1978) have identified at least one Mendelian factor that controls presence or absence of PMG activity in *D. melanogaster*. It maps about 2 c.o. units from *Amy*. A similar gene controlling activity patterns in the AMG has also been tentatively mapped close to *Amy* (DOANE 1977). Despite extensive effort, we have been unable to isolate any single Mendelian factor that controls amylase activity in any region of the midgut in *D. pseudoobscura*. However, we have learned more about the genetic control of the patterns from these attempts.

Table 4 shows the results of 164 single-pair crosses in which both parents and progeny were assayed for midgut amylase activity. In F_2 and backcrosses (to the parental strains, not the actual parents since they were dissected by six days post-eclosion), simple Mendelian segregation was seldom observed. When ratios approximated Mendelian expectations (in 18 of 164 crosses), attempts to repeat the results were unsuccessful.

TABLE 3

Realized heritabilities from selection experiments

	PMG +		PMG —			AMG —		
Generation	HAMP	FLAG	HAMP	HAMP-SUC	FLAG	HAMP	HAMP-SUC	FLAG
0 → 1	0.50	0.57	0.13	0.42	0.34	0.25	0.19	0.16
1 → 2	0.16	0.56	0.26	0.28	0.19	0.06	0.28	0.26
2 → 3	0.25	—1.0	—0.05	0.04	—0.05	0.12	0.10	0.14

"+" means selection for activity in a region, "—" is selection for absence of activity in region. See text and Figure 2 for more details.

TABLE 4

Results of 164 single-pair crosses of D. pseudoobscura *in which both parents and offspring were surveyed for amylase midgut activity patterns*

	AMG 2	AMG 3	PMG 1
Both parents with activity	93% (73)	91% (92)	94% (128)
One parent with activity	79% (64)	82% (55)	88% (27)
Both parents without activity	31% (27)	22% (17)	43% (9)

The percentage of offspring showing activity in the region considered are given; *i.e.*, in 73 crosses in which both parents showed AMG 2 activity, 93% of the progeny showed AMG 2 activity. The numbers in parentheses are the number of crosses of each type; between 4 and 22 progeny per cross were assayed.

Despite this inability to find Mendelian factors, the results in Table 4 do give further evidence that midgut activity patterns are at least partially genetically determined. Furthermore, it appears that, in general, presence of activity in a region tends to be dominant over absence of activity.

The HAMP population is fixed for the third chromosome gene arrangement, ST, and FLAG for AR. Heterozygotes between these strains show much reduced third chromosome recombination. By using parents homozygous for different electrophoretically detectable *Amy* alleles, we could detect "linkage" between the genes controlling midgut patterns and the inversions. Table 5 shows typical results for PMG activity. Similar results have been obtained for AMG regions 2 and 3. We have been unable to demonstrate unambigously a complete linkage of midgut pattern with third chromosome gene arrangements. However, results such as in Table 5 are repeatable and have been observed several times. Thus, we can conclude that at least some of the genes controlling amylase midgut activity patterns reside on the third chromosome.

However, genes on the third chromosome are not the only factors controlling amylase activity. Lines made homozygous for a single third chromosome, using marker stocks with an inversion, still show variation in midgut activity patterns. This is true for chromosome *2* as well. While it appears that homozygosis for a

TABLE 5

Results indicating partial linkage of third chromosome inversions (AR and ST) with control of PMG amylase activity

Parents ♀(AR)		Parents ♂(ST)		F_1		Back cross (to parental strain and phenotype)	
PMG	*Amy*	PMG	*Amy*	PMG	*Amy*	PMG	*Amy*
10	F/F	00	S/S	13 with PMG10	F/S	36 with PMG00	S/S
				1 with PMG00	F/S	13 with PMG10	S/S
						7 with PMG00	F/S
						36 with PMG10	F/S

Amy F marks AR and *S* marks ST. These allozymes probably correspond to PRAKASH and LEWONTIN's (1968) *Amy 0.84* and *1.00*, respectively. Only backcrosses of F_1 females to males of the parental strain with a PMG 00 phenotype and *AMY S/S* were scored.

chromosome reduces the variation, it is difficult to assess this quantitatively since a strain homozygous for one chromosome has other chromosomes that are likely to be recombinants with the marker strain.

Other enzyme studies: It is conceivable that the genetic system controlling the tissue-specific expression of amylase in midguts coordinately controls several other enzymes, especially those serving a digestive function. To test for this possibility, we have stained midguts of *D. pseudoobscura* for other enzyme activities. The procedure was the same as for amylase except, instead of layering the starch-agar suspension over the gut, we added one of the staining solutions described in MATERIALS AND METHODS. The guts were continuously observed for one to two hours. About 100 flies from four different populations were stained for each enzyme activity. The results were:

Esterases: Using either α-naphthyl acetate or β-naphthyl acetate as a substrate, strong esterase activity could be detected throughout the whole midgut, including MMG. No variation among individuals was detected.

Acid and alkaline phosphatase: Acid phosphatase activity was throughout the gut with no variation. Alkaline phosphatase activity was observed throughout the AMG and PMG, but not in MMG. Again, no variation in this pattern was observed.

Peptidase: Peptidase activity was not uniform throughout the midgut. The anterior end (cardia) and MMG showed no activity. There appeared to be a gradient of activity increasing toward the posterior, beginning about one-third down the length of the AMG, with a gap in the MMG. The PMG was very heavily stained, being especially dark where the malphigian tubules attach at the posterior end. There appeared to be some small variation among individuals with respect to the point in the AMG where activity began. Nevertheless, the activity pattern of peptidase is clearly different from that of amylase.

We conclude that, at least for the enzymes tested, amylase tissue-specific control is not coordinately controlling tissue specificity of other enzymes in the midgut.

DISCUSSION

All of our results indicate that the polymorphism for tissue-specific expression of amylase in *Drosophila pseudoobscura* is analogous to that in *D. melanogaster* studied by ABRAHAM and DOANE (1978). The age effects are similar, the patterns are similar, and both are under genetic control. ABRAHAM and DOANE were able to locate at least one gene, *map* (for midgut activity pattern), close to *Amy*. They worked with strains made homozygous for all three major chromosomes simultaneously. To obtain such strains, they used a multiply marked stock, the analog of which is not available in *D. pseudoobscura*. So far we have been unable to locate any single genes using techniques that are available for *D. pseudoobscura*.

Nevertheless, we have demonstrated by a variety of tests that the variation in *Amy* expression is genetically controlled, at least in part. We have not been able to detect any environmentally induced effects.

We will defer further discussion to the second paper in this series (POWELL 1979).

We thank S. H. BRYANT and W. W. ANDERSON for Drosophila stocks and W. W. DOANE for advice and information. W. W. ANDERSON and two anonymous reviewers made many helpful comments on an early version of this paper.

LITERATURE CITED

ABRAHAM, I. and W. W. DOANE, 1978 Genetic regulation of tissue-specific expression of amylase structural genes in *Drosophila melanogaster*. Proc. Natl. Acad. Sci., U.S. **75**:4446–4450.

AYALA, F. J. (editor), 1976 *Molecular Evolution*. Sinauer Press, Sunderland, Massachusetts.

DOANE, W. W., 1969 *Drosophila* amylases and problems in cellular differentiation. In: *Problems in Biology: RNA in Development*. Edited by E. W. HANLY, Univ. Utah Press, Salt Lake City. ——, 1977 Further evidence for temporal genes controlling *Amy* expression in *Drosophila melanogaster*. Genetics **86**: 515–516. ——, 1978 Control genes for temporal/spatial expression of amylase structural loci in postembryonic differentiation of *Drosophila melanogaster*. Int. Conf. Mol. and Devel. Bio. Insects (abstract).

DOANE, W. W., I. ABRAHAM, M. KOLAR, R. MARTENSON and G. DIEBLER, 1975 Purified *Drosophila* α-amylase: genetical biochemical, and molecular characterization. In: *Isozymes IV. Genetics and Evolution*. Edited by C. L. MARKERT, Academic Press, New York.

DOBZHANSKY, TH. and J. R. POWELL, 1975 *Drosophila pseudoobscura* and its American relatives, *Drosophila persimilis and Drosophila miranda*. In: *Handbook of Genetics*, Vol. 3: *Invertebrates of Genetic Interest*. Edited by R. C. KING, Plenum Press, New York.

FALCONER, D. S., 1960 *Introduction to Quantitative Genetics*. Ronald Press, New York. ——, 1965 The inheritance of liability to certain diseases estimated from the incidence among relatives. Ann. Human. Genet. **29**: 51–76.

KIDD, K. K. and L. L. CAVALLI-SFORZA, 1973 An analysis of the genetics of schizophrenia. Social Biol. **20**: 254–265.

KIMURA, M. (editor), 1977 *Molecular Evolution and Polymorphism*. Natl. Inst. Genet., Mishima, Japan.

LEWONTIN, R. C., 1974 *Genetic Basis of Evolutionary Change*. Columbia Press, New York.

NEVO, E., 1978 Genetic variation in natural populations: patterns and theory. Theoret. Pop. **13**: 121–177.

POWELL, J. R., 1978 The founder-flush speciation theory: an experimental approach. Evolution **32**: 465–474. ——, 1979 Population genetics of Drosophila amylase II. Geographic patterns in *D. pseudoobscura*. Genetics **92**: 613–622.

ROBERTSON, A. and I. M. LERNER, 1949 The heritability of all-or-none traits: viability of poultry. Genetics **34**: 395–411.

WILSON, A. C., 1976 Gene regulation in evolution. In: *Molecular Evolution*. Edited by F. J. AYALA, Sinauer Press, Sunderland, Massachusetts.

Corresponding editor: W. W. ANDERSON