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Inheritance of Enzymes and Blood Proteins in the Leopard Frog, *Rana pipiens*: Three Linkage Groups Established

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Individuals from natural populations of the leopard frog, Rana pipiens, *were analyzed for electrophoretic differences in blood proteins and enzymes from an amputated digit. The proteins examined represent products of 72 loci. Presumptive heterozygotes at multiple loci were selected for experimental crosses. Mendelian inheritance of 18 protein variations were demonstrated in the offspring. Tests for linkage or independent assortment were performed for 75 locus pairs. Three linkage groups were established. Linkage group 1 contains two loci, aconitase-1* (Acon1) *and serum albumin* (Alb), *with a 19% recombination frequency between them. Linkage group 2 contains four loci, glyoxalase* (Gly), *acid phosphatase-1* (Ap1), *acid phosphatase-2* (AP2), *and esterase-5* (Est5). *The data show the relationships* Gly–*21.1%*–AP1–*0%*–AP2–*6.3%*–Est5, *and* Gly–*25.6%*–Est5. *Linkage group 3 consists of four closely linked esterase loci. The data,* Est1–*5.1%*–Est6, Est6–*1.8%*–Est10–*1.9%*–Est4 *and* Est6–*3.0%*–Est4, *do not establish a complete order but suggest that* Est10 *is between* Est4 *and* Est6. *These results, with data demonstrating apparent independent assortment of 67 other locus pairs, provide a foundation for establishing the frog genetic map.*

KEY WORDS: electrophoretic variants; *Rana pipiens*; enzyme inheritance; linkage map.

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INTRODUCTION

In amphibians, as in all other organisms examined, allelic variants at a number of loci controlling the electrophoretic mobility of enzymes exist. The use of these electrophoretic variants is a powerful tool which, until now, has not been exploited for the formal study of genetic inheritance of these loci or to perform genetic linkage studies in amphibians. Many studies utilizing the variability of enzyme mobility of amphibians have been systematic or population studies examining large numbers of animals, sometimes for relatively few enzymes (Rogers, 1973; Dessauer *et al.*, 1975, 1977; Guttman, 1975; Hedgecock, 1976; Case, 1978; Larson and Highton, 1978) or blood proteins (Platz and Platz, 1973). Genetic interpretations for electrophoretic variants have, for the most part, been based on analogy with similar systems in *Drosophila* or mammals.

A number of studies (Johnson and Chapman, 1971; Wright and Subtelny, 1971; Gallien *et al.*, 1973; Wall and Blackler, 1974; Elinson, 1975) have taken advantage of the fact that closely related amphibian species have some electrophoretically distinguishable enzyme variants. Analysis of hybrid offspring enabled the examination of the expression of genes controlling the production of specific proteins during early embryogenesis. Where backcross hybrids were analyzed, the variants appeared to be inherited in a Mendelian manner as codominant alleles (Wright, 1975; Szymura and Farana, 1978). Only one study (Wright, 1975) suggested a possible linkage between glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase loci. Studies of this kind are limited to the enzymes which vary between species and by the frequent early lethality of hybrid embryos, precluding examination of enzymes or blood proteins fully expressed only in metamorphosed individuals.

Linkage relationships may be tested by crossing animals with genetic differences at two or more loci. The F_1 offspring are then backcrossed to one or both parents, and backcross offspring are analyzed for segregation and recombination of the genes of interest. Traditional genetic linkage studies of this sort in amphibians have been impeded by the relatively long generation times of many species and the lack of defined strains having identifiable genetic variants at multiple loci.

An alternative to production of sexually mature F_1 individuals and subsequent analysis of backcross hybrid offspring is the analysis of offspring of naturally occurring heterozygotes (Wright *et al.*, 1976). In the present study we took advantage of enzyme polymorphisms in natural populations of the leopard frog, *Rana pipiens*. Using a screening procedure involving many different enzyme systems, we selected for experimental crosses individuals heterozygous at several loci. Individuals heterozygous at up to nine enzyme and protein loci were crossed with individuals of the same species homozygous

at these loci. Thus, in a single generation, a formal analysis of the genetic inheritance of these loci was conducted simultaneously with a test cross to examine genetic linkage.

MATERIALS AND METHODS

Screening Adults for Biochemical Variants

A toe and a blood sample from five gravid females and one male *Rana pipiens* from each of two populations, Wisconsin and Vermont, were collected at the University of Michigan Amphibian Facility and shipped in dry ice to Houston. Blood was drawn from the musculocutaneous vein into a capillary tube and centrifuged to separate the blood cells and plasma.

Processing for electrophoretic analysis generally followed the procedures of Siciliano and Shaw (1976). Toes (approximately 0.1–0.2 g each) were minced on a glass plate resting on ice and homogenized by hand in a 1.0-ml-capacity glass homogenizer (Duall, Kontes). For each 0.1 g of tissue, 0.15 ml of homogenizing buffer (HM) consisting of 0.01 M tris-HCl, *p*H 7.5, containing 0.001 M EDTA and 0.001 M β-mercaptoethanol was used. After the adults had produced progeny, a portion of the liver was removed and homogenized in 0.30 ml of buffer per 0.1 g tissue. Homogenates were centrifuged at 15,000*g* for 20 min, and the supernatant fluids were subjected to vertical starch gel electrophoresis in either a tris–citrate (TC) buffer system, *p*H 7.0 (0.13 M tris–0.043 M citrate electrode buffer and 0.009 tris–0.003 M citrate in the gel), or a tris–versene–borate (TVB) buffer system, *p*H 8.0 (0.5 M tris–0.016 M EDTA–0.65 M borate electrode buffer and 0.05 M tris–0.0016 M EDTA–0.065 M borate in the gel), Connaught starch (Fisher Scientific) at 90 or 95 g/600 ml gel buffer was used. Two drops of β-mercaptoethanol was added to the 600 ml gel buffer mixture after boiling and degassing. The gel molds used are described in Siciliano and Shaw (1976).

After electrophoresis each gel was sliced to produce multiple gel slabs (six to eight from a 9.0-mm-thick gel) for enzyme staining. The enzymes examined in this preliminary screen are listed in Table I. The procedures for staining are found in Siciliano and Shaw (1976) or Harris and Hopkinson (1976).

Blood samples were analyzed by slab polyacrylamide gel electrophoresis modified from the method of Davis (1964). Red blood cell samples were lysed by freezing and thawing. An equal volume of homogenizing buffer (HM) was added to the blood cells and the mixture centrifuged at 15,000*g* for 20 min. The pellet containing nuclei was discarded. Five microliters of blood cell extract or plasma was combined with 5 μl of a mixture containing 0.125 M tris-HCl buffer, *p*H 6.7, 0.1 M β-mercaptoethanol, 20% glycerol, and 0.01% bromophenol blue and applied to sample slots in a slab gel 1.5 mm thick. The

Table I. Protein Systems Tested for Variants

Enzymes and abbreviations	E.C. number[a]	Buffer system[b]	Loci
1. Acid Phosphatase (AP)	3.1.3.2	TC	3
2. Aconitase (Acon)	4.2.1.3	TC	2
3. Adenylate kinase (AK)	2.7.4.3	TVB	1
4. Adenosine deaminase (ADA)	3.5.4.4	TC	1
5. Aldolase (Ald)	4.1.2.7	TVB	2
6. Creatine Kinase (CK)	2.7.3.2	TVB	2
7. Esterase (EST)	3.1.1.1	TC, TVB	13
8. Fumarase (Fum)	4.2.1.2	TVB	1
9. α-Galactosidase (α-Gal)	3.2.1.22	TVB	1
10. Glucose-6-phosphate dehydrogenase (G6PD)	1.1.1.49	TVB	1
11. Glucosephosphate isomerase (GPI)	5.3.1.9	TVB	2
12. α-Glucosidase (α-GSD)	3.2.1.20	TVB	3
13. β-Glucosidase (β-GSD)	3.2.1.21	TVB	3
14. β-Glucuronidase (β-GUR)	3.2.1.31	TVB	2
15. Glutamate oxaloacetate transaminase (GOT)	2.6.1.1	TVB	2
16. Glyceraldehyde-3-phosphate dehydrogenase (G3PD)	1.2.1.12	TC	1
17. α-Glycerophosphate dehydrogenase (α-GPD)	1.1.1.8	TVB	2
18. Glyoxalase I (Gly)	4.4.1.5	TVB	1
19. Glutathione reductase (GSR)	1.6.4.2	TVB	1
20. Hexosaminidase (HA)	3.2.1.30	TVB	2
21. Hexokinase (HK)	2.7.1.1	TVB	1
22. Isocitrate dehydrogenase (IDH)	1.1.1.42	TC	2
23. Lactate dehydrogenase (LDH)	1.1.1.27	TC	2
24. Malate dehydrogenase (MDH)	1.1.1.37	TC	2
25. Malic enzyme (ME)	1.1.1.40	TC	2
26. Mannosephosphate isomerase (MPI)	5.3.1.8	TVB	1
27. α-Mannosidase (α-Man)	3.2.1.24	TVB	1
28. Nucleoside phosphorylase (NP)	2.4.2.1	TC	1
29. Peptidase (PepLA and PepLGG)	3.4.3.1	TVB	3
30. 6-Phosphogluconate dehydrogenase (6PGD)	1.1.1.44	TC	1
31. Phosphoglucomutase (PGM)	2.7.5.1	TC	3
32. 2,3-Phosphoglycerate mutase (PGAM)	5.4.2.1	TVB	2
33. Tetrazolium oxidase (TO)	1.15.1.1	TVB or TC	1
34. Triosephosphate isomerase (TPI)	5.3.1.1	TVB or TC	1
Blood proteins and abreviations			
35. Hemoglobin (Hb)		PAGE	2
36. Albumin (Alb)		PAGE	1

[a] E.C. number according to Commission on Biochemical Nomenclature (1972).
[b] Buffer system used for electrophoresis as abbreviated in text.

resolving gel contained 10% polyacrylamide (30 parts acrylamide, 0.8 part bisacrylamide) in 0.37 M tris-HCl, *p*H 8.9. A 4% polyacrylamide stacking gel was used buffered with 0.125 M tris-HCl, *p*H 6.7. The electrode buffer was 0.028 M tris–0.192 M glycine, *p*H 8.3. Electrophoresis was at 100 V for approximately 4.5 hr. Positions of hemoglobins were marked by poking a hole in the gel or photography before fixation with 50% trichloroacetic acid and

staining with 0.1% Coomassie blue. The gels were cleared by soaking in 5% methanol–7% acetic acid.

Analysis of Biochemical Variants in Offspring

Adult animals with multiple heterozygous loci were chosen as parents and appropriate crosses were made. Fertilizations were done *in vitro* according to procedures described in Rugh (1962) and Nace *et al.* (1974). Offspring were reared through metamorphosis in Ann Arbor. Live juveniles were shipped to Houston. Animals were anesthetized in tricaine methanesulfonate (Finquel, Ayerst Laboratories), 0.2 mg/ml. Sex was determined by inspection of the gonads under a dissecting microscope. Blood was collected from a cut in the heart ventricle in a heparinized glass capillary tube, diluted 1:1 in amphibian citrate saline (*p*H 7.5) (4.9 g NaCl, 8.0 g Na-citrate·$2H_20$ in 1 liter) and centrifuged at 300*g*. Blood cells were sometimes washed by resuspending in amphibian citrate saline.

Whole leg extracts of juvenile frogs were prepared in the same way as adult toe extracts. Liver was homogenized using a ratio of 1 g tissue to 4 ml HM buffer (15 mg liver to 60 μl HM). Juvenile frog tissues were analyzed electrophoretically only for those proteins which were variant in one of the parents.

Note on Nomenclature

Several enzymes occurred as multiple isozymes. Isozymes were numbered so that the most anodal form was designated "1." The genetic locus controlling the isozyme is designated by the same number. This is consistent with the system generally used in the mouse. Exceptions to this rule are those systems for which a prior nomenclature has been established such as for the hemoglobins (Gillespie and Crenshaw, 1966). Alleles at a locus were assigned letters alphabetically. While the tendency was to call the most anodal form "*a*," the letter assignment in general reflects the order of discovery of an allelic form and not its relative mobility or its relative frequency. In some cases alleles are indicated as *a* and *a′* or *b* and *b′*, indicating alleles having products with very close electrophoretic mobilities.

RESULTS

Electrophoretic Patterns of Enzyme Variants

The protein products of 72 different loci were examined (Table I). Eighteen of these showed electrophoretic variants, the details of these variants are discussed below.

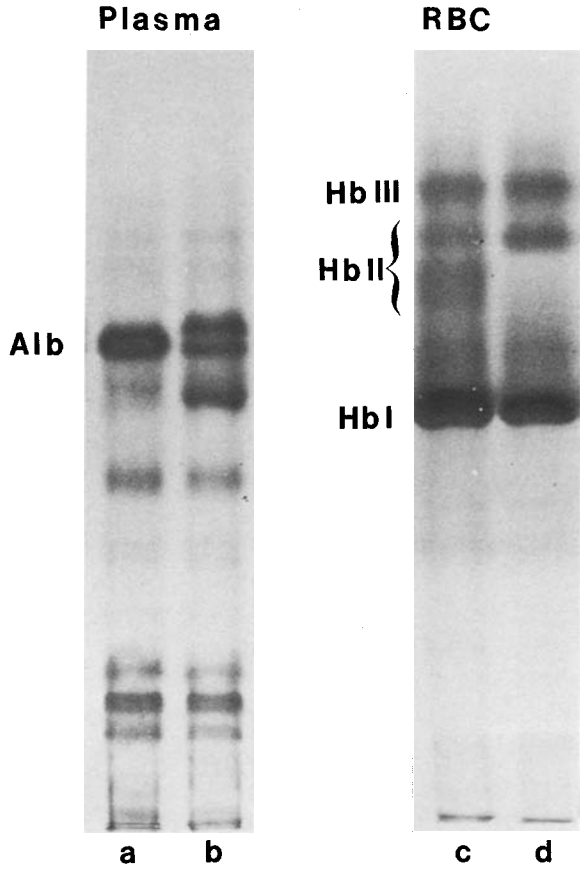

Fig. 1. Photographs of portions of a polyacrylamide gel stained for protein. Columns (a) and (b) show albumin genotypes *b'/b* and *a/b*, respectively, in plasma. Samples (c) and (d) are red cell lysates showing HBII genotypes *a/b* and *a/a*, respectively.

Blood protein patterns produced by polyacrylamide gel electrophoresis (PAGE) are shown in Fig. 1. The product of the *b'* allele at the albumin (*Alb*) locus has a slightly faster electrophoretic mobility than the product of the *b* allele (Fig. 1a), while the *a* allele product is still faster (Fig. 1b). Three hemoglobin zones are seen clearly after staining with Coomassie blue (Fig. 1c,d). HbI is the most abundant form. The genetic variant occurred in the HbII band. HbIIa is reduced in HbII *a/b* heterozygotes (Fig. 1c), and an HbII *b/b* homozygote (not shown) has only the HbIIb band in this region. Hemoglobins HbI and HbII appear to correspond to those forms described by

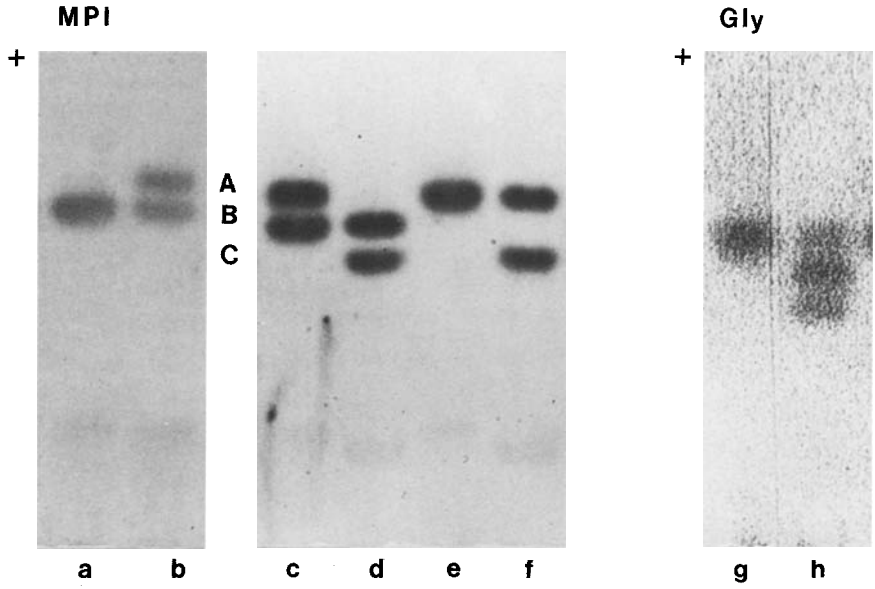

Fig. 2. Photographs of portions of starch gels stained for mannose-6-phosphate isomerase (MPI) or glyoxalase (Gly). MPI genotypes of samples (a) and (b) from cross 200 are (a) *b/b* and (b) *a/b* and of samples (c)–(f) from cross 600 are (c) *a/b*, (d) *b/c*, (e) *a/a*, (f) *a/c*. Gly genotypes (g)–(h) are (g) *a/a* and (h) *a/b*.

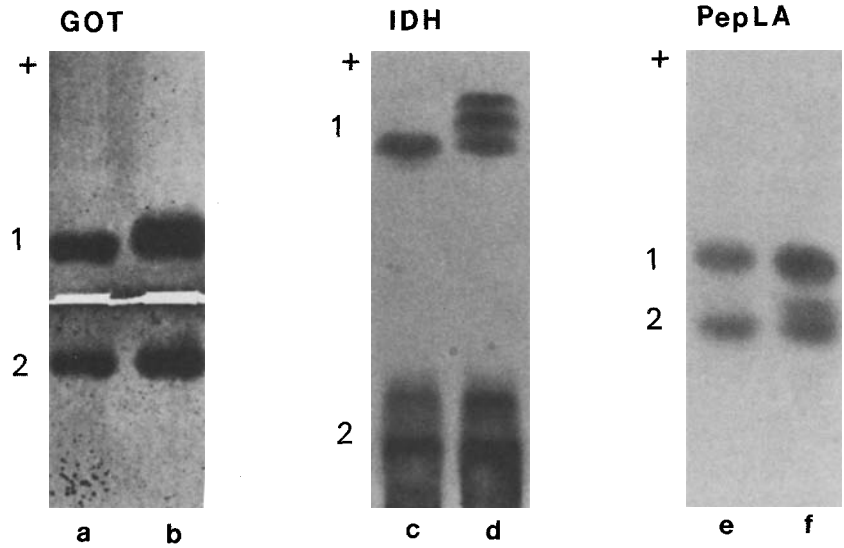

Fig. 3. Photographs of portions of starch gels stained for glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), and peptidase which cleaves the leucylalanine substrate (PepLA). *GOT1* genotypes are: (a) *b/b* and (b) *a/b*; IDH 1 genotypes are (c) *b/b* and (d) *a/b*; *PepLA2* genotypes are: (e) *b/b* and (f) *a/b*.

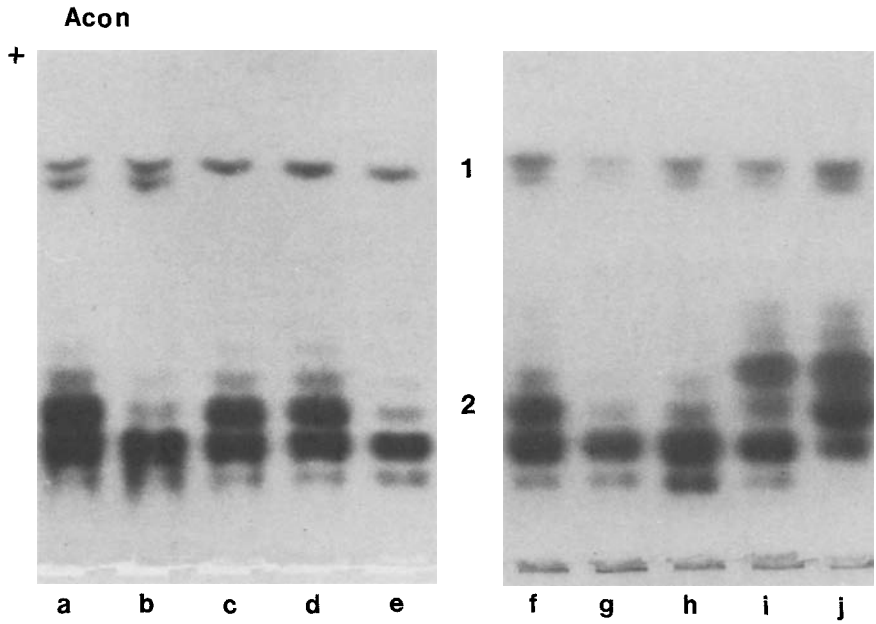

Fig. 4. Photographs of portions of starch gels showing segregation of alleles at aconitase (*Acon1* and *Acon2*) loci. Samples (a)–(e) are from cross 200, (f)–(j) from cross 300. The genotypes of the sample are (a) *Acon1 a/b, Acon2 a/b*; (b) *Acon1 a/b, Acon2 b/b*; (c) *Acon1 a/a, Acon2 a/b*; (d) *Acon1 a/a, Acon2 a/b*; (e) *Acon1 a/a, Acon2 b/b*; (f) *Acon1 a/b, Acon2 a/b*; (g) *Acon1 a/b, Acon2 b/b*; (h) *Acon1 a/b, Acon2 b/b*; (i) *Acon1 a/b, Acon2 c/b*; (j) *Acon1 a/b, Acon2 c/a.*

Gillespie and Crenshaw (1966). HbIII is usually detectable only in newly metamorphosed animals and probably represents a larval hemoglobin. The genetic basis of the variations in albumin and HbII is verified by the pattern of inheritance seen in the offspring of the putative heterozygotes.

Several enzyme systems contain multiple isozymes. Evidence for the control of multiple isozymes by separate genetic loci in *Rana pipiens* is the heritable variation in the electrophoretic mobility in one form but constancy or independent variation in the other forms. Subunit composition can be deduced from the number of electrophoretic forms in heterozygotes. Most of the enzyme systems in the frog are similar to those reported in mammalian systems (Harris and Hopkinson, 1976) as to subunit composition and number of structural loci.

Mannosephosphate isomerase (MPI) and glyoxalase (Gly) in the frog are each coded for by a single genetic locus. MPI is a monomer so that heterozygotes produce only two forms (Fig. 2a,b). Gly is a dimer enzyme and produces a three-band pattern in heterozygotes (Fig. 2g,h).

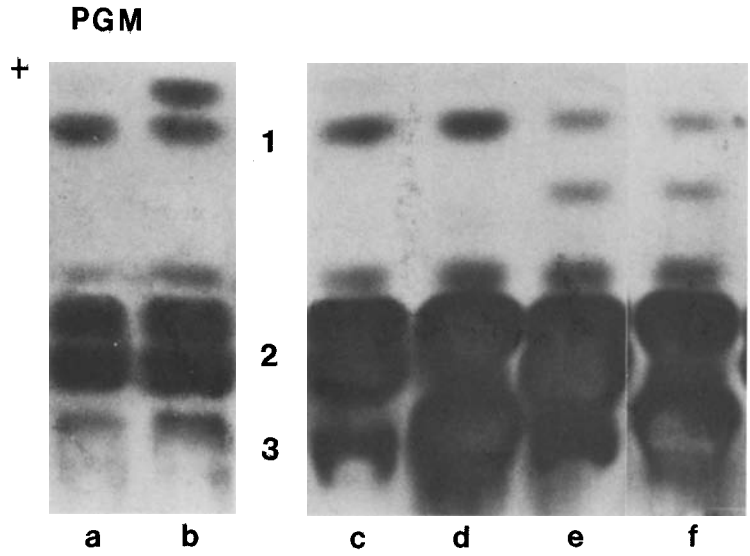

Fig. 5. Photographs of portions of starch gels stained specifically for phosphoglucomutase (PGM). Samples (a) and (b) are from cross 400, (c)–(f) from cross 500. The genotypes of the samples are (a) *PGM1 b/b, PGM2 a/b*; (b) *PGM1 a/b, PGM2 a/b*; (c) *PGM1 b/b, PGM2 a/b*; (d) *PGM1 b/b, PGM2 a/d*; (e) *PGM1 b/d, PGM2 a/b*; (f) *PGM1 b/d, PGM2 a/d.*

Glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), and peptidase, which cleaves the leucylalanine substrate (PepLA), are each coded for by two separate genetic loci, but in each of these cases a variant was found at only one locus (Fig. 3). Aconitase (Acon) also is coded for by two separate genetic loci and independent variants were found at both loci (Fig. 4). Phosphoglucomutase (PGM) and acid phosphatase (AP) are each coded for by three genetic loci and variants were found at two of the loci in each case (Figs. 5 and 6).

Esterases (Est) are detected using α-napthyl esters. Examination of several tissues, blood cells, and plasma identified 13 esterase zones. Not all forms are found in all tissues and not all forms are resolved in each buffer system. The patterns seen in the leg extracts of offspring and toe extracts of the parents of one of the crosses are seen in Fig. 7. Variants reported here include Est1 and Est5, both heterozygous in the male, and Est4, Est6, and Est10, heterozygous in the female. Use of two buffer systems and analysis of several tissues are required to resolve all of the esterase loci. A detailed analysis of esterase variants in *R. pipiens* will be published separately.

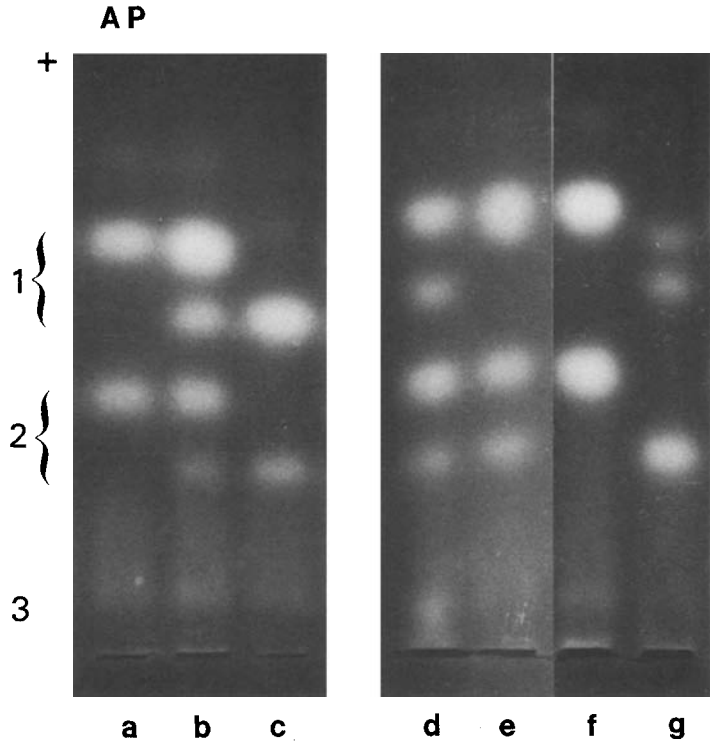

Fig. 6. Photograph of a starch gel stained for acid phosphatase (AP). Samples (a)–(c) are from cross 400, (d)–(g) from cross 600. The genotypes of the samples are (a) *AP1 a/a, AP2 a/a*; (b) *AP1 a/b, AP2 a/b*; (c) *AP1 b/b, AP2 b/b*; (d) *AP1 a/b, AP2 a/b*; (e) *AP1 a/a', AP2 a/b*; (f) *AP1 a/a, AP2 a/a;* (g) *AP1 a'/b, AP2 b/b.*

Inheritance of Enzyme Patterns

The crosses made and the enzymes showing electrophoretic variants in the parents are listed in Table II. Allelic segregation data for each enzyme variant in each cross are shown in Tables III, IV, and V. Table III presents data on the inheritance of enzyme loci from a heterozygous female parent. Table IV contains data on the inheritance of enzyme loci from a heterozygous male parent. Both tables record the number of offspring of each genotype which inherit the alternative alleles from the heterozygous parent. Most crosses are of the test-cross type, yielding homozygotes and heterozygotes, as, for example, those involving the *Acon1* locus. Note that for *Est5* the female parent was homozygous for the *b* allele in cross 300 and the *a* allele in cross 400.

In other crosses a third allele is involved, but the inheritance of the

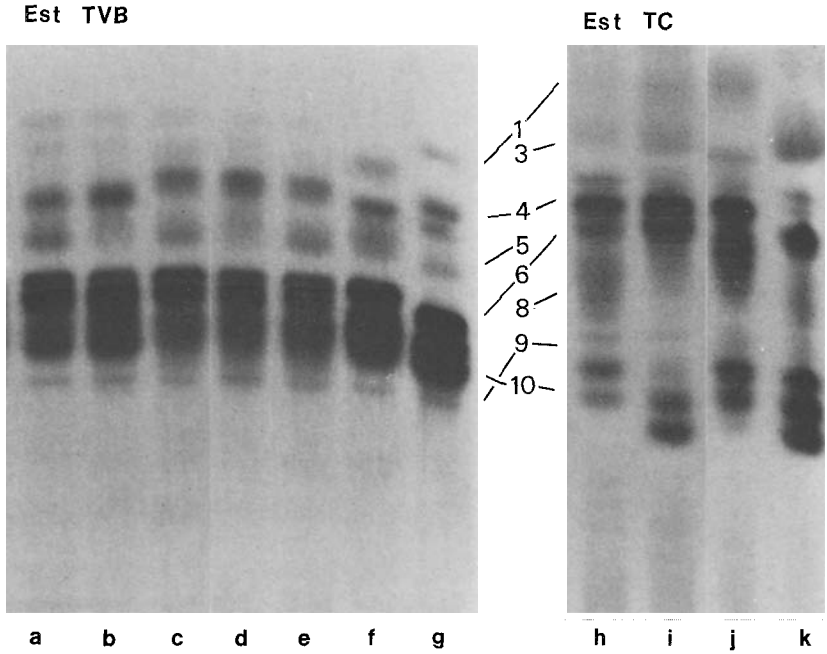

Fig. 7. Photographs of starch gels stained for esterases (Est) using α-naphthyl substrates. Columns (a)–(g) are from a starch gel using the TVB buffer system, columns (h)–(k), the TC buffer system. Columns (a)–(e) and (h)–(i) are from leg extracts of offspring of cross 300; columns (f) and (j) are toe extracts from the father of cross 300, male 32699, and columns (g) and (k) are toe extracts of the mother, female 31041. The positions of the various esterases are indicated by number. *Est1*, *Est4*, *Est5* are read on TVB, *Est6* and *Est10* on TC. *Est1* genotypes are (a) *a*/*b*, (b) *a*/*b*, (c) *a*/*c*, (d) *a*/*b*, (e) *a*/*c*, (f) *b*/*c*, (g) *a*/*a*. *Est4* genotypes are (a) *b*/*c*, (b) *b*/*c*, (c) *a*/*b*, (d) *a*/*b*, (f) *b*/*b*, (g) *a*/*c*. For *Est5* the genotypes are (a) *b*/*b*, (b) *a*/*b*, (c) *b*/*b*, (d) *a*/*b*, (e) *b*/*b*, (f) *a*/*b*, (g) *b*/*b*. *Est6* genotypes are (h) *a*/*d*, (i) *a*/*c*, (j) *a*/*a*, (k) *c*/*d*. *Est10* genotypes are (h) *b*/*b*, (i) *c*/*b*, (j) *b*/*b*, (k) *c*/*b*.

alternative alleles of the heterozygous parent can be clearly distinguished. For example, the male in crosses 300, 400, and 600 was homozygous at the albumin (*Alb*) locus (*b*/*b*) while the three females were heterozygous (*a*/*b*′). Offspring were of two phenotypes *a*/*b* and *b*/*b*′. Other crosses of this type were *Est4* (600), *Est6* (200, 300, 400), *Est10* (500), *PGM2* (500), and *Est1* (300).

Other crosses in which both parents are heterozygous but at least one allele is not shared enable the analysis of segregation of alleles in both parents. For example, in cross 600 the father was *a*/*b* and the mother *a*/*c* for MPI. Segregation of both parental sets of alleles can be distinguished. An *a*/*b* heterozygote (Fig. 2c) must have received the *b* allele from its father and the *a* allele from its mother. Similarly a *b*/*c* heterozygote (Fig. 2d) received the *b* allele from its father and the *c* from its mother. In an *a*/*a* homozygote (Fig. 2e) both parents contributed an *a* allele and an *a*/*c* heterozygote (Fig. 2f) received

Table II. Adult Frogs Heterozygous at Multiple Loci[a]

Animal No.	Sex	Genotype at heterozygous loci[b]		Parent in cross No.
31040	Female	*Acon1 a/b*	*Est6 b/c*	200
		AP1 a/b	*Est10 a/b*	
		AP2 a/b	*PGM1 a/b*	
		Est4 a/b	*GOT1 a/b*	
		Est5 a/b		
31041	Female	*Acon2 c/b*	*Est6 c/d*	300
		Alb a/b'	*Est10 c/b*	
		Est4 a/b	*HbII a/b*	
31042	Female	*Acon1 a/b*	*Est6 b/c*	400
		Alb a/b'	*IDH1 a/b*	
		AP1 a/b	*PepLA2 a/b*	
		AP2 a/b	*PGM1 a/b*	
		Est1 a/b		
31043	Female	*AP1 a/b*	*Est10 a/c*	500
		AP2 a/b	*PGM1 b/d*	
		Est4 a/b	*PGM2 b/d*	
		Est6 a/b		
31044	Female	*Alb a/b*	*GOT1 a/b*	600
		AP1 a/a'	*HBII a/b*	
		AP2 a/b	*MPI a/c*	
		Est4 a/b	*PepLA a/b*	
		Est5 a/b		
32699	Male	*Acon2 a/b*	*Est5 a/b*	200, 300, 400,
		AP1 a/b	*Gly a/b*	500, 600
		AP2 a/b	*MPI a/b*	
		Est1 b/c		

[a] See Table I for description of abbreviations.
[b] At all other loci examined, these animals were homozygous.

the *a* from the father and the *c* from the mother. Others of this type are *AP1* (600) and *Acon2* (300).

Table V contains data on the inheritance of enzyme loci where both parents were heterozygous for the same two alleles, yielding data similar to an F_2 cross. Loci in this category include *AP1* in crosses 200, 400, and 500, *AP2* in crosses 200, 400, and 600, and *Est5* in crosses 200 and 600. The similarity of numbers for *AP1* and *AP2* in crosses 200, 400, and 500 are accounted for by a very tight linkage. (See below.)

The data on segregation of all 18 of the variants whose inheritance was tested show no significant deviation ($p < 0.01$) from the expected normal Mendelian ratio. No sex linkage was detected.

Evidence for Control of *AP1* and *AP2* by Closely Linked Separate Loci

In cross 300 (Table IV) a male heterozygous at both *AP1* and *AP2* was

Table III. Inheritance of Alleles in Offspring of Heterozygous Females[a]

Locus	Cross	Parental genotypes: Female	Parental genotypes: Male		Offspring	χ^2
Acon1	200	*a/b*	*a/a*		46*a/a* 61*b/a*	2.10
	400	*a/b*	*a/a*		75*a/a* 62*b/a*	1.23
				Total	121*a/a* 123*b/a*	0.02
Acon2	300	*c/b*	*a/b*		56*c/-* 55*b/-*	0.009
Alb	300	*b′/a*	*b/b*		64*b′/b* 46*a/b*	2.95
	400	b′/a	b/b		63*b′/b* 79*a/b*	1.80
	600	*b′/a*	*b/b*		65*b′/b* 48*a/b*	2.56
				Total	192*b′/b* 173*a/b*	0.99
AP1	600	*a/a′*	*a/b*		64*a/-* 52*a′/-*	1.24
Est1	400	*a/b*	*b/c*		69*b/-* 61*a/-*	0.49
Est4	200	*a/b*	*b/b*		39*b/b* 41*a/b*	0.05
	300	*a/b*	*b/b*		62*b/b* 53*a/b*	0.70
	500	*a/b*	*b/b*		28*b/b* 24*a/b*	0.31
	600	*a/b*	*b/b*		45*b/b* 58*a/b*	1.64
				Total	173*b/b* 176*a/b*	0.03
Est6	200	*b/c*	*a/a*		43*b/a* 39*c/a*	0.20
	300	*d/c*	*a/a*		48*d/a* 65*c/a*	2.56
	400	*b/c*	*a/a*		62*b/a* 68*c/a*	0.28
	500	*b/a*	*a/a*		28*b/a* 24*a/a*	0.31
Est10	200	*a/b*	*b/b*		36*a/b* 30*b/b*	0.55
	300	*b/c*	*b/b*		60*c/b* 49*b/b*	1.11
	500	*a/c*	*b/b*		26*a/b* 26*c/b*	0.0
GOT1	200	*a/b*	*b/b*		50*a/b* 56*b/b*	0.34
	600	*a/b*	*b/b*		55*a/b* 61*b/b*	0.31
				Total	105*a/b* 117*b/b*	0.65
IDH1	400	*a/b*	*b/b*		85*a/b* 66*b/b*	2.39
MPI	600	*a/c*	*a/b*		56*a/-* 59*c/-*	0.08
PepLA2	400	*a/b*	*b/b*		61*a/b* 81*b/b*	2.82
	600	*a/b*	*b/b*		58*a/b* 58*b/b*	0.0
				Total	129*a/b* 139*b/b*	0.37
PGM1	200	*a/b*	*b/b*		61*a/b* 54*b/b*	0.43
	400	*a/b*	*b/b*		80*a/b* 71*b/b*	0.54
	500	*b/d*	*b/b*		25*d/b* 27*b/b*	0.08
				Total	166-*/b* 152*b/b*	0.62
PGM2	500	*b/d*	*a/a*		28*a/b* 24*a/d*	0.31
HbII	300	*a/b*	*a/a*		50*a/a* 51a/b	0.01
	600	*a/b*	*a/a*		56*a/a* 57*a/b*	0.01
				Total	106*a/a* 108*a/b*	0.02

[a] See Table I for description of abbreviations.

Table IV. Inheritance of Alleles in Offspring of Heterozygous Male

Locus	Cross	Parental genotypes			Offspring		χ^2
		Female	Male				
Acon2[a]	200	*b/b*	*a/b*		49*b/b*	48*a/b*	0.01
	300	*c/b*	*a/b*		60*b/-*	51*a/-*	0.73
	400	*b/b*	*a/b*		70*b/b*	66*a/b*	0.12
	500	*b/b*	*a/b*		19*b/b*	26*a/b*	1.09
	600	*b/b*	*a/b*		55*b/b*	58*a/b*	0.08
				Total	253*b/-*	249*a/-*	0.03
AP1	300	*a/a*	*a/b*		64*a/a*	48*a/b*	2.29
AP2	300	*a/a*	*a/b*		64*a/a*	48*a/b*	2.29
Est1	300	*a/a*	*b/c*		48*a/b*	39*a/c*	0.93
	400	*a/b*	*b/c*		69*−/b*	63*-/c*	0.27
				Total	117*-/b*	102*-/c*	1.03
Est5	300	*b/b*	*a/b*		44*b/b*	59*a/b*	2.18
	400	*a/a*	*a/b*		79*b/a*	57*a/a*	3.56
				Total	123*b/-*	116*a/-*	0.21
Gly	200	*a/a*	*a/b*		50*a/a*	65*a/b*	1.96
	300	*a/a*	*a/b*		47*a/a*	55*a/b*	0.63
	400	*a/a*	*a/b*		73*a/a*	68*a/b*	0.18
	500	*a/a*	*a/b*		27*a/a*	25*a/b*	0.08
	600	*a/a*	*a/b*		64*a/a*	51*a/b*	1.47
				Total	261*a/a*	264*a/b*	0.02
MPI	200	*b/b*	*a/b*		57*b/b*	59*a/b*	0.03
	300	*b/b*	*a/b*		50*b/b*	62*a/b*	1.29
	400	*b/b*	*a/b*		80*b/b*	65*a/b*	1.55
	500	*b/b*	*a/b*		18*b/b*	33*a/b*	4.41
	600	*a/c*	*a/b*		61*b/-*	54*a/-*	0.43
				Total	266*b/-*	273*a/-*	0.09

[a] See Table I for description of abbreviations.

test-crossed to a female homozygous for the *a* alleles at both loci. Among the 112 offspring analyzed, there were 64 *AP1 a/a, AP2 a/a*; and 48 *AP1 a/b, AP2 a/b* and no recombinants (*AP1a/a, AP2 a/b*; or *AP1 a/b, AP2 a/a*). In three other crosses (200, 400, 500, Table V) both parents were *AP1 a/b, AP2 a/b*. Among the combined 306 offspring, all were accounted for as *AP1 a/a, AP2*

Table V. Inheritance of Alleles, Both Parents Heterozygous

Locus	Cross	Genotypes of offspring			χ^2
		a/a	*a/b*	*b/b*	
Apl[a]	200	23	68	23	4.24
	400	28	72	43	2.16
	500	12	29	8	2.31
		63	169	74	4.14
AP2	200	23	68	23	4.24
	400	28	72	43	3.16
	500	12	29	8	2.31
	600	33	55	28	0.74
		96	224	102	1.78
Est5	200	22	26	15	3.48
	600	16	46	17	2.16
		38	72	32	0.54

[a] See Table I for description of abbreviations.

a/a; *AP1 a/b*, *AP2 a/b*; or *AP1 b/b*, *AP2 b/b* (Fig. 6a,b,c). No recombinants were found.

Separate *AP* loci are indicated by the independent variation of *AP1* and *AP2* found in one of the females (31044) used in cross 600 (Table III). Originally, we believed that she was a recombinant type having one band in the *AP1* (*a/a*) region and two in the *AP2* (*a/b*). Her pattern was the same as that of the offspring shown in Fig. 6e. However, among offspring of this female with a male having the *AP1 a/b*, *AP2 a/b* phenotype as in Fig. 6d, four types of offspring were seen. These include those having the parental patterns (6d, 6e) and one homozygous for the *a* alleles at both *AP1* and *AP2*. The fourth type, seen in Fig. 6g, had the *AP1 b* allele of the father and an *AP1 a'* allele having a slightly slower mobility than the *a* allele product. This animal was also homozygous at the *AP2* locus with the normal *AP2 b/b* pattern. Seeing the *a'/b* phenotype also indicated that the broad band shown in Fig. 6e was actually an *AP1 a/a'* genotype. Only the four genotypes shown in Fig. 6d–g were found in the offspring of the 600 cross. Out of 116, there were 33 *AP1 a/a*, *AP2 a/a*; 24 *AP1 a/a'*, *AP2 a/b*; 31 *AP1 a/b*, *AP2 a/b*; and 28 *AP1 a'/b*, *AP2 b/b*.

The tight linkage between the *AP1* and *AP2* loci made it possible to

identify the parental contribution to the *AP1-2* chromosomal genotype. *AP1–2 aa* indicates *AP1 a*, *AP2 a* linked on the same chromosomes. Those shown in Figure 6d–g are as follows: (d) *AP1-2 aa* from the mother, *bb* from the father: (e) *aa* from the father, *a'b* from the mother, (f) *aa* from both parents: (g) *a'b* from the mother, *bb* from the father.

Linkage Analysis

Frogs used as parents were heterozygous at six to nine loci each enabling the testing of many linkages. Since no recombination was found between *AP1* and *AP2*, these loci are considered together for the purpose of tabulation as the complex, *AP1-2*. Progeny of female 31040, heterozygous for eight loci, including the *AP1-2* complex (Table II) could be tested for all possible linkages (between locus pairs), a total of 28. These same animals could be tested for 15 linkages among the enzyme variants carried by the father. In this particular cross the father was heterozygous at two of the same loci as the mother.

The linkage analysis for 75 pairs of loci are presented in Table VI. The genotypes recorded for each animal at each locus were tabulated for each locus pair. Usually four genotypes, two parental and two recombinant, were found. For example, in a cross of *GOT1 a/b*, *PepLA2 a/b* female with a *GOT1 b/b*, *PepLA2 b/b* male, the offspring genotypes *GOT1 b/b*, *PepLA2 b/b* plus *GOT1 a/b*, *Pep LA2 a/b* were combined as parentals and *GOT1 a/b*, *PepLA2 b/b* and *GOT1 b/b*, *PepLA2 a/b* were combined as recombinants. The χ^2 values were calculated based on numbers expected for independent assortment. Included in the data on linkage are the two types of homozygous offspring from crosses involving parents both heterozygous at that locus (Table V). For example, linkage of *Acon1* and *AP1* was tested by counting *Acon1 a/a*, *AP1 a/a* plus *Acon1 a/b*, *AP1 b/b* as parentals and *Acon1 a/a*, *AP1 b/b* plus *Acon1 a/b*, *AP1 a/a* as recombinants. Offspring heterozygous at the *AP1* locus from two heterozygous parents could not be used in linkage analysis since the source of the *a* or *b* allele could not be determined. The designations "parental" or "recombinant" are used arbitrarily since for most locus pairs the offspring did not include the parental genotypes, and, for those that did, the phase (coupling or repulsion) was not known. The statistically greater class was designated "parental" and the small class "recombinant." In this way both linkage and phase can be tested.

Using the significance level of $p < 0.01$, three linkage groups were identified from these data (Fig. 8). Linkage group 1 includes the locus controlling the aconitase 1 isozyme (*Acon1*) and the locus controlling serum albumin (*Alb*), recombination fraction 0.190. Linkage group 2 includes four loci, glyoxalase (*Gly*), acid phosphatase 1 (*AP1*), acid phosphatase 2 (*AP2*), and

Table VI. Linkage Analysis[a]

Locus pair	Cross	"Parental"	"Recombinant"	χ^2	P^b	R.F.[c]
Acon1–Alb	**400**	***111***	***26***	***52.7***	***p < 0001***	***0.190***
Acon1–AP1-2	400	32	40	0.89		
	200	19	27	1.39		
Acon1–Est1	400	70	59	0.94		
Acon1–Est4	200	38	32	0.51		
Acon1–Est6	200	39	31	0.91		
	400	60	66	0.29		
Acon1–Est10	200	33	28	0.41		
Acon1–GOT1	200	45	58	1.64		
Acon1–IDH	400	67	72	0.18		
Acon1–PepLA2	400	66	71	0.18		
Acon1–PGM1	200	59	47	1.36		
	400	65	73	0.46		
Acon2–Alb	300	52	59	0.44		
Acon2–AP1-2	200	23	17			
	300	46	65			
	400	42	25			
	500	10	11			
	600	56	56			
	Total[d]	168	174	0.11		
Acon2–Est1	300	37	50			
	400	66	62			
	Total[d]	103	112	0.38		
Acon2–Est4	300	56	53	0.08		
Acon2–Est5	300	59	44	2.18	$p > 0.1$	
	400	58	73	1.72		
	Total[d]	117	117	0.0		
Acon2–Est6	300	56	55	0.01		
Acon2–Est10	300	57	54	0.08		
Acon2–Gly	200	44	53			
	300	48	54			
	400	53	76			
	500	21	24			
	600	61	51			
	Total[d]	227	258	1.98		
Acon2–MPI	200	52	45			
	300	59	52			
	400	37	32			
	500	19	26			
	600	48	42			
	Total[d]	215	197	0.79		

Table VI. Continued

Locus pair	Cross	"Parental"	"Recombinant"	χ^2	P^b	R.F.[c]
Acon2–HbII	300	53	47	0.36		
Alb–APl-2	400	31	38	0.71		
	600	68	45	4.68	$p>0.03$	
Alb–Est1	400	76	54	3.72	$p\sim0.05$	
Alb–Est4	300	57	53	0.15		
	600	49	52	0.09		
Alb–GOT1	600	64	49	1.99	$p\sim0.15$	
Alb–IDH1	400	69	71	0.03		
Alb–MPI	600	57	55	0.04		
Alb–PepLA2	400	75	67	0.45		
	600	44	67	4.77	$p\sim0.03$	
Alb–PGM1	400	84	58	4.76	$p\sim0.03$	
Alb–HbII	300	52	49	0.09		
	600	54	60	0.32		
AP1-2–Est1	300	45	42	0.10		
AP1-2–Est4	600	49	54	0.24		
AP1-2–Est5	300	100	6			
	400	63	5			
	Total[d]	***163***	***11***	***132.78***	***$p<0.0001$***	***0.063***
AP1-2–Est6	200	16	18	0.12		
	400	29	35	0.56		
	500	13	10	0.39		
AP1-2–Est10	200	12	11	0.04		
	500	11	11	0.00		
AP1-2–Gly	200	38	8			
	300	77	25			
	400	57	14			
	500	18	4			
	600	90	24			
	Total[d]	***280***	***75***	***118.38***	***$p<0.001$***	***0.211***
AP1-2–GOT1	200	16	29	3.76	$p\sim0.05$	
	600	61	55	0.31		
AP1-2–IDH	400	48	32	3.20	$p>0.06$	
AP1-2–MPI	200	32	14			
	300	54	58			
	400	32	39			
	500	11	12			
	600	57	57			
	Total[d]	186	180	0.10		
AP1-2–PepLA2	400	38	30	0.94		
	600	62	52	0.88		
AP1-2–PGM1	200	33	40	0.67		
	400	19	27	1.39		
	500	13	9	0.73		

Table VI. Continued

Locus pair	Cross	"Parental"	"Recombinant"	χ^2	P^b	R.F.[c]
AP1-2–HbII	600	53	61	0.56		
Est1–Est5	300	41	38			
	400	52	67			
	Total[d]	93	105	0.73		
Est1–Est6	***400***	***111***	***6***	***94.23***	***$p<0.0001$***	***0.051***
Est1–Gly	300	43	36			
	400	72	57			
	Total[d]	115	93	2.33	$p>0.1$	
Est1–IDH1	400	71	57	1.53		
Est1–PGM1	400	73	59	1.48		
Est4–Est6	200	78	3			
	300	107	2			
	500	50	2			
	Total[e]	***235***	***7***	***214.8***	***$p<0.0001$***	***0.030***
Est4–Est10	200	52	2			
	300	109	1			
	500	51	1			
	Total[e]	***212***	***4***	***200.3***	***$p<0.0001$***	***0.019***
Est4–GOT1	200	48	41	0.55		
	600	52	51	0.01		
Est4–MPI	600	55	48	0.48		
Est4–PepLA2	600	59	45	1.88		
Est4–HbII	600	50	50			
Est4–PGM1	200	42	37	0.32		
	500	26	25	0.02		
Est4–PGM2	500	34	17	5.67	$p<0.015$	
Est5–Gly	200	30	7			
	300	72	26			
	400	104	34			
	500	10	4			
	600	22	11			
	Total[d]	***238***	***82***	***76.05***	***$p<0.0001$***	***0.256***
Est5–MPI	200	17	20			
	300	48	55			
	400	70	67			
	500	18	20			
	600	17	15			
	Total[a]	170	177	0.14		

Table VI. Continued

Locus pair	Cross	"Parental"	"Recombinant"	χ^2	P^b	R.F.[c]
Est6–Est10	200	65	0			
	300	111	1			
	500	47	3			
	Total[e]	***223***	***4***	***211.3***	***$p<0.0001$***	***0.018***
Est6–PepLA	400	71	58	1.31		
Est6–PGM1	200	51	42	0.87		
	400	69	61	0.49		
Est6–PGM2	500	33	18	4.41	$p>0.03$	
Est10–PGM1	200	27	38	1.86		
	500	28	23	0.49		
Est10–PGM2	500	32	19	3.31	$p>0.06$	
Est10–HbII	300	56	44	1.44		
Gly–MPI	200	55	60			
	300	48	55			
	400	72	69			
	500	17	34			
	600	62	54			
	Total[d]	254	272	0.62		
GOT1–MPI	600	55	60	0.22		
GOT1–PepLA2	600	57	58	0.01		
GOT1–PGM1	200	55	55	0		
GOT1–HbII	600	54	58	0.14		
IDH1–PepLA2	400	68	74	0.25		
IDH1–PGM1	400	86	65	2.92	$p>0.08$	
MPI–PepLA2	600	67	48	3.14	$p>0.07$	
MPI–HbII	600	57	55	0.04		
PepLA2–PGM1	400	73	68	0.18		
PepLA2–HbII	600	55	56	0.01		
PGM1–PGM2	500	27	25	0.08		

[a] Boldface italics are used to indicate linked loci. Data for analysis of linkage pairs in fewer than 40 individuals are not included. See Table I for description of abbreviations.

[b] Probabilities (p) are entered for χ^2 values greater than 2.

[c] Recombination frequencies (R.F.) were calculated only for data having a probability (p) less than 0.01 of being due to chance.

[d] Total indicates totals for crosses where the locus pairs being tested are in the same individual male crossed with different females.

[e] Total indicates locus pairs being tested were in different individual females used for several crosses.

esterase 5 (*Est5*). The recombination frequencies between these loci established their order: *Gly–Ap1-2*, 0.211; *AP1–AP2*, 0.00; *AP1-2–Est5*, 0.063; *Gly–Est5*, 0.256. The order of *AP1* and *AP2* in the complex *AP1-2* will not be known until recombinants are found between these closely linked loci. Link-

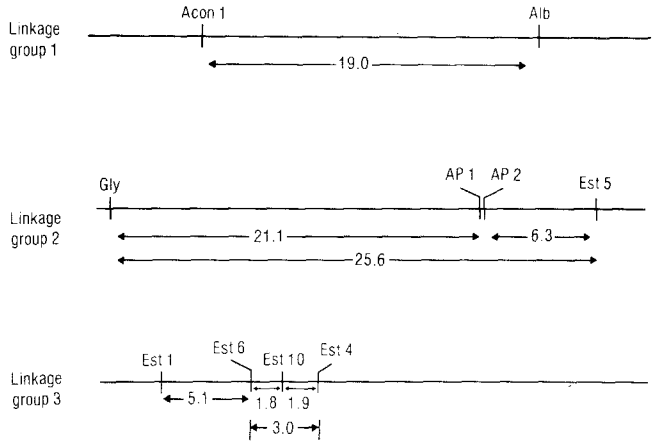

Fig. 8. Linkage maps for linkage groups 1, 2, and 3. Relative distances between loci are in percent recombination.

age group 3 contains four esterase loci. The data show the relationships: *Est1*–5.1%–*Est6*, *Est6*–1.8%–*Est10*–1.9%–*Est4* and *Est6*–3.0%–*Est4*. Since all linkage pairs were not testable in the present series of crosses, the order of the loci is not determined except that *Est10* is probably between *Est6* and *Est4*. Further work on this linkage group is in progress.

Examination of the data in Table VI suggests independent assortment of the remaining loci with only a few exceptions. Of particular interest is the possibility that, with additional data, *PGM2* may map in linkage group 3 with *Est4*, *Est6*, and *Est10*. Other locus pairs to be followed in future crosses for a loose linkage relationship are *IDH1–PGM1*, MPI–*PepLA2*, *Alb–Est1*, *Alb–PGM1*, and *AP1-2–IDH1* with p values < 0.1.

DISCUSSION

The results reported here established the Mendelian inheritance of 18 electrophoretic enzyme or blood protein variants in *Rana pipiens*. This is not surprising, but it tends to validate the use of electrophoretic variants as gene products in population and taxonomic studies of *Rana* and related amphibians.

The method of using natural heterozygotes as parents in studies of enzyme inheritance and linkage circumvents requirements for defined laboratory stocks and production of F_1 and backcross hybrid generations. For a limited number of crosses these studies have yielded a surprisingly large amount of data! The efficiency of data collection depends on selection of potential parents with maximum numbers of heterozygous loci. The difficulty that the phase, coupling or repulsion, is unknown at the start is overcome by

statistical evaluation of the data, setting high confidence limits. The procedures employed here might be used in other species with long generation times or those difficult to maintain as laboratory animals. With new procedures being published for detection of gene product variants, particularly for starch gel electrophoresis, this approach to genetics could be applied especially easily to those species having high average heterozygosities (Guttman, 1975; Fuerst *et al.*, 1977).

Genetic mapping in amphibians has been very limited. Cowan and Lloyd (1975) constructed physical maps of lampbrush chromosomes of several species showing the position of centromeres, loops, and the nucleolar organizer. With the exception of the nucleolar organizer the locations of genes controlling biochemical or morphological traits on the physical chromosome maps of amphibians are unknown. A number of mutant genes, primarily pigment-pattern variants and developmental anomalies, have been described in amphibian species commonly used in the laboratory, namely the axolotl (Malacinski and Brothers, 1974), *Xenopus laevis* (Gurdon and Woodland, 1975), *Rana pipiens* (Browder, 1968, 1975), and *R. nigromaculata* (Nishioka, 1977). In only a single instance has linkage between two mutant genes been found (Humphrey, 1959, 1975). Analysis of gynogenetic offspring of heterozygous mothers has been used to map the distance between mutant genes and the kinetochore (Volpe, 1970; Nace *et al.*, 1970), but the small number of mutants available limited the usefulness of this approach in mapping.

In *Rana pipiens* there are 13 pairs of chromosomes, $2N=26$ (DiBerardino, 1962). With the present demonstration of ten loci in three linkage groups and the apparent independent assortment of eight other loci, it should be possible to map other variant loci in *Rana pipiens*, including the pigmentation genes kandiyohi (*K*), burnsi (*B*), and melanoid (m), since enzyme polymorphisms occur in the same populations as these pigment mutants. It is expected that additional enzyme variants will be found by screening larger numbers of animals and in particular by sampling different populations. Enzyme loci which are apparently unlinked on the basis of recombination of approximately 50% may be found to be in the same linkage group if a locus is found that lies between them on the linkage map. Likewise, even the linkage groups defined here should be regarded as tentative since two of them could lie at opposite ends of a very large metacentric chromosome.

Whereas it is difficult to compare data on the genetics of unshared morphological traits, data on linkage relationships of enzyme loci in amphibians can be compared directly to linkage relationships of homologous loci in other vertebrates. Eventually a statement might be made concerning the conservation of linkages in evolution. Linkage data are available for enzyme loci in fish (Morizot *et al.*, 1977; Siciliano and Wright, 1976) as well as mice (Green, 1975) and humans (King, 1975). Linkage homologies have been

demonstrated between mouse and human chromosomes (Lalley *et al.*, 1978). The linkage of albumin to aconitase-1 in *Rana pipiens* is of special interest in comparing the linkage of genetic loci for each of these proteins in other species. Albumin variants have been described both in humans (Weitkamp *et al.*, 1969) and in the mouse (Petras, 1972). Serum albumin has been assigned to mouse chromosome 5 linked to *PGM1* (Nichols *et al.*, 1975). In *Rana pipiens Alb–PGM1* may show a loose linkage, as indicated earlier. Aconitase-1 variants have been found in the axolotl (Pavlick, 1976) and in humans (Slaughter *et al.*, 1975). A human chromosome 9 assignment for the Acon1 isozyme has been established (Povey *et al.*, 1976). The cluster of esterases in *Rana pipiens* linkage group 3 is comparable to a cluster of esterases in mouse chromosome 8 (Green, 1975), rat linkage group V (Womack and Sharp, 1976), and linkage of two esterase loci in platyfish and swordtails (Siciliano and Wright, 1976).

It has recently been demonstrated that frogs have a major histocompatibility region relatively distant from the kinetochore (Roux and Volpe, 1975; Richards *et al.*, 1980). It is of considerable evolutionary interest whether this locus might have linkage relationships to other loci, particularly for proteins analogous to those present in mammals (Klein, 1977). The glyoxalase gene in both humans and mice is linked to the major histocompatibility complex on chromosome 6 in humans (Giblett and Lewis, 1976) and chromosome 17 in mice (Meo *et al.*, 1977; Leinwand *et al.*, 1978). In the mouse both *H-2* and glyoxalase are linked to an acid phosphatase processing locus (Womack and Eicher, 1977). In humans two acid phosphatases *AcP1* and *AcP2* are located in chromosomes 2 and 11, respectively (King, 1975). These facts make the enzyme loci (*Gly*, *Ap1*, *Ap2*, *Est5*) in our linkage group two very interesting. Methods have been derived to test the linkage of enzyme variants to the major histocompatibility complex using skin grafting from mothers to their gynogenetic progeny to identify gynogenetic animals homozygous and heterozygous for the major histocompatibility complex. Further investigation will determine whether the major histocompatibility region in the frog is included in this linkage group.

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