

This is to certify that the
dissertation entitled
GENETIC AND BIOCHEMICAL ANALYSIS
OF PURINE RESISTANCE
IN DROSOPHILA MELANOGASTER

presented by

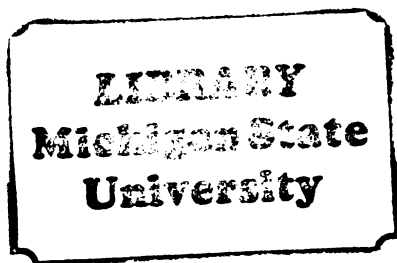
Daniel Henry Johnson

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Zoology

Thomas B. Friedman
Major professor

Date 2/21/83





RETURNING MATERIALS:
Place in book drop to
remove this checkout from
your record. FINES will
be charged if book is
returned after the date
stamped below.

--	--	--

**GENETIC AND BIOCHEMICAL ANALYSIS
OF PURINE RESISTANCE
IN DROSOPHILA MELANOGASTER**

By

Daniel Henry Johnson

A DISSERTATION

**Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of**

DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

1983

ABSTRACT

GENETIC AND BIOCHEMICAL ANALYSIS OF PURINE RESISTANCE IN *DROSOPHILA MELANOGASTER*

By

Daniel Henry Johnson

Purine (7 H-Imadizo[4,5-d]pyrimidine) is toxic to wild type *Drosophila*. Purine induced lethality appears to result from the conversion of purine to a toxic nucleotide since purine resistant mutants of *Drosophila* are deficient in adenine phosphoribosyl-transferase activity [APRT, AMP:pyrophosphate phosphoribosyl-transferase E.C. 2.4.2.7]. In order to use this chemical selection procedure for the study of gene organization and control in *Drosophila*, the relationship between purine resistance and deficiency in APRT activity was investigated. Genetic and cytogenetic mapping as well as complementation analysis indicate that purine resistance and deficiency of APRT activity in five independantly isolated mutants result from heritable alterations at a single locus, *Appt* (3:3.03). APRT activity shows allele-dose dependence in heterozygotes of *Appt* and in flies which are haploid for different *Appt* alleles. Chemical and physical analysis of APRT molecules purified from two variant strains of *Drosophila* demonstrate that APRT is a dimer composed of apparently identical 23,000 dalton subunits having a different isoelectric point in each variant strain. Cytogenetic mapping indicates that the locus specifying the isoelectric point of APRT lies

Daniel H. Johnson

within the region 62B7-12 of *Df(3L)R⁺E* which contains the *Aprt* locus. These results show that APRT is a dimer composed of identical polypeptides and that *Aprt* is the structural gene for this enzyme.

In addition to purine toxicity mediated through APRT activity, results presented here suggest that purine is also converted to a toxic nucleotide by an alternate pathway. This pathway appears to involve the conversion of purine to purine riboside by inosine phosphorylase [Purine nucleoside:orthophosphate ribosyltransferase E.C. 2.4.2.1] followed by phosphorylation of purine riboside to a toxic nucleotide by adenosine kinase [ATP:adenosine 5'-phosphotransferase E.C. 2.7.1.20]. The significance of this pathway to establishing a direct selection procedure for wild type recombinants between different *Aprt* mutant alleles is discussed.

ACKNOWLEDGEMENTS

I thank Dr. Thomas Friedman for his friendship, confidence and generous support during the course of this work. I greatly appreciate Dr. James Asher's invaluable criticism and the free access to his laboratory and library. I thank Dr. Stephen Bromley and Dr. T. M. Rizki for continued interest in my work. Many thanks to Leos Kral and Dr. Leonard Robbins for helpful discussions and to Dr. Robert Robbins for many hilarious ones. I thank all my professors and mentors, past and present for their unselfish contribution to my training and appreciation of science. A most special thanks to Susan Masta, Dianne Hallinen and Dianne Kobylarz for their skillful and dedicated technical assistance.

TABLE OF CONTENTS

	Page
List of Tables	iv
List of Figures	v
Introduction	1
Text Organization and Nomenclature	3
The Biochemical Defect in Purine Resistant Mutants of <i>Drosophila</i> ..	5
Genetic Characterization of <i>Aprt</i>	14
APRT: A Single Polypeptide Encoded by <i>Aprt</i>	31
Temporal Expression and Spatial Distribution of APRT Activity	54
Alternate Pathways of Purine Salvage in <i>Drosophila</i>	58
Summary and Discussion	67
Appendix 1: Enzyme and Protein Assays	74
Appendix 2: Sources and Culture of <i>Drosophila</i>	97
Appendix 3: Selection of Purine Resistant Mutants	99
Appendix 4: Gel Electrophoresis and Isoelectric Focusing	106
Appendix 5: Gel Filtration	115
Appendix 6: Purification of <i>Drosophila</i> APRT	118
Appendix 7: Preparation and Partial Characterization of Anti-APRT IgG	135
List of References	147

LIST OF TABLES

Table	Page
1. HGPRT and APRT Activity in Human Cells and <i>Drosophila</i>	10
2. Segregation Analysis of <i>Aprt</i> ¹	15
3. Complementation Analysis of <i>Aprt</i> ¹ , <i>Aprt</i> ² and <i>Aprt</i> ³	18
4. Recombination Mapping of <i>Aprt</i> on Chromosome Three	21
5. Complementation Analysis of <i>Aprt</i> Mutants and <i>Df(3L)R</i> ^{+E}	26
6. Complementation Analysis of <i>Aprt</i> Alleles and <i>Df(3L)R</i> ^{+E}	28
7A. APRT Activity During Early Development	56
7B. APRT Activity in Various Tissues	56
8. Purine Base Salvage Enzyme Activities in <i>Drosophila</i>	59
9. Separation of Purine Bases, Nucleosides and Nucleotides on Polygram Cel 300 PEI	75
10. Adenosine Kinase Activity in <i>Drosophila</i>	94
11. Summary of APRT Purification Procedure	121
12. Immunization of a Rabbit with <i>Drosophila</i> APRT	136
13. Immunoprecipitation of Hamster and <i>Drosophila</i> APRT	141

LIST OF FIGURES

Figure	Page
1. Purine Nucleotide Biosynthetic Pathways	6
2. APRT Activity in Mixtures of <i>Drosophila</i> Extract	11
3. Summary of Genetic and Cytogenetic Mapping of <i>Aprt</i>	23
4. Double Reciprocal Plot of APRT Velocity vs. Adenine Concentration	33
5. Double Reciprocal Plot of APRT Velocity vs. PrPP Concentration	34
6. Autoradiograms of <i>Drosophila</i> APRT in Isoelectric Focusing Gels	36
7. Autoradiograms of APRT Activity from Mammals and <i>Drosophila</i> in an Isoelectric Focusing Gel	40
8. Gel Filtration of <i>Drosophila</i> APRT	43
9. SDS Polyacrylamide Gel Electrophoresis of Purified <i>Drosophila</i> APRT	45
10. Two-Dimensional Analysis of Purified <i>Drosophila</i> APRT	48
11. Isoelectric Focusing of Purified <i>Drosophila</i> APRT	50
12. Temporal Profile of APRT Activity During Development in <i>Drosophila</i>	55
13. Biosynthesis of AMP in <i>Drosophila</i>	62
14. Purine Riboside Sensitivity in <i>Drosophila</i>	64
15. APRT Velocity vs. pH	77

16. APRT Velocity vs. Adenine Concentration	79
17. APRT Velocity vs. PrPP Concentration	81
18. APRT Velocity vs. Magnesium Concentration	83
19. Comparison of Magnesium and Manganese as Metal Cofactors of APRT Activity	85
20. Dependence of APRT Activity on Incubation Time and Protein Concentration	89
21. Selection of Purine Resistant Mutants	100
22. Purine Sensitivity in <i>Drosophila</i>	101
23. Removal of <i>Aprt</i> Linked Recessive Lethal Mutations	105
24. pH Gradient in an Isoelectric Focusing Gel	114
25. SDS Polyacrylamide Gel Electrophoresis of Purified and Partially Purified <i>Drosophila</i> APRT	122
26. AMP Agarose and Blue Agarose Chromatography of <i>Drosophila</i> APRT	125
27. Octyl Agarose Chromatography of <i>Drosophila</i> APRT	128
28. Purification of IgG by Protein A Agarose Chromatography	137
29. Dilution of <i>Drosophila</i> APRT Antiserum	139
30. Western Blot of <i>Drosophila</i> APRT	143

INTRODUCTION

Present concepts of gene organization and control emerged initially from the investigation of induced mutations in procaryotic organisms. The ability to isolate specific gene alterations through direct selection procedures was a critical consideration when choosing particular gene loci for study. Despite the usefulness of the approach, direct mutant selection has seldom been possible in whole, developmentally complex eucaryotes. Consequently, not a single gene locus in any higher organism is as thoroughly understood with respect to both its organization and control as any of several procaryotic gene loci. This situation recommends the investigation of gene loci in higher eucaryotes for which direct mutant selection appears to be possible.

Glassman first observed that *Drosophila* is sensitive to killing by purine (7 H-Imidazo[4,5-d]pyrimidine) and that xanthine dehydrogenase [XDH, xanthine:NAD⁺ oxidoreductase E.C. 1.2.1.37] deficient mutants *ry* (3:52.0) and *ma-1* (1:64.8) were much more sensitive to purine toxicity than wild type flies (1). He proposed that purine is a substrate for XDH and that the product of this reaction has reduced toxicity. Duck (2) attempted to use the differential sensitivity to purine of wild type and the XDH deficient mutant *ma-1* to isolate supressor mutations of *ma-1* alleles by selection for purine resistance

in mutagen treated *ma-1* flies. His search uncovered a single purine resistant mutant, *Pur*, which remained deficient in XDH activity. Although Duck failed to identify the mechanism of purine resistance conferred by *Pur*, he demonstrated that this mutation was dominant to *Pur*⁺ with respect to purine resistance and that this affect was independent of, but additive to, the affect of XDH activity in promoting purine resistance (2). Two additional purine resistant mutations, *Pur*^r and *Pur*^{r-1} were independently isolated by W. Gelbart and A. Chovnick during investigations of the *ry* locus (Chovnick, personal communication).

T. Friedman proposed that purine resistance conferred by the *Pur* mutations might result from a defective purine base salvage enzyme which prevents the conversion of purine to a toxic product in the purine-resistant mutants. This hypothesis was based on the observation that mutants of cultured eucaryotic cells selected for resistance to a variety of toxic purine base analogues are invariably deficient in activity of a purine base salvage enzyme (3-12). The investigation presented here was initiated to analyze the biochemical mechanisms involved in purine toxicity and to identify the primary biochemical defect in purine resistant mutants of *Drosophila*.

Text Organization and Nomenclature

This presentation is generally organized and written to reflect the chronological order and manner in which hypotheses were developed and tested in an effort to understand the genetics and biochemistry of purine resistance in *Drosophila melanogaster*. The detailed description of methods and materials and the process of their development have been placed exclusively in appendices of this dissertation which are referenced accordingly in the text.

Evidence is presented here which support the conclusion that *Ap^rt* is the structural gene for adenine phosphoribosyltransferase and that mutations in this gene confer resistance to purine killing due to a deficiency in APRT activity. The trivial name *Pur* was originally assigned by the discoverers of the first three purine resistant mutants. That name has been changed here to *Ap^rt* in conformance with the standard conventions for genetic nomenclature of loci encoding the structural genes of enzymes in *Drosophila* (13). Alleles of *Ap^rt* are distinguished by two different criterion: (i) *Ap^rt* alleles isolated by purine selection which result in a deficiency of APRT activity are identified with a numeral superscript of the symbol *Ap^rt* to indicate the order in which they were discovered and (ii) two naturally occurring variant alleles of *Ap^rt* which confer sensitivity to purine killing but which alter the specific activity and the isoelectric

point of APRT are designated by the letter superscripts A and B of *Ap^rt* to indicate the relative acidic and basic isoelectric point of APRT molecules produced by each homozygous variant strain.

The Biochemical Defect in Purine Resistant Mutants of *Drosophila*

Most organisms synthesize purine nucleotides by two basically different metabolic pathways (Figure 1). The *de novo* pathway elaborates the purine base upon a ribose sugar moiety by a series of enzymatic steps requiring amino and carboxylic acid precursors. The salvage pathways of nucleotide synthesis take advantage of preformed, free purine bases and nucleosides by converting them in a single enzymatic step to a nucleotide (Figure 1). The nucleotides so formed participate as essential chemical intermediates in a variety of synthetic processes and as the subunit precursors of the nucleic acids. The manner in which the nucleotide and its purine base precursors interact with other molecules is obviously dependent upon their unique chemical structures. A particular purine base may be recognized by a salvage enzyme which converts that base to a nucleotide. The resulting nucleotide must then be correctly recognized by a variety of other enzymes and molecules in order to correctly perform its function. An error of recognition in either the synthesis step or subsequent steps may have deleterious consequences for the organism.

Three decades ago Szybalski (14) cultured human cells in the presence of the guanine analogue 8-azaguanine and discovered that this analogue was toxic. He also found that human cells could be isolated

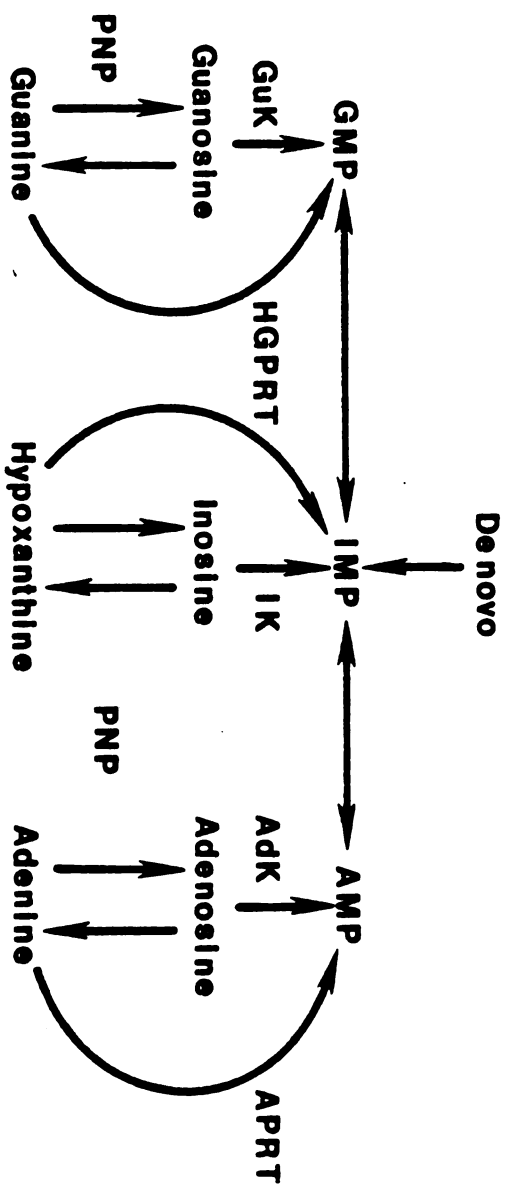


Figure 1. Purine Nucleotide Biosynthetic Pathways

Figure 1. Legend

GuK: Guanine Kinase

PNP: Purine Nucleoside Phosphorylase

HGPRT: Hypoxanthine-Guanine Phosphoribosyltransferase

IK: Inosine Kinase

AdK: Adenosine Kinase

APRT: Adenine Phosphoribosyltransferase

which are insensitive to the toxicity of this guanine analogue by culturing the cells in its presence. A variety of toxic analogues of natural purine bases have since been used for the selection of resistant lines of cultured eucaryotic cells (10). Subsequent investigations have shown that resistance to a particular purine base analogue results from deficiency in activity of a specific purine base salvage enzyme. The inability of cells to salvage a particular purine base appears to confer resistance to the toxic analogue of that base since the analogue fails to be converted to a nucleotide in the mutant cell line which is deficient in the salvage enzyme activity (3,15-18). Studies of mutant hamster cell lines deficient in HGPRT activity by Fenwick and co-workers (8) and in APRT activity by Taylor and co-workers (19) suggest that resistance of these cell lines to analogues of guanine and adenine respectively, result from mutations within the structural genes for these enzymes. In both cases this conclusion is based on the observations that the resistant cell lines show variation in the kinetic and physical properties of HGPRT and APRT in comparison to the analogue-sensitive parent cell lines (18-19).

Purine resistance in *Drosophila* appeared to result from a single mutation and therefore, was probably due to a single defective gene product. Since mutants of cultured eucaryotic cells are resistant to the toxic affects of unusual purine bases due to a deficiency in activity of a specific purine base salvage enzyme, it appeared likely that the purine-resistant *Drosophila* mutant *Aprt*¹ would show deficiency for a purine base salvage activity which was present in

purine-sensitive flies. Furthermore, because the number of major salvage enzymes (Figure 1) for purine bases is relatively small, it was reasonable to test this hypothesis by simply assaying the relevant enzymes in both purine-resistant and purine-sensitive flies.

The initial experiments involved the assay of hypoxanthine-guanine phosphoribosyltransferase [HGPRT, IMP:pyrophosphate phosphoribosyltransferase E.C. 2.4.2.8] and adenine phosphoribosyltransferase [APRT, AMP:pyrophosphate phosphoribosyltransferase E.C. 2.4.2.7] according to modifications (Appendix 1) of Merrill's procedure (20). The results of these assays (Table 1) indicate that the purine-resistant mutant *Ap^{rt}*¹ is deficient in APRT activity in contrast to purine-sensitive flies (21). In order to test whether the deficiency of APRT activity in the *Ap^{rt}*¹ mutant was due to the unique presence of an inhibitor of APRT activity in this strain, mixing experiments were performed (Figure 2). The data show that the level of APRT activity in mixtures of crude homogenates from the purine-sensitive wild type and the purine-resistant mutant is strictly additive and dependent upon the amount and source of the APRT activity in the mixture. Therefore, the presence of a diffusible inhibitor of APRT activity in the mutant is unlikely. The results suggest that purine resistance and deficiency of APRT activity in the mutant are causally related and due to the same mutant defect.

This conclusion was corroborated by the subsequent observations that cultured human fibroblasts, resistant to the adenine analogue diaminopurine, are also resistant to purine toxicity and are deficient in APRT activity. In contrast, the wild type parent cell line having

Table 1. HGPRT and APRT Activity in Human Cells and Drosophila

Source of Enzyme ¹	HGPRT Activity ²	APRT Activity ³
<u>Drosophila Aprt^A/Aprt^A</u> (purine sensitive)	0.0	54.3
<u>Drosophila Aprt¹/Aprt¹</u> (purine resistant)	0.0	1.5
Human 253 J (wild type)	163.7	318.7
Human 253 J DAP ^{r-1} (2,6-diaminopurine resistant)	155.8	0.0
Human 253 J 6TG-1 (6-thioguanine resistant)	0.0	100.6

¹Enzymes were assayed in crude extracts (Appendix 1).

²nanamoles (8¹⁴C)IMP formed per minute per milligram protein x 10⁻²

³nanamoles (8¹⁴C)AMP formed per minute per milligram protein x 10⁻²

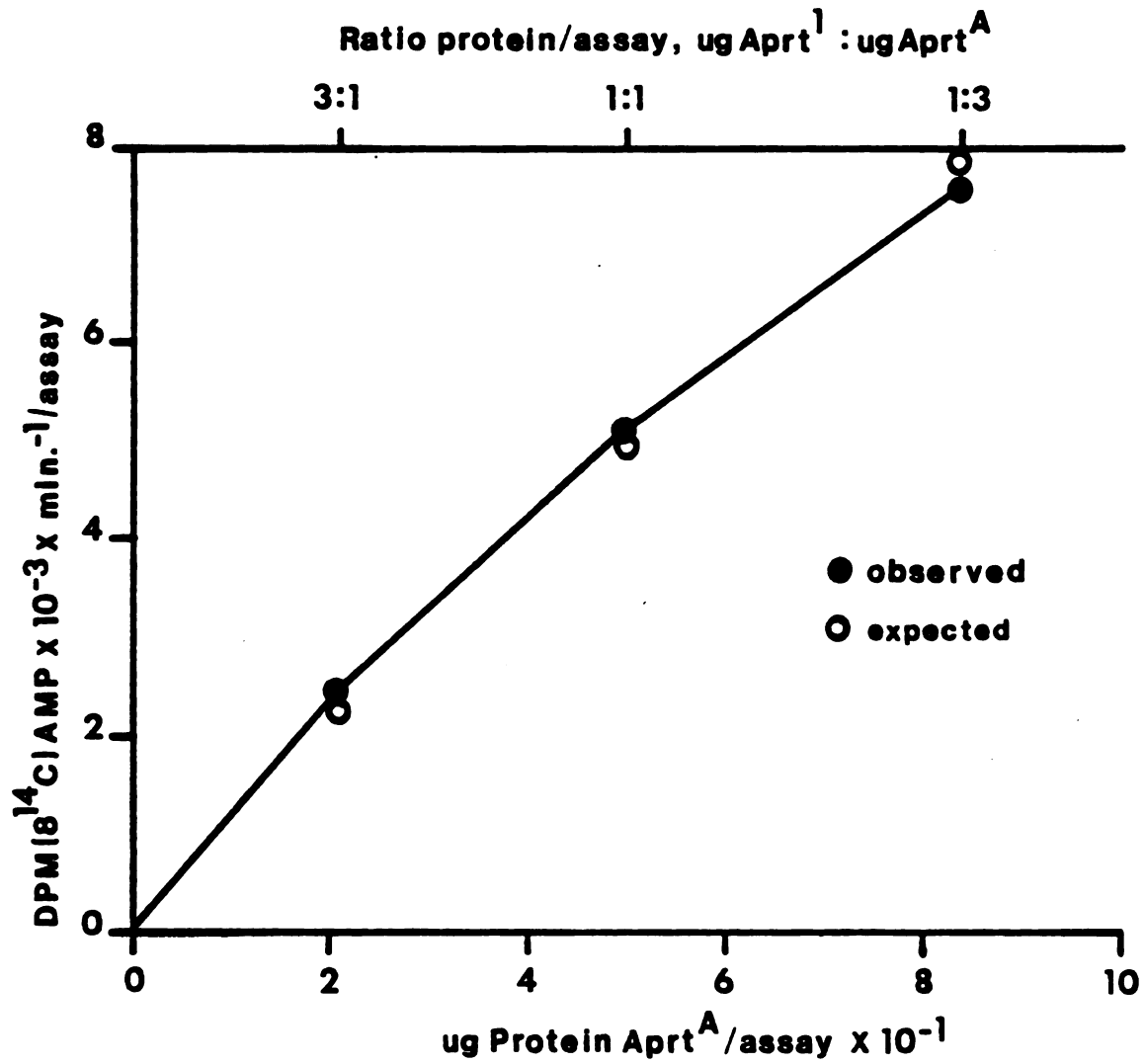


Figure 2. APRT Activity in Mixtures of Drosophila Extract

Figure 2. Legend

APRT activity was measured in crude extracts from adult flies (Appendix 1). The expected values assume that APRT activity is strictly additive with the magnitude of activity being dependent upon the source of the extract in the mixture.

both APRT and HGPRT activities, as well as a 6-thioguanine resistant cell line having APRT activity but no detectable HGPRT activity, are both sensitive to purine killing (22) (Table 1). These results are consistent with the observation that purine is a potent inhibitor of mammalian APRT activity (23). Thus, purine appears to be an analogue of adenine and a substrate of APRT. In addition, Becker has shown that a *Drosophila* cell line selected for resistance to the adenine analogue 8-azaadenine is deficient in APRT activity in contrast to the analogue sensitive parent cell line (24). The data presented in Table 1 also show that HGPRT activity is absent in both purine-sensitive and purine-resistant *Drosophila*. The significance of this observation for understanding the biochemical mechanisms of purine base salvage in *Drosophila* is discussed in the final chapter dealing with alternate mechanisms of purine salvage.

In order to confirm that purine resistance in the *Aprt*¹ mutant of *Drosophila* is due only to a deficiency in APRT activity it was necessary to demonstrate that both deficiency in APRT activity and purine resistance assort genetically with the same locus in the mutant. This was accomplished by three different procedures: (i) genetic mapping of the purine resistance locus followed by testing for segregation between the phenotypes of purine resistance and deficiency of APRT activity, (ii) complementation analysis between different purine-resistant mutants and (iii) purine selection of mutations newly induced with ethylmethane sulfonate followed by genetic mapping and comparison of APRT activity in the mutant to the purine-sensitive parent strain.

Genetic Characterization of *Aprt*

The genetic mapping of the purine resistance locus was performed to confirm the location of the *Aprt*¹ mutation assigned by Duck. The results presented in Table 2 indicate that both the purine resistance mutation and a locus resulting in deficiency of APRT activity are linked to chromosome three. This result was unexpected since Duck (2) previously reported that this purine-resistant mutation resides at approximately position 78 to the right of the recessive mutant *c* (2:75.5). On the other hand, this result was interesting since the purine-resistant mutants *Aprt*² and *Aprt*³, which were initially examined as balanced lethal stocks of chromosome three, showed only a modest increase in purine resistance in the case of *Aprt*² and none at all in the case of *Aprt*³ in comparison to the purine-sensitive wild type strain. Moreover, the *Aprt*² and *Aprt*³ balanced lethal stocks exhibited approximately half the level of APRT activity of that found in a purine-sensitive wild type strain. Since the *Aprt*¹ mutation was found to reside on chromosome three and not on chromosome two, it seemed likely that the *Aprt*² and *Aprt*³ mutations were alleles of *Aprt*. Therefore, the *Aprt*² and *Aprt*³ mutations must be in trans to a wild type allele of *Aprt* in the balanced lethal stock. This would explain both the nominal resistance of these stocks to purine killing and their approximate half wild type level of APRT activity (Table 2).

Table 2. Segregation Analysis of Aprt¹

Cross No.	Sex	Genotype ¹ of Parental Cross	Phenotype of Purine Survivors ² Genotype of Flies Assayed ³	APRT Activity ⁴	n ⁵	s ⁶
1	M	Pm/Pm ⁺ ; Sb/Sb ⁺	Cy ⁺ Pm ⁺ Sb ⁺ D ⁺	2.0	3	1.5
	F	Cy/Cy ⁺ ; D/D ⁺	Cy Pm ⁺ Sb ⁺ D ⁺	2.0	3	1.2
			Cy ⁺ Pm Sb ⁺ D ⁺	2.3	3	0.3
			Cy Pm Sb ⁺ D ⁺	2.0	3	1.0
2	M	.ci ^D /ci ^{D+}	ci ^{D+} ey ^{D+}	2.3	1	-
	F	ey ^D /ey ^{D+}	ci ^D ey ^{D+}	2.0	1	-
			ci ^{D+} ey ^D	2.0	1	-
			ci ^D ey ^D	2.0	1	-
3	M	c/c ⁺ ; Aprt ¹ ry ² /Aprt ^A ry ⁺	c ry ²	-	-	-
	F	c/c ⁺ ; Aprt ¹ ry ² /Aprt ¹ ry ²	c ⁺ ry ²	-	-	-
4		same as cross 3	c ry ⁺	83.5	10	12.8
		no purine in the media	c ⁺ ry ²	2.4	10	0.4
		Cy/Pm; Sb/D		78.3	3	4.0
		ey ^D /ci ^D		85.8	3	7.0
		Aprt ^A ry ⁺ /Aprt ^A ry ⁺		109.5	3	6.0
		c/c; Aprt ¹ ry ² /Aprt ¹ ry ²		2.0	3	0.5

Table 2. Legend

- ¹The mutations *Pm* and *Cy* are dominant markers linked to chromosome two. The mutations *Sb* and *D* are dominant markers linked to chromosome three. The mutations *ci*^D and *ey*^D are dominant mutations in chromosome four. The mutations *c* and *ry*² are recessive mutations linked to chromosomes two and three respectively. Chromosomes containing the wild type alleles of the markers in crosses 1 and 2 were derived from *Aprt*¹ homozygotes. Dominant markers carried by the female parents in crosses 1-2 are contained within balancer chromosomes to prevent the recovery of recombinant progeny.
- ²Progeny from the crosses indicated were reared on purine containing media (4 mg purine/10 ml media) to confine survival to *Aprt*¹ homozygotes.
- ³The *Cy/Pm; Sb/D* stock and the *ey*^D/*ci*^D stock are both purine-sensitive.
- ⁴APRT activity is expressed as DPM(8¹⁴C) AMP formed in the assay per minute per microgram protein.
- ⁵_n is the number of independent determinations for the APRT assay.
- ⁶_s is the standard deviation for the APRT assay.

If this hypothesis was correct it was expected that the three purine-resistant mutations would behave as alleles and fail to complement in trans to the *Aprt*¹ mutation with respect to either purine resistance or deficiency in APRT activity. To test this hypothesis, matings were made and the progeny reared on purine containing media as presented in Table 3. The results indicate that only the third chromosome homologues in trans to the *InP18 Aprt*^B *Ubx ny*⁴¹ chromosome are present in the purine-resistant survivors in crosses of the *Aprt*² and *Aprt*³ balanced lethal stocks to *Aprt*¹ homozygotes. Moreover, the *Aprt*¹/*Aprt*² and *Aprt*¹/*Aprt*³ purine-resistant trans-heterozygotes are deficient in APRT activity in comparison to the parental balanced lethal stocks or the the wild type purine-sensitive *Aprt*^B homozygotes. These observations support several conclusions: (i) all three purine-resistant mutations are non-complementing alleles of a single locus, *Aprt* which resides on chromosome three, (ii) both purine resistance and deficiency in APRT activity are conferred by the same mutation at *Aprt* in the mutants, (iii) the simplest explanation for an absence of complementation between the mutants with respect to a deficiency in APRT activity is that *Aprt* is the structural gene for adenine phosphoribosyltransferase and (iv) non-complementing mutant alleles of *Aprt* can be readily isolated in trans to existing mutant alleles of *Aprt* by direct selection for purine resistance.

In order to develop an efficient genetic protocol for the isolation of non-complementing mutant alleles of *Aprt*¹ the following preliminary steps were taken: (i) the precise location of *Aprt* on

Table 3. Complementation Analysis of Aprt¹, Aprt² and Aprt³

Cross No.	Sex	Genotype ¹	Parental Cross	Phenotype of Purine Survivors ²	APRT Activity ⁴	n ⁵	s ⁶
Genotype of Flies Assayed ³							
1	M	InP18	Aprt ^B Ubx ry ⁴¹ (1 ⁺)				
		Aprt ²	Ubx ⁺ ry ⁺ (1)	Ubx ⁺ ry ⁺	6.5	3	0.6
	F	Aprt ¹	Ubx ⁺ ry ² (1 ⁺)				
		Aprt ¹	Ubx ⁺ ry ² (1 ⁺)				
2	M	InP18	Aprt ^B Ubx ry ⁴¹ (1 ⁺)				
		Aprt ³	Ubx ⁺ ry ⁺ (1)	Ubx ⁺ ry ⁺	1.2	3	0.3
	F	Aprt ¹	Ubx ⁺ ry ² (1 ⁺)				
		Aprt ¹	Ubx ⁺ ry ² (1 ⁺)				
		InP18	Aprt ^B Ubx ry ⁴¹ (1 ⁺)				
		Aprt ²	Ubx ⁺ ry ⁺ (1)		20.1	3	2.9
		InP18	Aprt ^B Ubx ry ⁴¹ (1 ⁺)				
		Aprt ³	Ubx ⁺ ry ⁺ (1)		13.0	2	3.2
		Aprt ¹	ry ² /Aprt ¹ ry ²		1.7	3	0.5
		Aprt ^B /Aprt ^B			39.8	3	3.5

Table 3. Legend

- ¹All of the markers referred to in the Table are linked to chromosome three. *InP18* is a balancer chromosome. The symbol (l) indicates the presence of a recessive lethal mutation in the chromosome. The symbol (l⁺) indicates the presence of a wild type allele of (l) in the chromosome.
- ²Progeny from the crosses were reared on purine containing media to confine survival to purine resistant trans-heterozygotes (4 mg purine/10 ml of media).
- ³The stocks of flies carrying *InP18* are balanced lethal stocks of the *Aprt*² and *Aprt*³ mutations.
- ⁴APRT activity is expressed as DPM(8¹⁴C)AMP formed in the assay per minute per microgram protein.
- ⁵n is the number of independent determinations for the APRT assay.
- ⁶s is the standard deviation for the APRT assay.

chromosome three was determined, (ii) based on the map location of *Aprt*, balancer chromosomes and marker chromosomes were chosen to allow the unequivocal identification of mutagen treated chromosomes and the construction of balanced lethal stocks of newly isolated mutations in *Aprt* and (iii) a protocol for the removal of linked recessive-lethal mutations from chromosomes containing newly isolated mutations in *Aprt* was developed. The details of these procedures are presented in Appendix 3.

The position of the *Aprt*¹ mutation and of the *Aprt*⁴ and *Aprt*⁵ purine-resistant mutations isolated in this study were determined by the procedures described in Table 4. Although these experiments were designed to map the locus of purine resistance in these mutants, stocks carrying the purine-selected recombinant chromosomes were constructed and assayed for APRT activity. In all cases the purine-selected recombinants were deficient in APRT activity. The data (Table 4 and ref. 25) place *Aprt*¹ at position 3.03, approximately 0.1 cM to the right of *R* on the left arm of chromosome three of Roberts revised map (26). This result led Friedman (25) to analyze the cytological position of *Aprt* using stocks carrying terminal translocations of a portion of the left arm of chromosome three onto a freely assorting *Y* chromosome. Males of these stocks are triploid for the translocated portion of chromosome three. The results of this analysis demonstrated that a wild type allele of *Aprt* was contained within the region 61E1-62D7 of the interstitial 3L translocation *Dp(T(Y;3)H141)* (Figure 3 and ref. 25).

Table 4. Recombination Mapping of Aprt on Chromosome Three

Sex	Genotype ¹ of Parental Cross	Phenotype of Recombinant F ₁ Survivors ²			Total Survivors
		% R	% ru ⁺	% h ⁺	
M	mmh ⁺ ve ⁺ R ⁺ Aprt ¹ h/mmh ⁺ ve ⁺ R ⁺ Aprt ¹ h	0.13*	-	-	4012
F	mmh ⁺ ve R Aprt ^A h ⁺ /mmh ⁺ ve ⁺ R ⁺ Aprt ¹ h				
M	ru Aprt ¹ h/ru Aprt ¹ h	-	0.7	26.5	431
F	ru Aprt ¹ h/ru ⁺ Aprt ^A h ⁺				
M	ru Aprt ⁴ h/ru Aprt ⁴ h	-	1.1	22.8	561
F	ru Aprt ⁴ h/ru ⁺ Aprt ^A h ⁺				
M	ru Aprt ⁵ h/ru Aprt ⁵ h	-	0.4	37.5	245
F	ru Aprt ⁵ h/ru ⁺ Aprt ^A h ⁺				

*Of the five R recombinants, three were fertile in a backcross to mmh and ve homozygotes which gave the genotype of the recombinant chromosomes. All three R recombinants were of the genotype mmh⁺ ve R Aprt¹ h.

Table 4. Legend

- ¹ All of the markers referred to in the Table are linked to chromosome three.
- ² Progeny from the crosses were reared on purine containing media to confine survival to purine resistant homozygotes (3.5 mg purine/10 ml media).

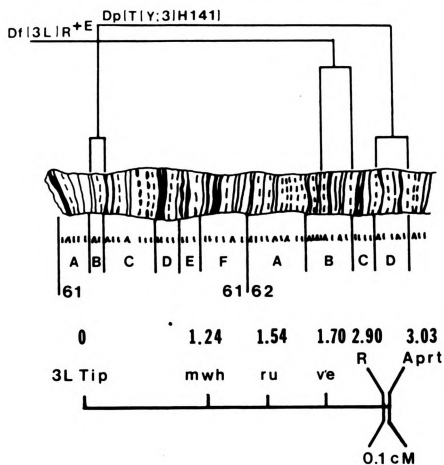
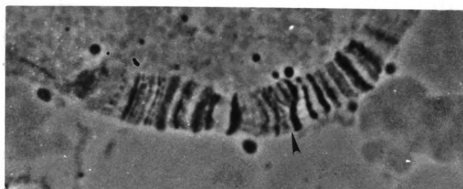


Figure 3. Summary of Genetic and Cytogenetic Mapping of Aprt

Figure 3. Legend

The photomicrograph shows the left terminal end of chromosome three in a salivary gland squash from mid-third instar larvae carrying *Df(3L)R^{+E}* and *Aprt¹*. The arrow indicates the location of the deletion in the deficiency homologue. The central drawing in the figure depicts the cytological map of the left terminal arm of chromosome three after Bridges (13). The vertical brackets indicate (i) the approximate left (61B1-3) and right (62D1-7) breakpoints for the interstitial translocation *Dp(T(Y;3)H141)* and (ii) the deleted region in *Df(3L)R^{+E}*. The lower drawing presents a linkage map of chromosome three for genes which lie within the cytological region presented above. The position of the mutants *mwh*, *ru* and *ve* is based on data from Roberts and Evans-Roberts (26). The position of *R* is based on the recombination distance between *ve* and *R* (13). The position of *Aprt* is based on data from Table 4.

A more precise localization of the cytological position of *Ap^rt* was made possible using a small interstitial deficiency *Df(3L)R⁺E* generously provided by Dr. J. Bonner (Appendix 2 and Figure 3). This deficiency was isolated by treating flies carrying the dominant mutation *R* with X-rays and screening the progeny for the *R⁺* phenotype resulting from deletion of the *R* allele (J. J. Bonner, personal communication). Since *Ap^rt*¹ is closely linked to *R* (Table 4) it was possible that this deletion might also include *Ap^rt*. This possibility was tested by two different methods of complementation: (i) crosses were made between the *Df(3L)R⁺E* balanced lethal stock and each of the homozygous purine-resistant mutants and the progeny reared on purine containing media and (ii) deficiency heterozygotes of all the alleles of *Ap^rt* were constructed and assayed for APRT activity (Table 5). The results show that *Df(3L)R⁺E* confers purine resistance in trans to the mutant alleles of *Ap^rt* and does not contribute to the level of APRT activity in deficiency heterozygotes of either the purine-resistant mutant or purine-sensitive wild type alleles of *Ap^rt*. This absence of complementation indicates that all or part of *Ap^rt* must lie within the six chromomere deficiency 62B7-12 in *Df(3L)R⁺E* (Figure 3).

In addition to the complementation analysis already presented, trans-heterozygotes for all of the alleles of *Ap^rt* and *Df(3L)R⁺E* were constructed and assayed for APRT activity (Table 6). These data show an absence of complementation between any of the alleles of *Ap^rt* or *Df(3L)R⁺E* and show an allele-dose-dependence in the level of APRT activity in the trans- and deficiency-heterozygotes. Dose affects of

Table 5. Complementation Analysis of Aprt Mutants and Df(3L)R⁺E

Cross No.	Sex	Genotype ¹	Parental Cross	Phenotype of Purine Survivors ²	APRT Activity ⁴	n ⁵	s ⁶
				Genotype of F1ies Assayed ³			
1	M	TM3 ru ⁺ h ⁺ /Df(3L)R ⁺ E	ru ⁺ h	ru ⁺ h	1.0	2	0.1
	F	ru Aprt ¹ h/ru Aprt ¹ h					
2	M	TM3 ru ⁺ h ⁺ /Df(3L)R ⁺ E	ru ⁺ h	ru ⁺ h	4.0	2	2.3
	F	ru Aprt ² h/ru Aprt ² h					
3	M	TM3 ru ⁺ h ⁺ /Df(3L)R ⁺ E	ru ⁺ h	ru ⁺ h	0.5	2	0.3
	F	ru Aprt ³ h/ru Aprt ³ h					
4	M	TM3 ru ⁺ h ⁺ /Df(3L)R ⁺ E	ru ⁺ h	ru ⁺ h	0.0	2	-
	F	ru Aprt ⁴ h/ru Aprt ⁴ h					
5	M	TM3 ru ⁺ h ⁺ /Df(3L)R ⁺ E	ru ⁺ h	ru ⁺ h	0.5	2	0.3
	F	ru Aprt ⁵ h/ru Aprt ⁵ h					
				TM3 ru ⁺ h ⁺ /Df(3L)R ⁺ E	ru ⁺ h	38.5	2 6.8
				* TM3 ru ⁺ h ⁺ /ru ⁺ R h		89.2	2 6.0

* Df(3L)R⁺E ru⁺ h was induced with X-rays in the ru⁺ R h chromosome.

Table 5. Legend

- ¹The *TM3* chromosome is a balancer chromosome.
- ²Progeny from the crosses were reared on purine containing media to confine survival to purine-resistant deficiency heterozygotes (4.0 mg purine/10 ml media).
- ³Flies carrying *TM3* are purine sensitive.
- ⁴APRT activity is expressed as DPM(8^{14}C)AMP formed in the assay per minute per microgram of protein.
- ⁵ n is the number of independent determinations for the APRT assay.
- ⁶ s is the standard deviation for the APRT assay.

Table 6. Complementation Analysis of Aprt Alleles and Df(3L)R^{+E}

	Aprt ^A	Aprt ^B	Aprt ¹	Aprt ²	Aprt ³	Aprt ⁴	Aprt ⁵	Df(3LR) ^{+E}
Aprt ^A	1.00	68.0	61.0	57.0	50.0	44.0	45.0	43.0
Aprt ^B		45.0	23.9	26.5	24.9	17.4	21.2	23.0
Aprt ¹			2.0	7.5	1.5	1.5	1.0	1.0
Aprt ²				9.5	5.0	5.0	7.5	4.0
Aprt ³					0.5	0.0	0.5	0.5
Aprt ⁴						0.5	1.5	0.0
Aprt ⁵							0.5	0.5

Values in the table represent the percent APRT activity in flies of each genotype compared to Aprt^A homozygotes. Each data point is based on the average specific activity of APRT from at least two independent determinations.

this kind resulting from mutations at a single locus have been widely reported in *Drosophila* and provide strong evidence of structural gene alterations (27-32).

The results presented thus far demonstrate that five different mutations, independently isolated by selection for purine resistance appear to map to a single locus, *Aprt* (3:3.03), and do not complement with respect to purine sensitivity or deficiency in APRT activity. Each of the alleles of *Aprt* show a characteristic specific activity of APRT which is heritable in a dose-dependent fashion in trans-heterozygotes. Furthermore, *Df(3L)R⁺E* appears to delete the *Aprt* locus since this deletion confers resistance to purine when carried in trans to the purine-resistant mutant alleles and because the deficiency homologue does not contribute to the level of APRT activity in deficiency heterozygotes. Taken together these results suggest that *Aprt* is a structural gene for adenine phosphoribosyltransferase.

The ultimate goal of the investigation of *Aprt* is to use purine selection to isolate mutations affecting the expression of the *Aprt* gene product in whole *Drosophila*. These mutations might then be used to understand the molecular mechanisms which influence the expression of the *Aprt* gene product during the course of development. In order to achieve such a comprehensive understanding of *Aprt*, the following basic questions must be answered: (i) how do mutations in *Aprt* cause a deficiency of APRT activity, (ii) if *Aprt* is a structural gene for the enzyme APRT, are there any others or is this protein coded by a single gene, (iii) how is the APRT protein expressed temporally and spatially during *Drosophila* development and (iv) is it possible to counterselect

a deficiency in APRT activity in addition to purine mediated selection of a deficiency in APRT activity ? Counterselection of a deficiency in APRT activity would allow the direct isolation of rare wild type revertants and recombinants between different purine-resistant mutations which restore APRT activity.

APRT : A Single Polypeptide Encoded by *Appt*

The genetic analysis of *Appt* supports the conclusion that this locus is a structural gene for adenine phosphoribosyltransferase. An important prediction of this hypothesis is that some of the heritable alterations at *Appt* should be reflected as physical alterations in the APRT protein from that mutant strain.

The determinations of APRT activity in the purine-resistant mutants *Appt*¹ and *Appt*² (Table 3) indicate that these mutants have low but easily detectable levels of enzyme activity. These deficiencies of APRT activity might result from two different kinds of alterations assuming that *Appt* is the structural gene for this enzyme: (i) the mutation affects the structural element of *Appt* causing a change in the primary structure of APRT that modifies its specific activity and (ii) the mutation disrupts a controlling element adjacent to the structural element resulting in either reduced synthesis of APRT mRNA or impaired translation of the mRNA produced. The latter mutational mechanism would be expected to result in the synthesis of normal APRT polypeptides which are present in reduced quantities in the mutant in comparison to the wild type.

In order to distinguish between these two alternate explanations for a deficiency in APRT activity, a kinetic analysis of the APRT activity in the *Appt*¹, *Appt*² and *Appt*^A homozygotes was conducted

and the data plotted according to methods used by Fenwick and co-workers for comparing the kinetic properties of HGPRT activity from mutant and normal Chinese hamster ovary cells (8).

The APRT reaction results in the condensation of adenine with 5-phosphoribosyl-1-pyrophosphate (PrPP) to form the nucleotide 5'-AMP. Therefore, the affinity of APRT for both of these substrates was compared between the *Appt*¹ and *Appt*² mutants deficient in APRT activity and the wild type *Appt*^A. The affect of adenine concentration on the synthetic activities of APRT from these strains is presented in Figure 4 and the affect of PrPP concentration in Figure 5. From these results it is apparent that the APRT activity in the *Appt*¹ mutant has a much lower affinity for adenine than does APRT activity from either *Appt*² or the *Appt*^A strain which in turn have similar apparent affinities for adenine (Figure 4). On the other hand, the affinity of APRT activity from *Appt*² for PrPP is much lower than the affinity of APRT activity from either the *Appt*¹ or the *Appt*^A strain, which appear to have similar affinities for PrPP (Figure 5). These results suggest that the mutant alleles *Appt*¹ and *Appt*² alternately affect the affinity of the APRT protein they encode for adenine and PrPP respectively, in contrast to the wild type *Appt*^A. Although the results indicate that these mutant alleles cause physical alterations in the APRT protein, the data must be interpreted with some caution for the following reasons: (i) the kinetic analysis presented in Figures 4 and 5 was conducted on crude extracts of APRT activity and the observed differences might result from strain specific differences in the levels or kinetic properties of enzymes or

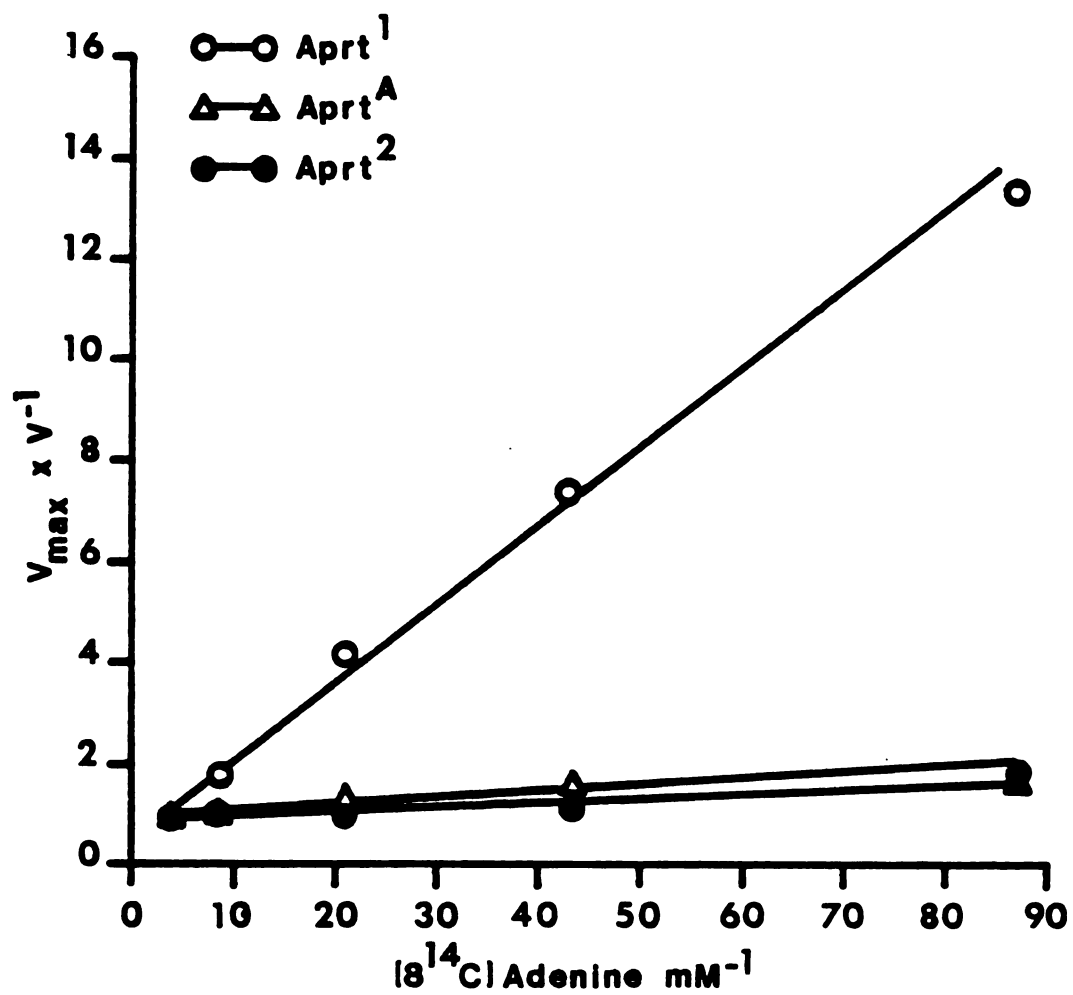


Figure 4. Double Reciprocal Plot of APRT Velocity vs. Adenine Concentration

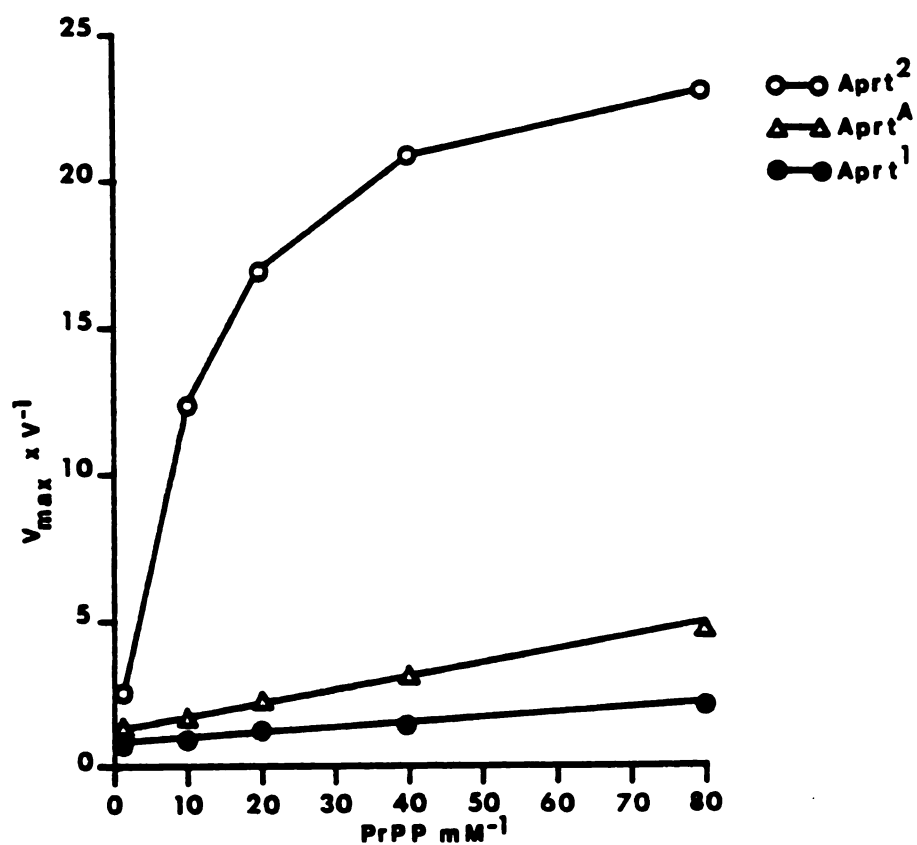


Figure 5. Double Reciprocal Plot of APRT Velocity vs. PrPP Concentration

molecules other than APRT which exhibit an affinity for either adenine or PrPP and (ii) it cannot be conclusively established that either the *Ap^rt*¹ or the *Ap^rt*² mutations were induced in the *Ap^rt*^A allele and so the specific comparison of these alleles is strictly arbitrary. In order to conclusively establish that heritable alterations at *Ap^rt* result in physical changes to the corresponding APRT protein a more direct analysis of the chemical and physical properties of APRT in two different strains of *Drosophila* was conducted.

The results of complementation analysis presented in Table 6 show that the specific activity of APRT in the purine-sensitive wild type strains *Ap^rt*^A and *Ap^rt*^B is characteristic of each strain and is allele-dose-dependent in heterozygotes. This suggests that differences in APRT specific activity between these natural variant strains is due to a physical difference in the APRT produced by each strain. This hypothesis was tested by non-denaturing isoelectric focusing of the APRT activity in these strains by methods developed for HGPRT activity (33) and APRT activity (34) in mammals as modified for *Drosophila* (Appendix 4). The isoelectric focusing autoradiograms of APRT activity (Figure 6) demonstrate the following: (i) APRT activity from the natural variant strains *Ap^rt*^A and *Ap^rt*^B exhibit different isoelectric point isozymes of APRT activity, (ii) the physical differences in APRT molecules produced by these strains probably accounts for the strain-specific differences in APRT specific activity rather than a difference in the concentration of enzyme molecules between the strains and (iii) the locus which specifies the

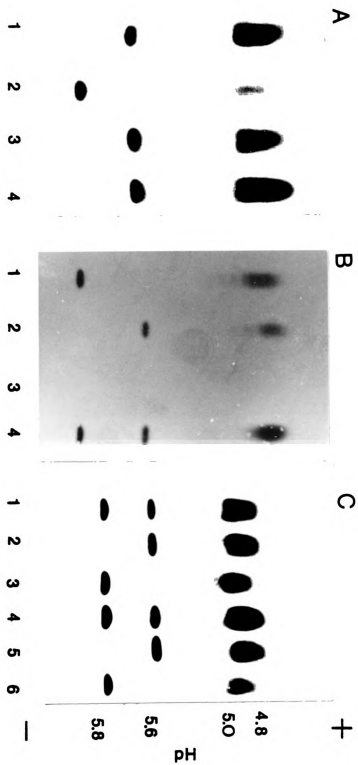


Figure 6. Autoradiograms of Drosophila APRT in Isoelectric Focusing Gels

Figure 6. Legend

Three separate non-denaturing isoelectric focusing autoradiograms of APRT activity were produced as described in Appendix 4. The pH gradient is from 4 (+) to 7 (-) in each gel and the pH of gel slices corresponding to APRT activity is shown for gel C. Lanes contained APRT from flies of the following genotypes: (A) 1. flies carrying the chromosome in which $Df(3L)R^{+E}$ was generated, ($Appt^A$ homozygote), 2. $Df(3L)R^{+E} / Appt^B$, 3. $Df(3L)R^{+E} / Appt^A$, 4. male flies homozygous for $Appt^1$ on the third chromosomes and carrying $Dp(T(Y;3)H141)$. (B) 1. flies homozygous for chromosome three derived from $Appt^B$ homozygotes but in which chromosomes one and four were derived from $Appt^A$ homozygotes, 2. flies homozygous for chromosome three derived from $Appt^A$ homozygotes but in which chromosome two was derived from $Appt^B$ homozygotes, 3. females of the $Dp(T(Y;3)H141)$ strain which are homozygous for $Appt^1$ on chromosome three but do not carry the translocation, 4. $Appt^A / Appt^B$. (C) 1. and 4. $Appt^A / Appt^B$, 2. and 5. $Appt^A / Appt^A$, 3. and 6. $Appt^B / Appt^B$.

isoelectric point of APRT lies at or near the *Ap^rt* locus. The latter conclusion is based on the following results: (i) the isozymes of *Ap^rt^A* and *Ap^rt^B*, regardless of isoelectric point, assort together in a Mendelian fashion with chromosome three (Figure 6B, lanes 1 and 2), (ii) a locus specifying the *Ap^rt^A* isozymes segregates with the *Y* chromosome carrying the third chromosome translocation *Dp T(Y;3)H141* (Figure 6A, lane 4 and 6B, lane 3) which was shown (25) to contain *Ap^rt* and (iii) the *Ap^rt^A* isozymes of APRT have been extinguished as the result of deletion in the *Df(3L)R^{+E}* chromosome (Figure 6A, lanes 1-3).

Non-denaturing isoelectricfocusing of APRT exposes a complex pattern of APRT activity with at least one acidic and one more-basic isozyme of APRT activity in each homozygous variant strain. The pattern of spots results only from APRT activity since *Ap^rt¹* homozygotes produce no exposure of the X-ray plate (Figure 6B, lane 3) nor do *Ap^rt^A* homozygotes when 5-phosphoribosyl-1-pyrophosphate is omitted from the gel incubation mixture. The simplest explanation of the isozyme patterns is that the most acidic bands (Figure 6, pH 4.8-5.0) of enzyme activity are produced by multimeric forms of APRT while the more basic bands of activity (pH 5.6 and 5.8) are produced by monomers. This is true for the following reasons: (i) there is no band of APRT activity at an intermediate pH between the more basic bands (pH 5.6 and 5.8) in heterozygotes as would be expected if heterodimers were formed (Figure 6B, lane 4 and 6C, lanes 1 and 4), (ii) the more acidic band of APRT activity (pH 4.8-5.0) appears to span this entire pH range in heterozygotes suggesting that it contains

both homodimers of each type and heterodimers (Figure 6C, lanes 1 and 4) and (iii) the more-basic bands (pH 5.6 and 5.8) have the same isoelectric point in homozygotes or trans-heterozygotes of the variants (Figure 6C)

Isoelectric focusing is a high-resolution procedure which, when performed under non-denaturing conditions, is expected to separate otherwise identical proteins based on conformational and/or quaternary structural differences (35). It is also conceivable that the isozymes of APRT in homozygotes reflect a heteromeric structure for this enzyme in *Drosophila*. Analysis of APRT purified from humans (36,37) and from rodents (12,19,38) indicates that in both cases APRT is a 40,000 dalton dimer composed of apparently identical subunits. Therefore, it was of interest to know how APRT activity from these organisms would behave when subjected to non-denaturing isoelectric focusing as performed with *Drosophila* APRT. The results of this analysis (Figure 7) indicates that isozyme formation under these conditions is characteristic of both *Drosophila* and mammalian APRT, including human erythrocyte APRT for which peptide fingerprint analysis indicates a homodimeric structure (37). In spite of these results it remains possible that *Drosophila* APRT is encoded by more than a single structural gene.

In order to determine the quaternary and subunit characteristics of *Drosophila* APRT a series of experiments were conducted. Gel filtration of APRT was performed in order to estimate its apparent native molecular weight. APRT was purified from *Aprt*^A and *Aprt*^B homozygotes and analyzed by one- and two-dimensional electrophoresis

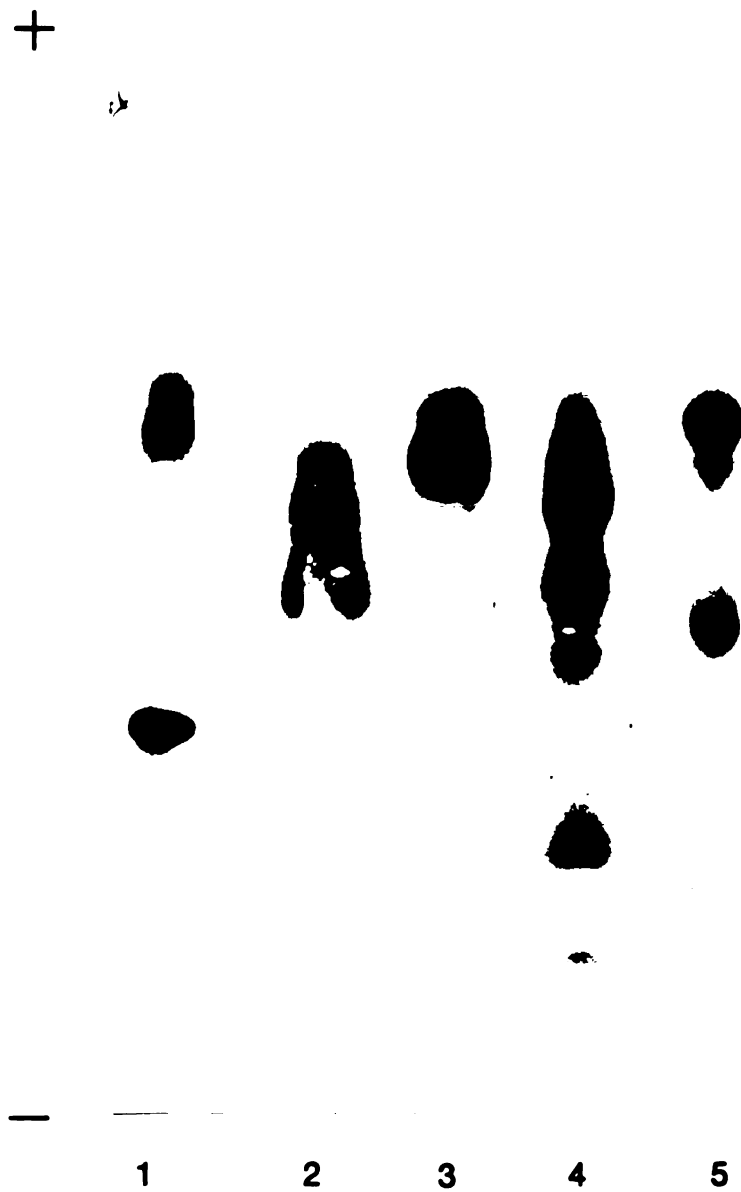


Figure 7. Autoradiograms of APRT Activity from Mammals and Drosophila in an Isoelectric Focusing Gel

Figure 7. Legend

The isoelectric focusing autoradiogram of APRT activity was produced as described in Appendix 4. Lanes contained APRT activity from crude extracts of (1) *Drosophila melanogaster* (*Appt*^A homozygotes), (2) hamster liver, (3) mouse liver, (4) rat liver, and (5) human erythrocytes (from the author, D.H.J.). The pH gradient was from 4 (+) to 7 (-).

of the purified enzymes to compare their subunit structure and estimate the subunit molecular weight.

Gel filtration of *Drosophila* APRT under non-denaturing conditions gives an apparent molecular weight of 38,000 daltons (Figure 8) while one-dimensional electrophoresis of APRT under denaturing conditions shows that the purified enzyme (Appendix 6) migrates as an apparently homogeneous protein with a subunit molecular weight of 23,000 daltons (Figure 9). These results are similar to those reported for sea urchin and mammalian APRT (12) and suggest that *Drosophila* APRT is active as a dimer. The genetic results inferred from non-denaturing isoelectric focusing of *Drosophila* APRT indicate that the most basic bands of APRT activity (Figure 6, pH 5.6 and 5.8) in each homozygous variant are due to active monomers, particularly since these bands have the same isoelectric point in either homozygotes or heterozygotes (Figure 6C). Although there is no evidence of APRT activity eluting from the gel filtration column at a point corresponding to its subunit molecular weight, a fraction of rat liver APRT purified by non-denaturing isoelectric focusing was active as a 20,000 dalton protein (38); yet when rat liver APRT is analyzed by gel filtration, without prior purification by isoelectric focusing, it behaves as a 40,000 dalton dimer (12). It is conceivable that the unique conditions which exist under non-denaturing isoelectric focusing either stabilizes the enzyme activity of monomers or that active monomers are produced from dimers under these conditions.

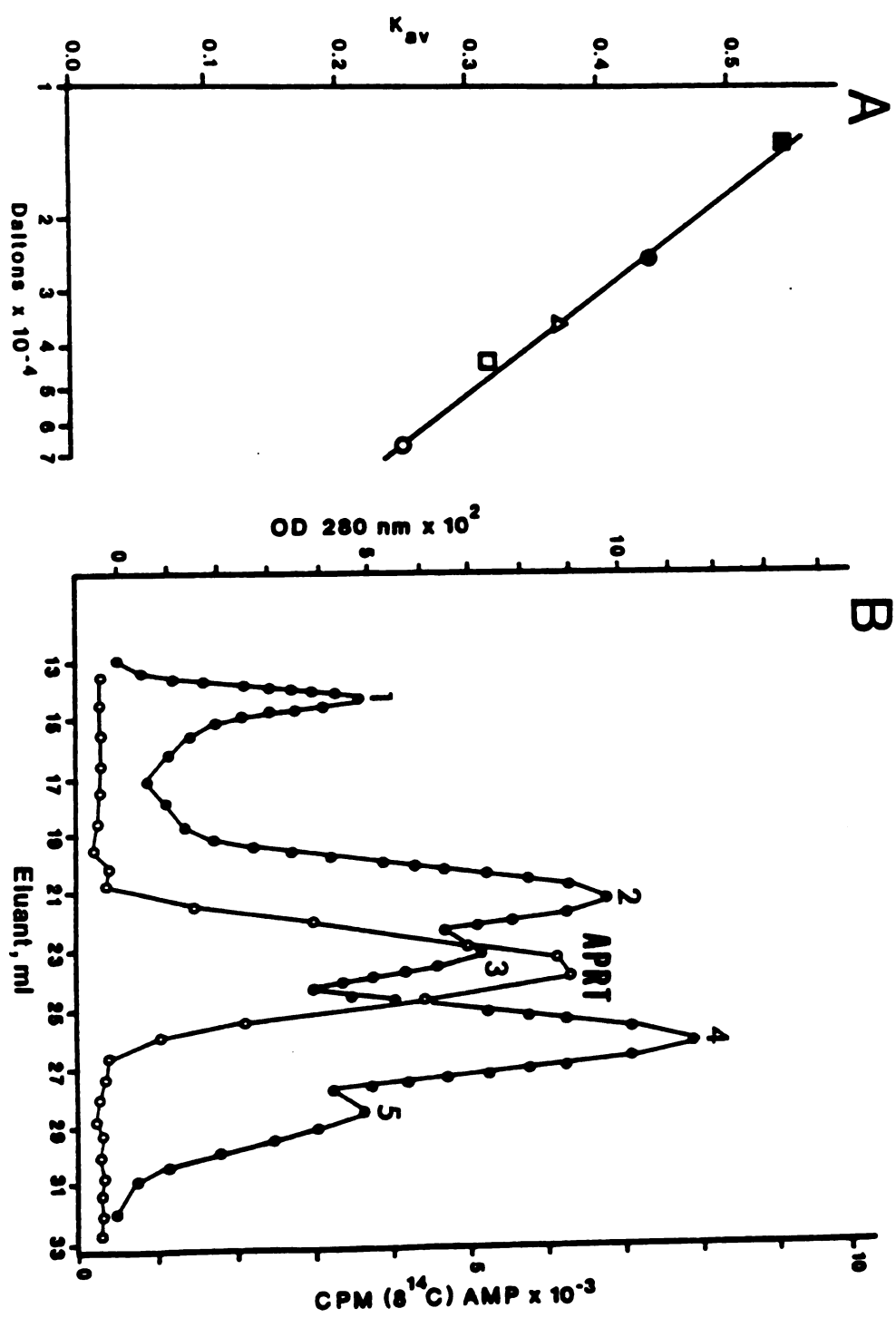


Figure 8. Gel Filtration of Drosophila APRT

Figure 8. Legend

Gel filtration of *Drosophila* APRT and standard proteins was performed as described in Appendix 5. The K_{av} of APRT (Δ) and standard proteins (symbols below) in the semi-log plot (A) is based on the mean from three separate chromatographic runs which did not vary by more than 2%. The elution pattern (B) of standard proteins and blue dextran 2000 ($\bullet\text{---}\bullet$) is reconstructed from separate chromatographic runs: peak 1 blue dextran 2000 (2×10^6 daltons), peak 2 (O) bovine serum albumin (67,000 daltons), peak 3 ($\square$) ovalbumin (43,000 daltons), peak 4 ($\bullet$) (chymotrypsinogen A (25,000 daltons), peak 5 ($\blacksquare$) ribonuclease A (13,700 daltons). The elution profile of APRT (O—O) represents the CPM(8^{14}C) AMP formed in the APRT assay after 5 minutes incubation by 10 microliters of each eluant fraction from the gel filtration of ammonium-sulfate precipitated APRT.

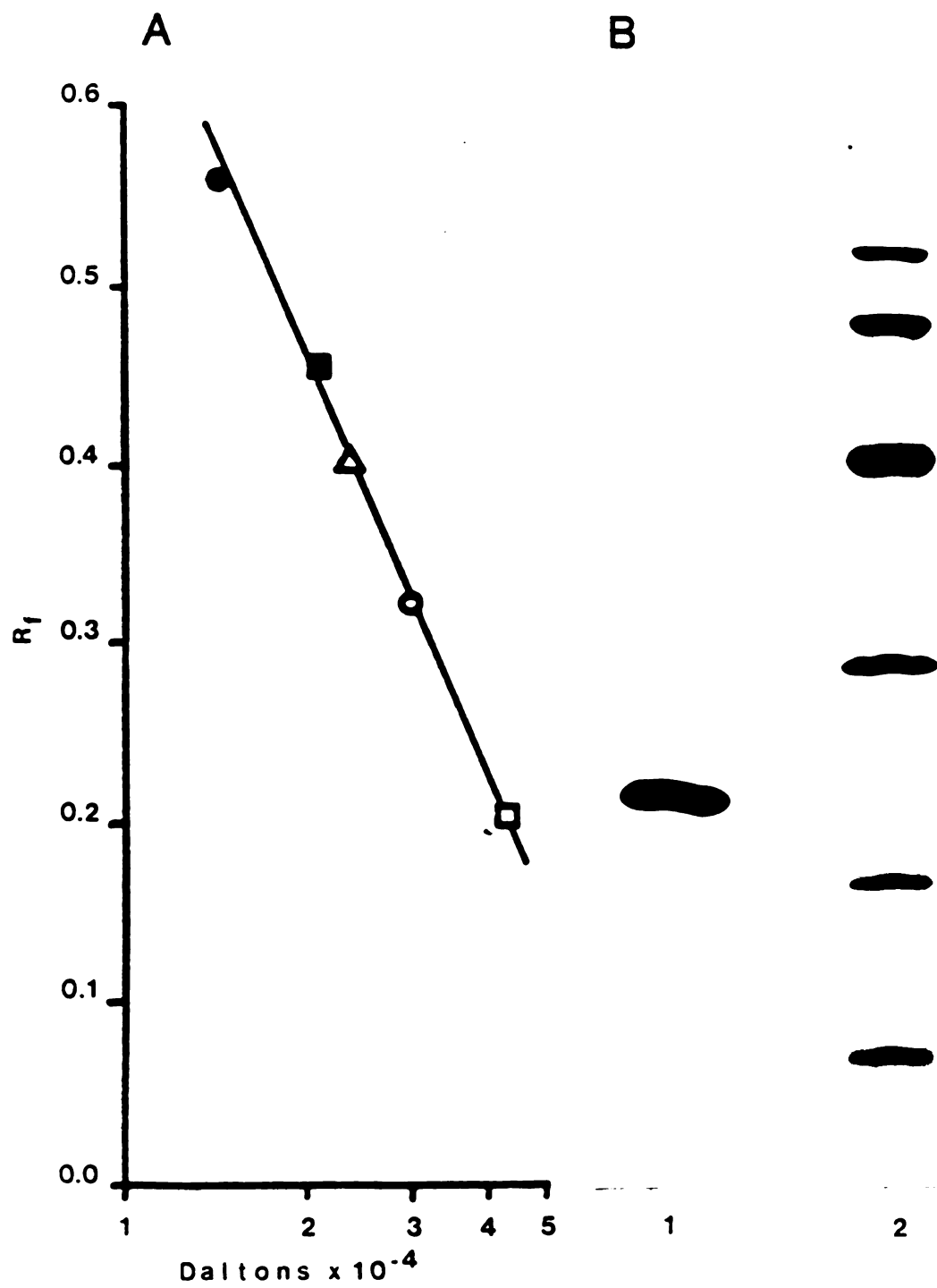


Figure 9. SDS Polyacrylamide Gel Electrophoresis of Drosophila APRT

Figure 9. Legend

Denaturing polyacrylamide gel electrophoresis and silver-staining of purified *Drosophila* APRT and standard proteins were performed as described in Appendix 4. The semi-log plot (A) is derived from the migration of standard proteins in the gel (B). Only those standard proteins with an R_f that produces a linear function of R_f vs. $\log$ protein molecular mass are plotted. Lane 1 of the gel contained *Drosophila* APRT (Δ) purified from *Appt*^A homozygotes. Lane 2 contained in descending order: phosphorylase b (94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin ($\square$) (43,000 daltons), carbonic anhydrase (O) (30,000 daltons), trypsin inhibitor ($\blacksquare$) (20,100 daltons), alpha-lactalbumin ($\bullet$) (14,400 daltons).

Two-dimensional electrophoresis of APRT purified from *Ap^rt^A* and *Ap^rt^B* homozygotes demonstrates that each variant produces APRT molecules of similar molecular weight but with a different isoelectric point (Figure 10). APRT purified from *Ap^rt^A* homozygotes on three separate occasions produced single bands in first-dimension gels which were consistently resolved into one major and one minor spot in the second-dimension gel as shown (Figure 10 A, B and C and Figure 11A). The resolution of the minor protein spot is probably due to lateral spreading of proteins which occurs in the second-dimension gel. In addition, several other minor silver-staining bands are frequently detected in first dimension gels containing purified APRT (Figure 11A, arrows). These minor bands are very faint in second-dimension gels, probably due to the general loss of protein during equilibration of the first-dimension gel prior to electrophoresis into the second-dimension gel (39). It appears that all or most of the silver staining material seen in these gels is APRT since: (i) the protein purified from *Ap^rt^A* homozygotes has the same isoelectric point as the activity of purified APRT from this strain (Figure 11 B and C), (ii) all of the protein in fractions of APRT purified from either variant, regardless of isoelectric point, has a molecular weight of approximately 23,000 daltons (Figure 9 and 10) and (iii) the intensity of the minor silver staining bands increases with consecutive freeze-thawing of the protein sample while the apparent molecular weight of the protein remains unchanged (Figure 10D). Relatively moderate methods of handling proteins, such as freezing a lyophilized

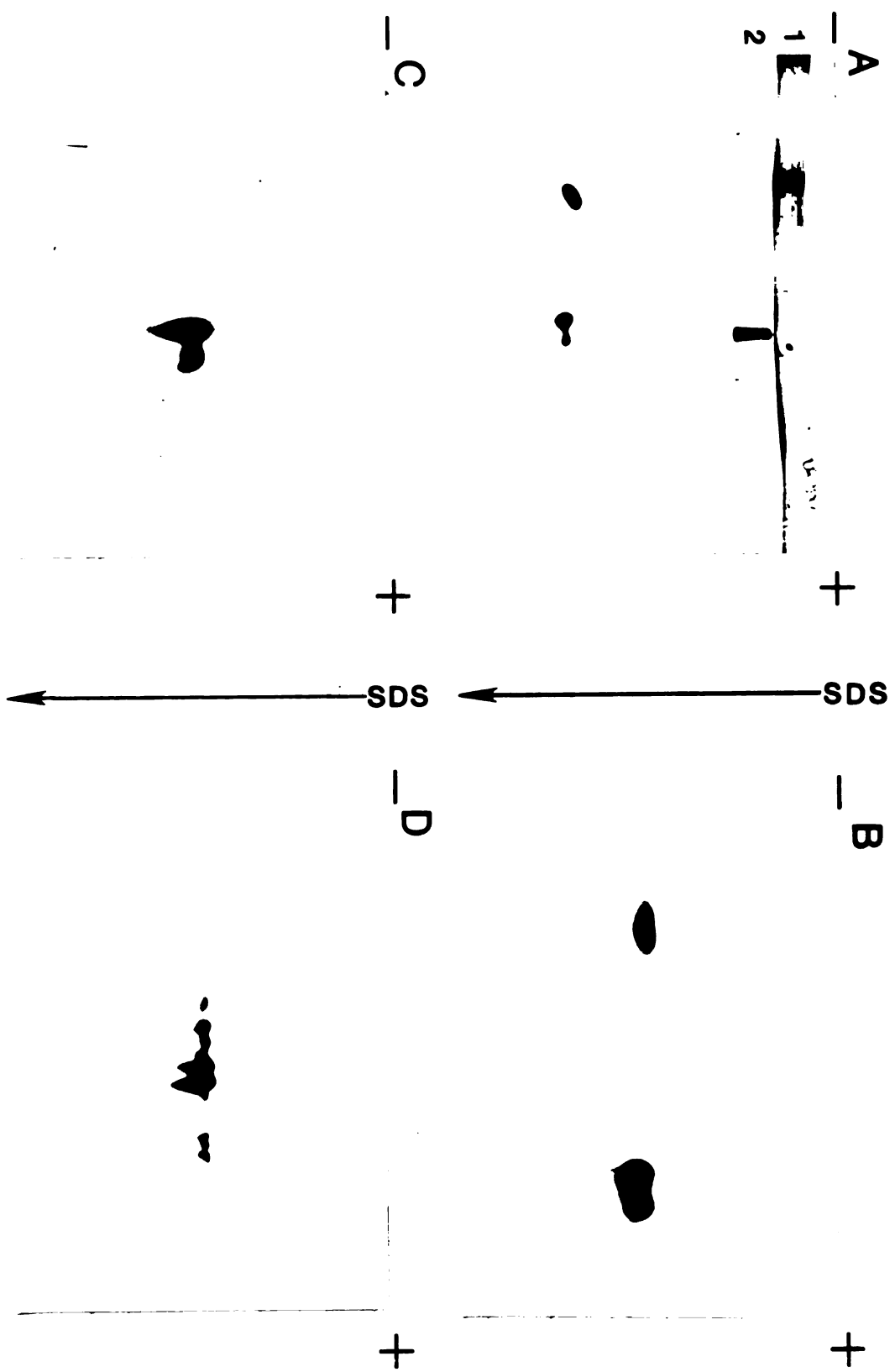


Figure 10. Two-Dimensional Analysis of Purified Drosophila APRT

Figure 10. Legend

Two-dimensional analysis of purified *Drosophila* APRT which employs denaturing conditions in both dimensions, and silver-staining of proteins were performed as described in Appendix 4. First-dimension gels containing purified APRT from *Appt*^B and *Appt*^A homozygotes are shown in gels 1 and 2 respectively which are aligned with each other according to pH above an equivalent two-dimension gel containing both purified enzymes (A). The isoelectric point of APRT from *Appt*^A and *Appt*^B variants is 6.0 and 6.3 respectively. Gel B is a second dimension gel equivalent to gel A. Gels C and D contained only APRT purified from the *Appt*^A variant.

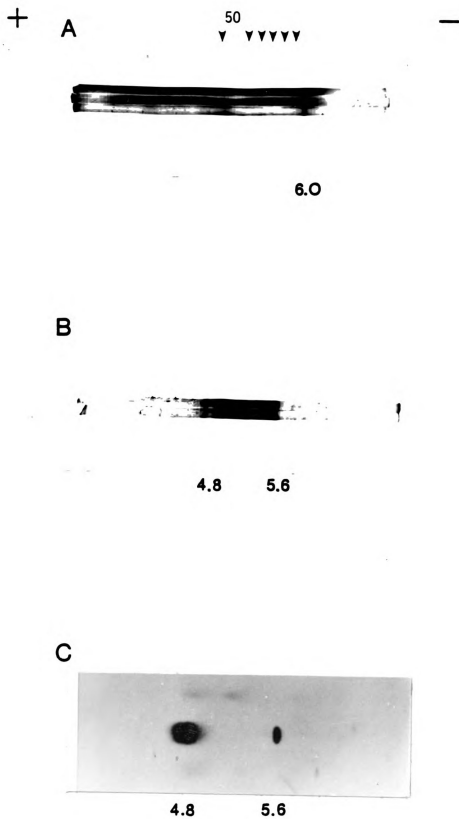


Figure 11. Isoelectric Focusing of Purified Drosophila APRT

Figure 11. Legend

Drosophila APRT purified from *Aprt^A* homozygotes was subjected to isoelectric focusing under the following conditions: (A) denaturing conditions according to O'Farrell (39) and the gels silver-stained for protein. (B) non-denaturing conditions and the gels silver-stained for protein. (C) non-denaturing conditions as in (B) in a gel fluorographed for APRT activity. The pH gradient was from 4 (+) to 7 (-) in gels from each panel and the pH of gel slices which correspond to the location of protein bands or APRT activity is presented below each panel.

sample (not done with APRT), can induce charge heterogeneity in a protein (39). Artifactual charge heterogeneity as reported by O'Farrell (39) results in a series of spots in second dimension gels which are of identical molecular weight but of lesser staining intensity than the major protein from which they are derived. Since this description matches the behavior of the APRT protein in this gel system and because APRT purified from the *Ap^rt^B* variant does not contain a minor protein spot equivalent to that seen in the *Ap^rt^A* protein (Figure 10 A, B and C), it appears that the minor protein spots seen in preparations of APRT from *Ap^rt^A* may be due to artifactual charge heterogeneity. It is also possible that the minor protein spot (Figure 10 A, B and C), which co-purifies only with APRT from the *Ap^rt^A* variant, results from *in vivo* modification of a portion of the APRT molecules in this strain. Alternatively, this minor spot could be due to simultaneous purification of a strain specific contaminating protein which has nearly identical physical, chemical and ligand specific affinity properties as APRT.

The results of non-denaturing isoelectric focusing of APRT activity demonstrate that the isoelectric point of APRT is specified by a locus within the region 62B7-12 of *Df(3L)R⁺E* and, therefore, this locus lies at or near *Ap^rt*. Two-dimensional electrophoresis of APRT under denaturing conditions show that *Ap^rt^A* and *Ap^rt^B* produce APRT molecules of similar molecular weight but with a different isoelectric point. When these observations are considered with the results of genetic mapping and tests of complementation between all of the alleles of *Ap^rt*, several important conclusions can be drawn: (i)

deficiency in APRT activity confers purine resistance, (ii) the APRT protein is composed of identical polypeptides encoded by a single structural gene (iii) the *Appt* locus is the structural gene for APRT and (iv) mutations in this structural gene can be readily isolated by direct selection for purine resistance in whole *Drosophila*.

Temporal Expression and Spatial Distribution of APRT Activity

Since *Aprt* represents a genetically and biochemically tractable gene-enzyme system, it was of interest to determine whether or not *Drosophila melanogaster* show any obvious stage- or tissue-specific differences in the level of APRT activity. APRT activity was assayed at various times during development from the newly laid egg up until the time of eclosion of the imago (Figure 12). No dramatic differences are seen in this temporal profile of APRT specific activity. The mutant *Aprt*¹ is essentially deficient in APRT activity throughout the life cycle while the *Aprt*^A wild type shows an initial peak of enzyme activity in the first instar larva which is approximately two fold greater than the activity seen prior to and after this time in development. The increase in APRT activity which occurs following fertilization and prior to hatching appears to result from activation of the paternally contributed *Aprt* allele following fertilization of the egg. This conclusion is drawn from the fact that eggs newly laid by homozygous *Aprt*¹ females mated with male *Aprt*^A homozygotes have essentially mutant levels of APRT activity while the newly hatched heterozygote larva has approximately half the level of APRT activity as newly-hatched *Aprt*^A homozygote larvae (Table 7A). The *Aprt* system may thus be useful for investigating the general process in which paternally contributed genes become synthetically

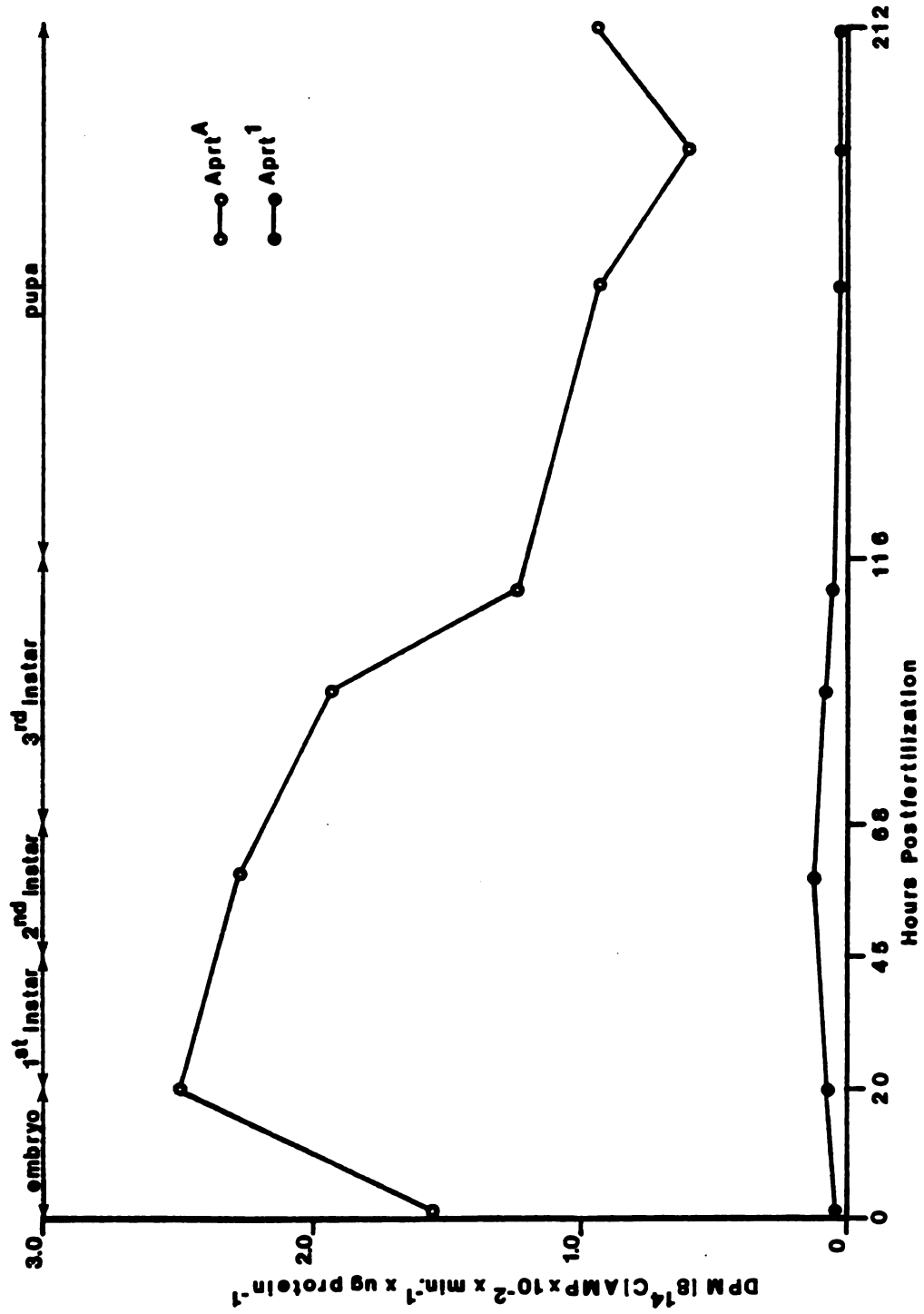


Figure 12. Temporal Profile of APRT Activity During Development in Drosophila

Table 7A. APRT Activity During Early Development

Genotype	APRT Activity [*]	
	Fertilized Egg	Newly Hatched Larva
Aprt ¹ /Aprt ¹	105	210
Aprt ^A /Aprt ¹	140	1596
Aprt ^A /Aprt ^A	2730	3297

* APRT Activity was assayed in crude extracts of eggs and larvae. Each assay contained 10 larvae or 10 egg equivalents of protein. Activity is expressed as DPM(8¹⁴C)AMP formed per minute of incubation time per assay.

Table 7B. APRT Activity in Various Tissues

Tissue	APRT Activity [*]
Brain	+
Thoracic Muscle	+
Gut	+
Malpighian Tubules	+
Ovary	+

* Crude supernatants of the homogenized tissues from Aprt^A adult homozygotes were subjected to nondenaturing isoelectric focusing. The + indicates the presence of the Aprt^A isozymes (Figure 6).

active during embryogenesis.

In addition to the apparently simple temporal pattern of APRT activity, this enzymes activity is present in all major adult tissues of *Drosophila* (Table 7B). These results are not particularly suprising since purine toxicity appears to be a gradual phenomenon and the efficacy of purine selection is dependent upon the presence of purine in the selection media during the majority of the larval feeding period. It appears that the wild type *Aprt* gene is constitutively active in *Drosophila* as appears to be the case in *E. coli* (40). However, the *Aprt*¹ mutant is deficient in APRT activity throughout the life cycle suggesting that this enzymes activity is not essential for viability of *Drosophila*.

Alternate Pathways of Purine Salvage in *Drosophila*

In addition to assays of HGPRT and APRT activity presented earlier in Table 1, several other salvage enzyme activities were assayed in *Drosophila* (Table 8, Appendix 1). The presence of inosine phosphorylase activity was previously reported in *Drosophila* (41). The enzyme was studied here since purine appears to be a substrate for xanthine dehydrogenase which Glassman attributed to the similarity of purine to hypoxanthine (1). Since previous workers only examined the conversion of inosine to hypoxanthine by inosine phosphorylase (41) it was of interest to know if the *Drosophila* enzyme could also perform the reverse of this reaction which might be important to the mechanism of purine toxicity. Although it is not known if *Drosophila* have inosine kinase activity, the results of feeding radiolabeled inosine (42) and the ability to make *Drosophila* cell growth conditional on the use of inosine as a nucleotide source (43) suggest that inosine may be converted to a nucleotide by this route. Therefore, it is possible that some purine may be converted to a toxic nucleotide through this hypothetical pathway assuming that inosine phosphorylase recognizes purine as hypoxanthine and converts it to purine riboside and assuming that purine riboside is toxic or is converted to a toxic product. In any case, this pathway is not involved in the biochemical defect in the purine-resistant mutants examined here since inosine phosphorylase

Table 8. Purine Base Salvage Enzyme Activities in Drosophila

Assay Code ¹	Enzyme Assayed ²	Activity in Homozygotes ³	
		Aprt ¹	Aprt ^A Aprt ^B
A	Inosine Phosphorylase	16.6	22.6 11.7
B	Purine Nucleoside Phosphorylase	0.0	0.0 0.0
C	HGPRT ⁴	0.0	0.0 0.0
D	Adenosine Kinase	-	21.4 -

- ¹A (8¹⁴C)Hypoxanthine + α-D-ribose-1-phosphate $\longleftrightarrow$ (8¹⁴C)Inosine + Pi
 B (8¹⁴C)Adenine + α-D-ribose-1-phosphate $\longleftrightarrow$ (8¹⁴C)Adenosine + Pi
 C (8¹⁴C)Hypoxanthine + 5-phosphoribosyl-1-pyrophosphate $\longrightarrow$ (8¹⁴C)IMP + PPi
 D (8¹⁴C)Adenosine + ATP $\longrightarrow$ (8¹⁴C)AMP + ADP

²Enzymes were assayed in crude extracts of adult flies (Appendix 1).

³Activity is expressed as DPM (8¹⁴C)Product formed per minute per μg protein.

⁴Homogenates were treated with norit prior to assay.

activity is present in both the purine-resistant mutant and the purine-sensitive wild type *Drosophila* (Table 8).

It was also of interest to determine if *Drosophila* extracts could effect the conversion of adenine to adenosine. This synthesis is generally attributed to the enzyme purine nucleoside phosphorylase (inosine phosphorylase), which converts a variety of purine bases to their corresponding nucleosides (44). Although hypoxanthine is converted to inosine by extracts of *Drosophila*, the conversion of adenine to adenosine was not observed in extracts of either the purine-resistant mutant or the wild type strains (Table 8). Therefore, this route does not appear to be involved in the toxification of purine in *Drosophila*. *Drosophila* does not appear to have an HGPRT activity (Table 1). This conclusion has been reached in the past by other investigators from both assays of HGPRT activity in extracts of *Drosophila* and from the observation that cultured *Drosophila* cells are insensitive to killing by the toxic guanine analogue 8-azaguanine (24,45). However, Becker reported that he was able to detect HGPRT activity in crude extracts of *Drosophila* following treatment of the extracts with norit (46). This experimental approach was repeated here without detection of HGPRT activity in *Ap^rt^A*, *Ap^rt^B* or *Ap^rt¹* homozygotes (Table 8).

The primary reason for assaying these enzymes was to define the biochemical defect in the purine resistant mutant which renders it insensitive to purine killing. Although it became apparent that a deficiency in APRT activity was the biochemical defect conferring resistance to purine in mutants of *Ap^rt*, it remained curious that the

*Aprt*¹ mutant was much more sensitive to purine toxicity when this mutant was also deficient for xanthine dehydrogenase activity in comparison to *Aprt*¹ flies having XDH activity (2,21). This observation became more significant when it was found that alanosine, an inhibitor of *de novo* AMP synthesis (Figure 13) would not effectively counterselect APRT deficiency in *Drosophila* (47) even so this is a standard method of selecting wild type revertants of APRT deficient eucaryotic cells in culture (10). In practice it was found that alanosine was nearly as toxic to *Aprt*^A wild type flies as it was to the APRT deficient mutants (47). This result suggested that *Drosophila* have a pathway for AMP synthesis in addition to the *de novo* pathway and the salvage pathway through APRT activity.

The observation that XDH-deficient, APRT-deficient double mutants of *Drosophila* were considerably more sensitive to purine killing suggested that the double mutant was converting purine to a toxic nucleotide by a pathway other than through APRT activity which was exacerbated in the double mutant due to its inability to detoxify purine through the XDH pathway. Since *Drosophila* does not appear to have HGPRT activity (Table 1 and 8), nor can they convert adenine to adenosine (Table 8); it appeared that the alternate pathway might involve the initial synthesis of purine riboside by inosine phosphorylase activity (Table 8). The purine riboside formed in this step might then be converted to a toxic nucleotide by a nucleoside kinase activity. If this hypothesis were correct it predicts that

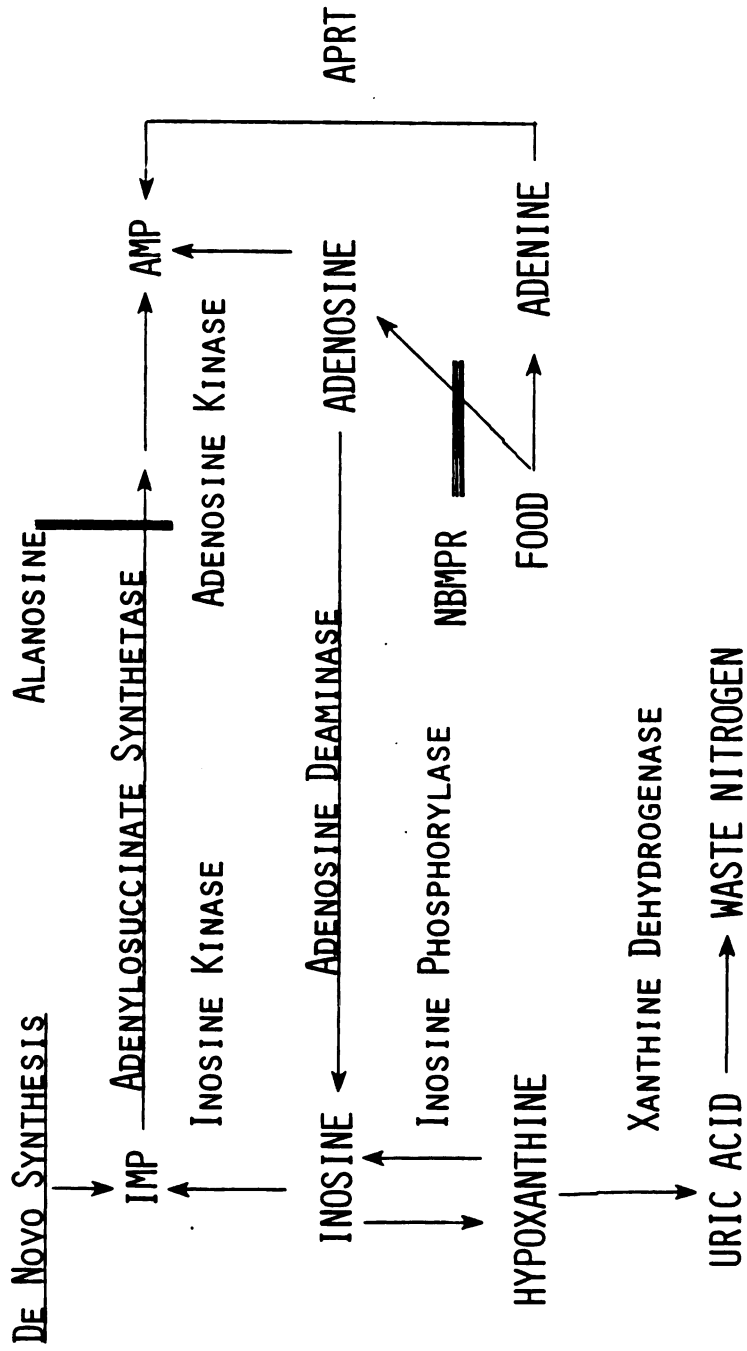


Figure 13. AMP Biosynthesis in *Drosophila*

purine-resistant mutants and the purine-sensitive wild type would be nearly equally sensitive to purine riboside toxicity since both genotypes would have this alternate pathway. The results presented in Figure 14 suggest that this alternate pathway of purine toxification does exist in *Drosophila* since both purine resistant and purine sensitive flies are about equally sensitive to purine riboside induced lethality. This result is particularly significant since cultured mammalian cell mutants, deficient in adenosine kinase activity, are resistant to purine riboside toxicity in contrast to purine riboside sensitive cells (48). Preliminary assays of adenosine kinase activity demonstrate that this enzyme activity is present in crude extracts of *Drosophila* (Table 8). Therefore, it appears that adenosine kinase provides both an alternate pathway for the toxification of purine, in addition to the activity of APRT and adenosine kinase activity provides an alternate route for the synthesis of AMP through adenosine salvage (Figure 13). This result also explains why alanosine fails to counterselect APRT deficiency in whole *Drosophila*.

Since counterselection of a deficiency in APRT activity in *Drosophila* would allow genetic fine structure analysis between *Aprt* mutant alleles, the adenosine kinase pathway is of considerable interest. Two hypothetical approaches seem possible for developing a protocol for the counterselection of a deficiency in APRT activity in *Drosophila*. The most obvious is to isolate an adenosine kinase deficient mutant by selection for resistance to purine riboside. This would be most economically accomplished in the XDH deficient mutant *ry*

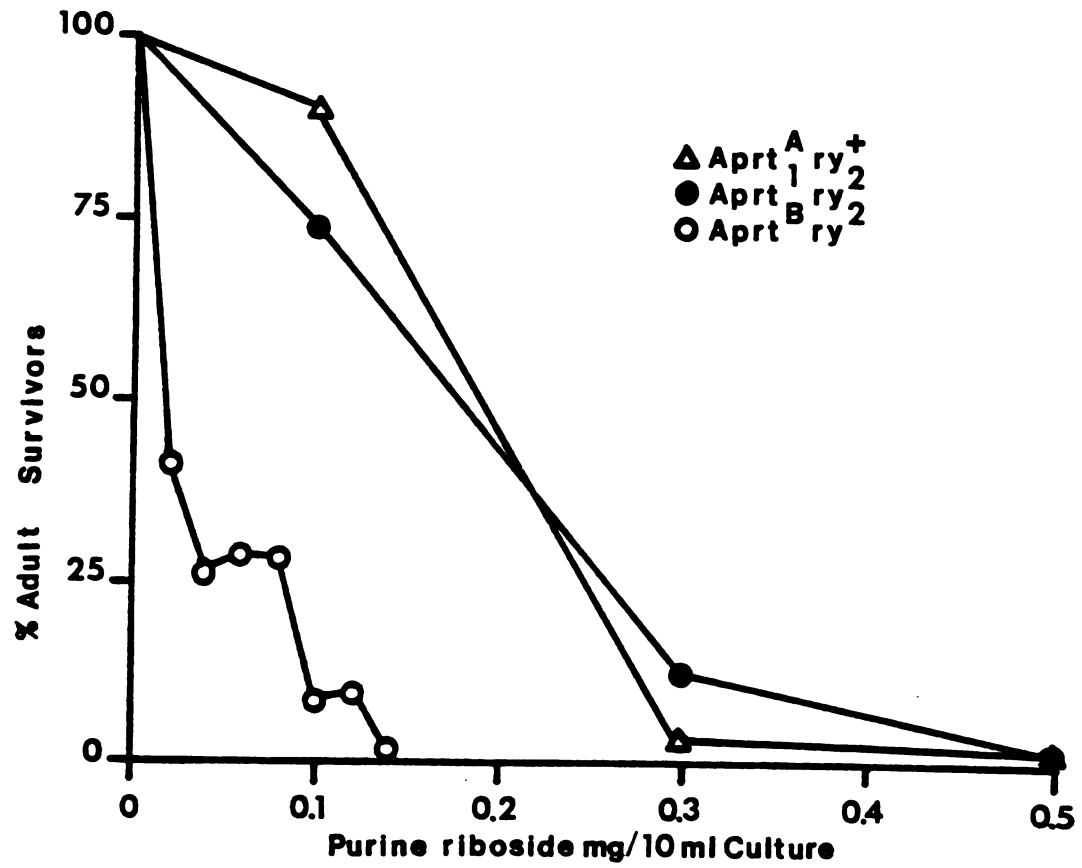


Figure 14. Purine Riboside Sensitivity in Drosophila

Figure 14. Legend

First instar larvae of the indicated genotypes were placed in shell vials containing 10 ml of culture media (25 larvae/vial). Purine riboside was then added to each vial dissolved in 60 microliters of distilled water. There were 4 replicate vials for each purine riboside concentration.

(Figure 14). The enhanced sensitivity of *ry* to purine riboside toxicity in comparison to the wild type, *Ap^{rt}^A*, probably results from the absence of xanthine dehydrogenase activity in *ry*. Inosine phosphorylase may convert some purine riboside to free purine, mistaking purine riboside for inosine (Figure 13). Since *ry* has no XDH activity, which detoxifies purine in wild type flies, the purine formed from purine riboside in *ry* flies can only be excreted or converted to a toxic product via APRT activity. However, in wild type *Ap^{rt}^A* homozygotes, the purine formed from purine riboside can be detoxified by XDH activity. Thus, the wild type is more resistant to purine riboside toxicity than *ry*. *Ap^{rt}¹ ry²* homozygotes have no XDH activity but are deficient in APRT activity as well and are, therefore, resistant to purine formed from the catabolism of purine riboside. The APRT deficient, adenosine kinase deficient double-mutant would theoretically be incapable of AMP synthesis in the presence of alanosine (Figure 13). An alternate approach to this protocol would take advantage of the observation that NBMPR (nitrobenzylthioinosine) apparently blocks adenosine transport and rescues purine riboside sensitive mammalian cells (49) as well as whole mice (50) from a lethal exposure to purine riboside. The inclusion of NBMPR in the culture media in addition to alanosine would theoretically block the synthesis of AMP in the *Ap^{rt}* mutants by all available routes. Either of these protocols should clearly distinguish between flies having APRT activity and deficient flies since in both cases survival is contingent upon the presence of APRT activity.

Summary and Discussion

A combination of genetic and biochemical techniques were used to analyze mutants and natural variants affecting purine sensitivity and the specific activity of adenine phosphoribosyltransferase in *Drosophila*. Genetic and cytogenetic mapping studies show that purine resistance and deficiency in APRT activity result from alterations at a single locus, *Appt* (3:3.03), which lies in the region 62B7-12 (Table 4 and Figure 3). The results of complementation tests support the conclusions from genetic mapping since all five purine-resistant mutants and the natural purine-sensitive variants fail to complement with respect to APRT activity and are thus confined to a single complementation group (Table 6). The level of APRT activity shows allele-dose-dependence in trans-heterozygotes and in flies which are haploid for different *Appt* alleles. In addition to the effects of *Appt* alleles on purine sensitivity and specific activity of APRT, mutants and natural variants of this locus also cause physical alterations of the APRT protein.

The preliminary kinetic analysis of APRT activity in the mutants *Appt*¹ and *Appt*² suggest that these mutations affect the affinity of APRT for adenine and 5-phosphoribosyl-1-pyrophosphate respectively, in comparison to each other and to the *Appt*^A wild type strain (Figure 4 and 5). Gel filtration of APRT under non-denaturing conditions

indicates that this enzyme has a native molecular weight of 38,000 daltons (Figure 8) while electrophoresis of the purified enzyme under denaturing conditions provides a subunit molecular weight of 23,000 daltons. These results suggest that APRT is normally active as a dimer. Moreover, the *Appt*^A and *Appt*^B natural variant strains are shown to produce APRT molecules with a similar molecular weight but with a different isoelectric point (Figure 10) which, when considered with the results of gel filtration, demonstrate that APRT is a dimer composed of identical subunits. The locus which specifies the isoelectric point of APRT lies within the region 62B7-12 of *Df(3L)R*^{±E} which contains the *Appt* locus (Figure 6 and Table 5).

The combined results suggest that APRT is a dimer composed of identical polypeptides encoded by a single structural gene, *Appt*. The conclusion that *Appt* is the structural gene for APRT is based primarily on the widely held assumption that a locus which affects the physical characteristics of an enzyme and which shows gene-dose effects on the level of that enzymes activity, must represent the structural gene for the enzyme (8,19,26-32). This argument is supported by evidence that loci which appear to code for post-translational modifiers of an enzyme or a trans-acting regulator of an enzymes structural gene, do not show gene-dose effects on that enzymes activity. For example, the *ma-1* locus appears to code a post-translational modifier of xanthine dehydrogenase in *Drosophila* (51,52). Homozygous *ma-1* mutants fail to exhibit XDH activity however, *ma-1/ma-1*⁺ heterozygotes have the same level of XDH activity as *ma-1* homozygotes (27). This is in contrast to the gene-dose effects of

ry⁺ alleles on the level of XDH activity (27) and *ry* has been shown to be the structural gene for XDH in *Drosophila* (27,53-55). In the case of alcohol dehydrogenase (ADH) in *Drosophila*, variants have been isolated from natural populations that affect the level of ADH activity and the concentration of ADH protein which map to chromosome three (56) while the ADH structural gene (*ADH*) is linked to chromosome two (57,58). The action of these apparent regulators of *ADH* is dominant, causing the same enhanced level of ADH activity in heterozygotes or homozygotes of a particular third chromosome genotype (56). Again, this is in contrast to the incremental increase in the level of ADH activity in response to increasing doses of *ADH*⁺ alleles (28,57). Ultimately, the unequivocal proof that a particular locus is the structural gene for a given protein is a demonstration of co-linearity between the amino acid sequence of the protein and the codon sequence of the putative structural gene.

The primary reason for this detailed investigation of *Ap^rt* and its product is that mutations of this locus can be directly isolated by purine selection. In this respect *Ap^rt* is unique among most gene loci that have been examined in higher eucaryotes. Only a few of these loci offer the investigator a method for the rapid isolation of allelic mutations potentially affecting both the regulatory and structural elements of a specific gene in an intact animal. This investigation began with a mutation conferring resistance to purine by an unknown biochemical mechanism. The approach taken here has been to assemble a fundamental genetic and biochemical framework of understanding from which to investigate the organization and control

of the gene *Appt* and its protein product. Several important steps toward the development of that framework have been made: (i) all of the five purine resistant mutations have been shown to be alleles of a single locus, *Appt*, (ii) the location of *Appt* within the *Drosophila* genome has been precisely determined, (iii) a series of protocols for the isolation and subsequent genetic manipulation of purine resistant mutants have been worked out, (iv) isoelectric point variants of APRT have been discovered and mapped to *Appt*, (v) the product of *Appt* has been identified as adenine phosphoribosyltransferase and shown to be a single enzymatically active polypeptide, (vi) the APRT protein has been purified and an antibody (Appendix 7) to the protein has been made, (vii) an alternate pathway for the synthesis of AMP via adenosine kinase in *Drosophila* has been described and (viii) a potentially useful direct chemical selection procedure for the isolation of adenosine kinase deficient mutants of *Drosophila* has been devised with important implications for the counterselection of a deficiency in APRT activity.

The procedures described for purine selection should allow the isolation of a library of mutations affecting the structural and regulatory elements of *Appt*. The eventual development of a procedure for the counterselection of a deficiency in APRT activity in whole *Drosophila* should allow the fine structure mapping of purine resistant mutations in *Appt*. This same procedure ought to allow for the cloning of *Appt* by transvection of *Drosophila* cells deficient in APRT activity with DNA containing a wildtype *Appt* gene. A preliminary test of this procedure by Wyss using a *Drosophila* cell line deficient in APRT

activity demonstrates the potential usefulness of the approach (59). Furthermore, this procedure was used successfully for the transvection cloning of the hamster APRT structural gene in mouse L cells deficient in APRT activity (60). Fine structure mapping in conjunction with sequence analysis of the cloned *Drosophila Aprt* gene will provide an understanding of the organization of this locus. This information will be important for discriminating between mutations which lie within the protein coding regions of *Aprt* and those which do not. Fine structure mapping has been used in this way to characterize mutations in the xanthine dehydrogenase structural gene by Chovnick and co-workers (61). Subsequent analysis of the latter kinds of mutations should yield an understanding of the molecular mechanisms involved in the control of *Aprt*.

In addition to the analysis of *Aprt* gene organization and control for its own sake, the molecular cloning and characterization of this gene offers opportunities for the study of other *Drosophila* gene loci as well. Rubin and Spradling have recently developed a technique for introducing cloned genes from *Drosophila* into *Drosophila* embryos followed by the isolation of flies which transmit the exogenous DNA in their germ line (62,63). This pioneering technique will ultimately allow the *in vivo* analysis of *in vitro* modified DNA, such as inter-gene hybrids between the putative controlling elements of one gene and the protein coding elements of another. Similar approaches have already proven valuable for studying the regulation of inducible and repressable gene loci in procaryotes (64,65). The usefulness of this new experimental approach for understanding the molecular

mechanisms of eucaryotic gene control is far reaching however, it will require the following elements: (i) the availability of many different cloned genes from *Drosophila*, (ii) the availability of cloned, easily detectable structural gene variants at a variety of loci, (iii) the availability of different kinds of regulatory genetic elements, both normal and mutant, from a variety of cloned genes which show different temporal and spatial modes of expression during *Drosophila* development, (iv) the availability of selectable genetic markers for the direct isolation and maintenance of transvected gene sequences and (v) the ability to compare the activity and effects of transvected gene sequences having novel mutations to the effect of those same mutations which have been previously isolated and studied *in situ* at their normal position in the *Drosophila* genome.

The *Aprt* gene-enzyme system exhibits uniquely important features in the latter respects. The *Aprt*^A and *Aprt*^B structural gene variants are easily distinguishable in crude extracts of homozygotes or heterozygotes by isoelectric focusing. It appears likely that a procedure for the counterselection of a deficiency in APRT activity can be developed in whole *Drosophila*. This procedure would be useful for the isolation and maintenance of *Drosophila* strains transvected for particular wild type alleles of *Aprt*. The APRT protein appears to be expressed at all times and in all tissues during development of *Drosophila* in contrast to many other gene loci which have been examined. Mutations in the *Aprt* structural element and presumably in its controlling element(s) can be isolated *in situ* on chromosome three in the intact organism which provides an important control for the

analysis of possible position affect variagation resulting from the transvection of cloned genes to new sites within the *Drosophila* genome. From these diverse apparent and theoretical standpoints this genetic and biochemical analysis of *Aprt* is a useful approach to a more detailed understanding of eucaryotic gene organization and control.

APPENDICES

Appendix 1: Enzyme and Protein Assays

APRT and HGPRT Assays

Two methods were used for the assay of APRT and HGPRT activities. A DEAE filter assay was used to monitor the purification of *Drosophila* APRT (Appendix 6) and to test the ability of rabbit sera to precipitate APRT (Appendix 7). A chromatography assay was used for precise determinations of HGPRT and APRT activity. DEAE is a strong anion exchanger which tightly binds the negatively charged nucleotide products of the HGPRT and APRT catalized reactions while allowing the relatively neutral purine bases to be washed from the filter. This assay is rapid, sensitive and more economical than the chromatography method. The chromatography assay is, however, more precise and more versatile than the DEAE filter assay since it results in the separation of a variety of purine bases and nucleotides from a mixture by thin-layer chromatography (Table 9). The chromatography assays of APRT and HGPRT activity used in this study are modifications of the procedures developed by Merril (20) for the assay of these enzymes in lysates of human erythrocytes.

Table 9. Separation of Purine Bases, Nucleosides and Nucleotides on Polygram Cel 300 PEI

Compound ¹	R _f
AMP ²	0.07
IMP ³	0.19
Adenosine ²	0.55
Inosine ³	0.82
Adenine ²	0.38
Hypoxanthine ³	0.64

¹Nucleotides and nucleosides were made up as 2% solutions in distilled water. Purine bases were made up as saturated solutions in distilled water. Five microliter aliquots of each solution were applied to the PEI TLC plate.

²Chromatography was performed with 0.1 M LiCl.

³Chromatography was performed with 0.3 M LiCl.

APRT Assay [E.C. 2.4.2.7]

The first step in developing the assay for *Drosophila* APRT activity was to optimize the chemical parameters which promote enzyme activity. The response of *Drosophila* APRT activity to different pH, (8^{14}C)adenine concentration, 5-phosphoribosyl-1-pyrophosphate (PrPP) concentration and magnesium ion concentration is shown in Figures 15, 16, 17 and 18 respectively. In addition to these tests, the ability of manganese ion to replace magnesium as a metal cofactor for *Drosophila* APRT activity was examined since it is reported that either of these divalent ions will promote APRT activity from human cells (66) and bacteria (67). The results presented in Figure 19 indicate that manganese ion results in an approximate three fold increase in the velocity of *Drosophila* APRT activity at a four fold lower concentration in comparison to magnesium ion. However, both manganese sulfate and manganese chloride cause a precipitate to form in the reaction mixture containing crude extracts of *Drosophila* and in crude extracts of *Drosophila* containing these salts at concentrations above 5 mM. The precipitate probably results from the denaturation of protein by heavy metal contaminants in the "reagent grade" lots of manganese salts which are 10 to 100 times greater than in lots of "reagent grade" magnesium salts (Malinkrodt). Therefore, magnesium was chosen as the metal cofactor in assays of *Drosophila* APRT activity.

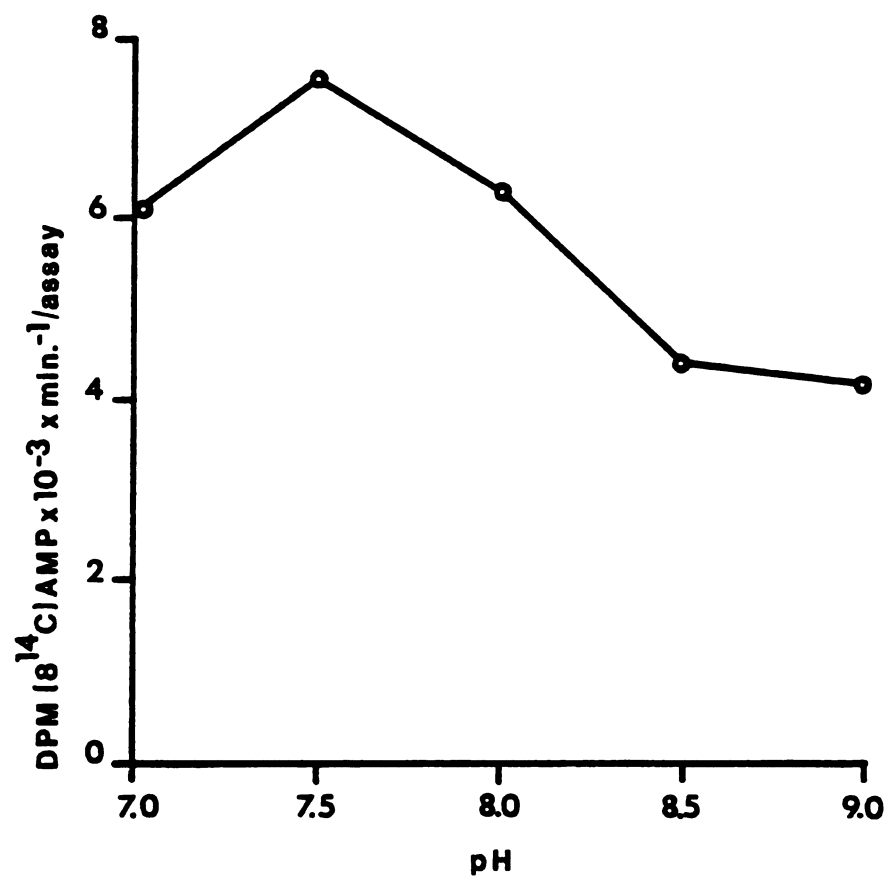


Figure 15. APRT Velocity vs. pH

Figure 15. Legend

Assay mixtures contained the optimal concentrations of reagents and crude extracts of adult *Aprt^A* homozygotes. Tris buffer (50 mM) was used for the pH range from 7.0-8.5. Caps buffer (50 mM) was used at pH 9.0. Conditions were as described for the TLC assay.

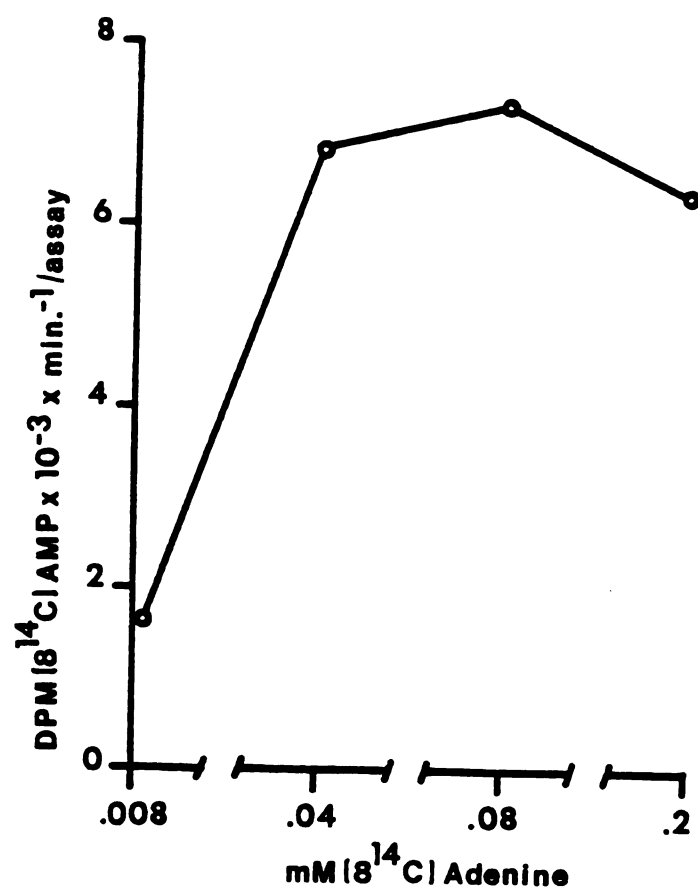


Figure 16. APRT Velocity vs. Adenine Concentration

Figure 16. Legend

Assay mixtures contained the optimal concentration of reagents and crude extract from adult *Aprt^A* homozygotes. Conditions were as described for the TLC assay.

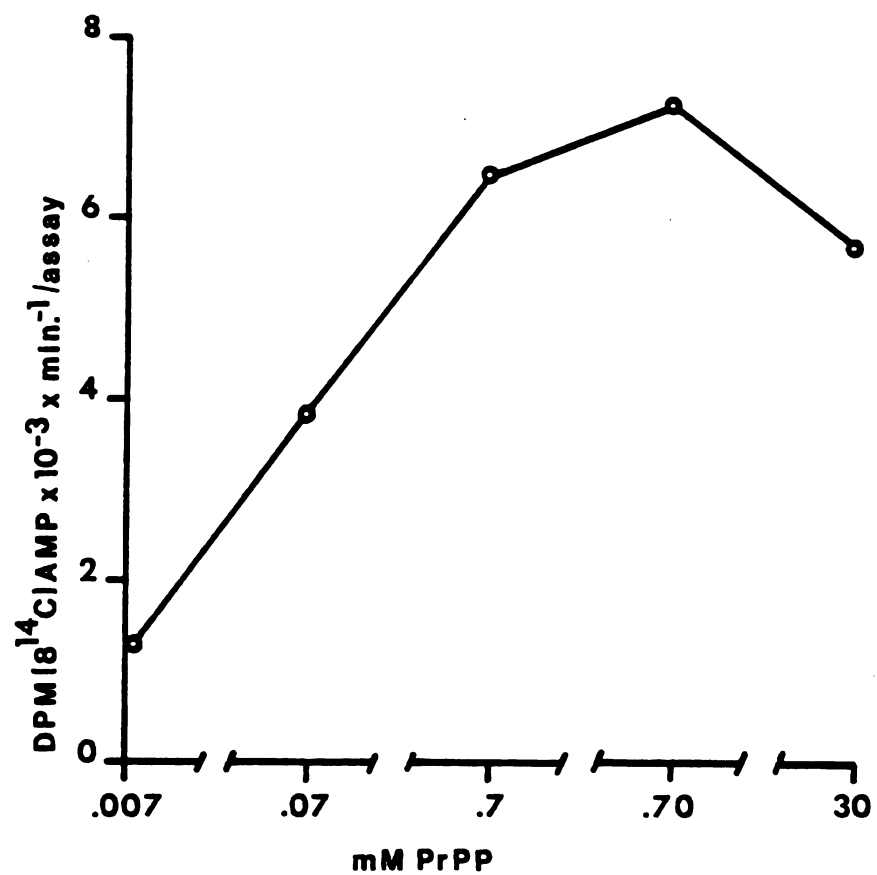


Figure 17. APRT Velocity vs. PrPP Concentration

Figure 17. Legend

Assay mixtures contained the optimal concentration of reagents and crude extract of adult *Aprt^A* homozygotes. Conditions were as described for the TLC assay.

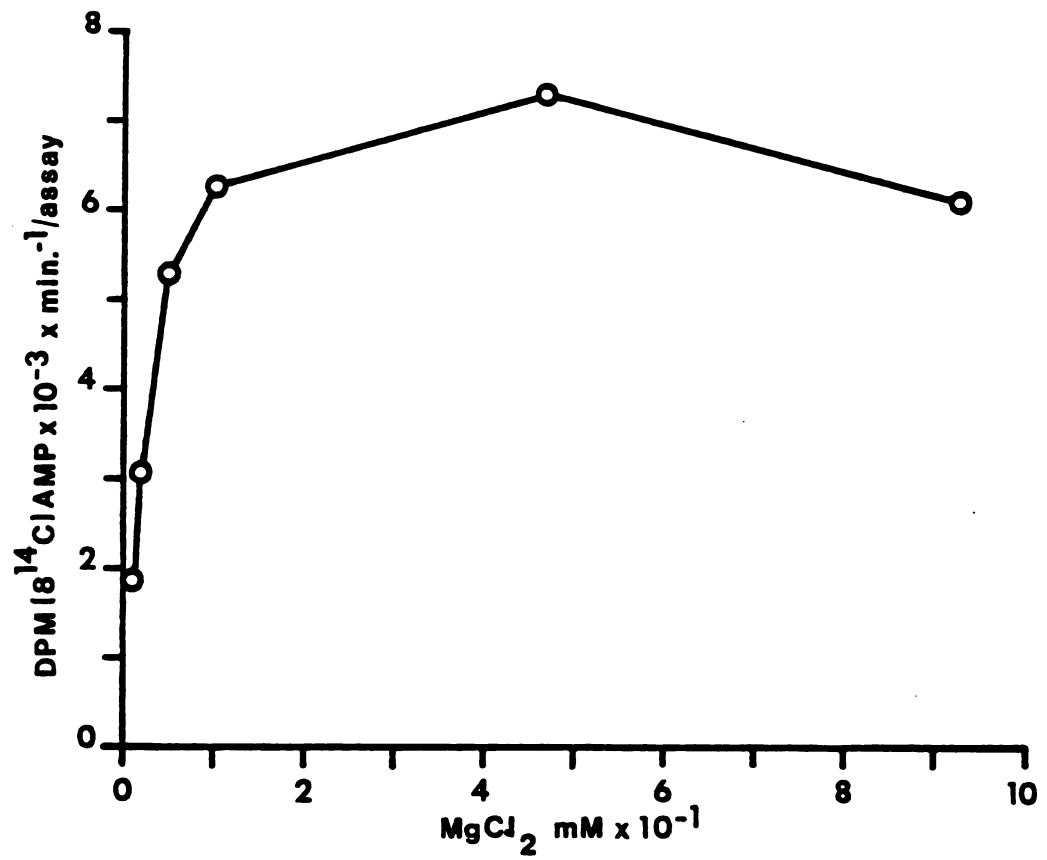


Figure 18. APRT Velocity vs. Magnesium Concentration

Figure 18. Legend

Assay mixtures contained the optimal concentration of reagents and crude extract from *Aprt*^A homozygotes. Conditions were as described for the TLC assay.

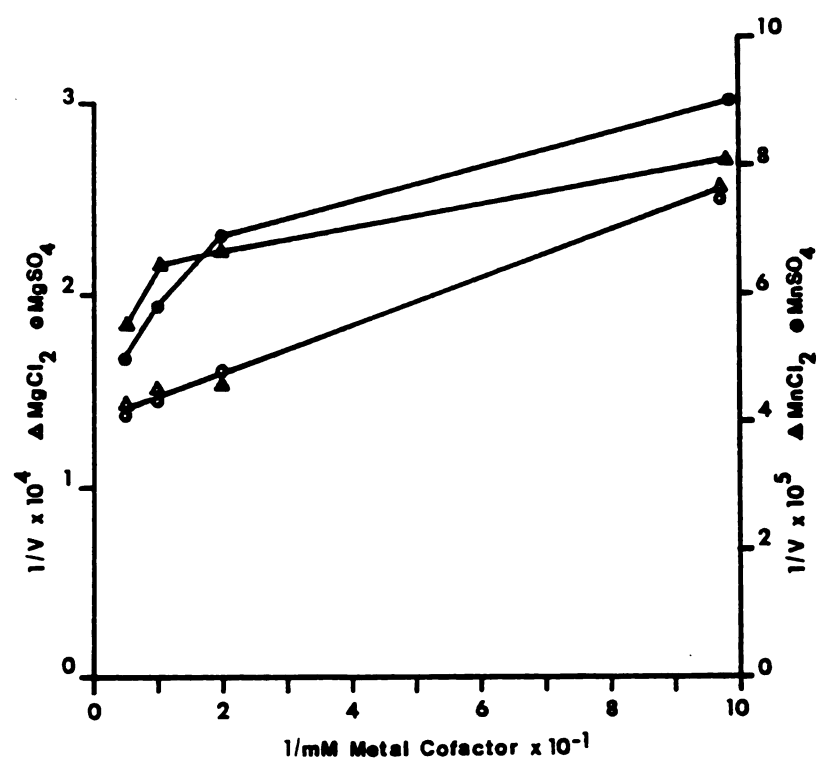


Figure 19. Comparison of Magnesium and Manganese as Metal Cofactors of APRT Activity

Figure 19. Legend

Assay mixtures contained the optimal concentration of reagents and crude extract from *Aprt*^A homozygotes. Conditions were as described for the TLC assay.

Based on the results presented in Figures 14-18 the following reaction mixture was adopted for the assay of *Drosophila* APRT activity: 60 mM tris-HCl pH 7.5, 0.1 mM Na-EDTA, 26 mM magnesium chloride, 2 mM dithiothreitol, 6.25 mM Na-PrPP, 7.6×10^{-5} M (8^{14}C)adenine (62 millicuries/millimole) and 10 microliters of enzyme in a total volume of 35 microliters. All chemicals were obtained from Sigma except magnesium chloride (Malinkrodt) and (8^{14}C)adenine (Amersham). Assays were performed in plastic micro test tubes which were incubated in a constant temperature bath at 25°C . Crude extracts of *Drosophila* were prepared by homogenizing 5 male and 5 female adult *Drosophila* in 100 microliters of distilled water in an all glass tissue grinder (Duell, Kontes) submerged in an ice bath. Enzyme assays were initiated by the addition of 10 microliters of homogenate to 25 microliters of the appropriately concentrated reaction mixture and the reaction vessel was immediately transferred to the 25°C water bath.

DEAE Assay Procedure

In this procedure, 10 microliter aliquots of the reaction mixture were removed at various times following initiation of the reaction (1-10 minutes) and spotted on 2.5 cm DEAE cellulose filter circles (Whatman, DE81 cellulose). The filters were washed with 50 milliliters of distilled water on a vacuum filtration manifold (Hoefer). The washed filters were thoroughly dried and the bound radioactivity determined by liquid scintillation spectrometry.

Chromatography Assay Procedure

Using the thin-layer chromatography method, 5 microliter aliquots of the reaction mixture were removed after 4 and 8 minutes of incubation and spotted in 2 cm wide lanes, 5 cm from the edge of a 20 cm x 20 cm plastic-backed Polygram Cel 300 PEI TLC plate (Brinkman) at sites previously overlaid with 5 microliters of a 2% solution of 5'AMP. The plate was then developed in 0.1 M LiCl until the solvent front had migrated at least 16 cm up the plate. The developed plate was dried and the position of the AMP marker determined by viewing the plate with a short-wave UV mineral light. The AMP containing spots were cut from the plate and the radioactivity on these chips was determined by liquid scintillation spectrometry. The synthesis of (8^{14}C)AMP from (8^{14}C)adenine and PrPP in the reaction is shown to vary linearly with incubation time and concentration of *Drosophila* protein in the assay (Figure 20).

HGPRT Assay [E.C. 2.4.2.8]

The same procedure used for the assay of *Drosophila* APRT activity was used for the assay of human cell APRT activity and HGPRT activity with the following modifications:

1. (8^{14}C)hypoxanthine (50 millicuries/millimole, New England Nuclear) replaced (8^{14}C)adenine in the reaction mixture at the same concentration.

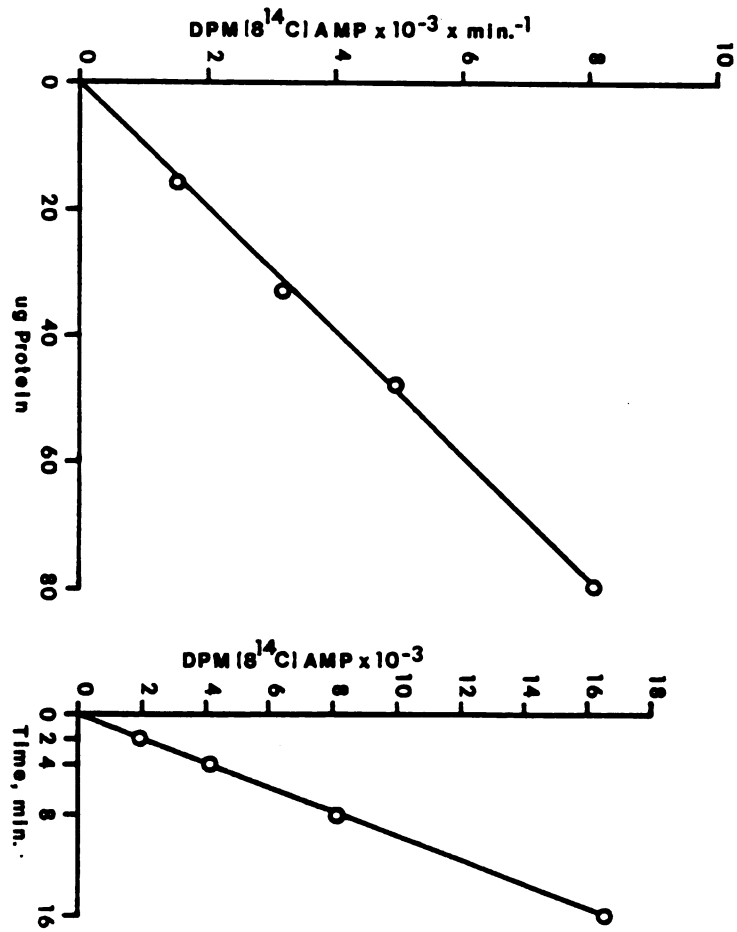


Figure 20. Dependence of APRT Activity on Incubation Time and Protein Concentration

Figure 20. Legend

Conditions were as described for the TLC assay of APRT activity.
Reaction mixtures contained crude extracts of adult *Ap^{rt}^A*
homozygotes.

2. PEI plates were marked with 5 microliters of a 2% IMP solution instead of AMP.

3. Assays of human cell extracts were incubated at 37°C instead of 25°C.

4. The reaction mixtures for human cell APRT and HGPRT activity included 5 mM TTP to inhibit 5'nucleotidase activity [5'-ribonucleotide phosphohydrolase, E.C. 3.1.3.5] which is present in human cell extracts but absent or insignificant in extracts of *Drosophila*.

Extracts of human cells were prepared by washing the cells free of culture media with 0.15 M NaCl. The flasks were broken open with pliers and the cells scraped from the walls with a rubber policeman, aspirated into a pasteur pipette and transferred to a plastic micro test tube. Cells were ruptured by freeze-thawing in a dry ice ethanol bath. The lysates were then centrifuged in a Brinkman microcentrifuge for 2 minutes and the supernatant was taken for assays.

Inosine Phosphorylase Assay [E.C. 2.4.2.1]

This enzyme was assayed using the same reaction mixture as described previously for APRT activity in *Drosophila* with the following modifications:

1. PrPP was replaced in the assay with alpha-D-ribose-1-phosphate at the same concentration.
2. (8¹⁴C)hypoxanthine replaced (8¹⁴C)adenine in the assay at the same concentration.

3. Inosine phosphorylase synthesizes inosine from hypoxanthine and alpha-D-ribose-1-phosphate. Therefore the TLC plate was spotted with a 2% solution of inosine instead of AMP.

4. The TLC plate was developed in distilled water prior to the application of the inosine marker since inosine migrates close to the front in 0.1 M LiCl chromatography. The front is badly streaked in unwashed plates which interferes with the resolution of the inosine spots.

The results in Table 8 (A and C) show that the synthesis of (8^{14}C)inosine from (8^{14}C)hypoxanthine is dependent upon the substitution of alpha-D-ribose-1-phosphate for PrPP in the reaction mixture. Therefore, it is concluded that the synthesis of inosine by extracts of *Drosophila* in this assay results from inosine phosphorylase activity.

Purine Nucleoside Phosphorylase Assay

This enzyme is generally considered to be synonymous with inosine phosphorylase. However, the name purine nucleoside phosphorylase is usually given to an enzyme which can convert a variety of purine bases to their corresponding nucleosides (44). For the purposes of this investigation it was of interest to know if *Drosophila* extracts could perform the synthesis of (8^{14}C)adenosine from (8^{14}C)adenine and

alpha-D-ribose-1-phosphate. This enzyme activity was assayed by the same procedure as for inosine phosphorylase with the following modifications:

1. (8^{14}C) hypoxanthine was replaced in the reaction with (8^{14}C) adenine at the same concentration.
2. The prewashed TLC plate was spotted with 5 microliters of a 2% solution of adenosine instead of inosine.

Adenosine Kinase Assay [E.C. 2.7.1.20]

The adenosine kinase assay in *Drosophila* is modeled after the procedure of Herschfield and Kredich (68) for the assay of human cell adenosine kinase. The assay of this enzyme in human cell extracts is complicated by the presence of a high level of adenosine deaminase activity [adenosine aminohydrolase, E.C. 3.5.4.4] which rapidly converts the (8^{14}C) adenosine substrate to (8^{14}C) inosine. The rapid depletion of (8^{14}C) adenosine results in a premature termination of (8^{14}C) AMP synthesis by adenosine kinase activity. This problem was solved by including EHNA (erythro 9-[3-(2-hydroxy-nonyl)]adenine) in the reaction mixture. At a concentration of 5 micromolar EHNA in the reaction, human adenosine deaminase activity is completely inhibited without affecting the activity of adenosine kinase (Dr. G. B. Elion, Wellcome Research Lab, personal communication and source of EHNA). Preliminary assays of adenosine kinase activity in extracts of *Drosophila* (Table 10) demonstrate that crude extracts of adult flies contain an ATP dependent enzyme activity which converts

Table 10. Adenosine Kinase Activity in Drosophila

Assay Condition ¹	DPM(8 ¹⁴ C)AMP	DPM(8 ¹⁴ C)Adenosine	DPM(8 ¹⁴ C)Inosine
- ATP	0	-	50,000
+ ATP	32,000	-	69,000
+ EHNA	37,000	38,000	106,000
+ Coformycin	21,000	147,000	1,000

¹The indicated compounds were included or removed from the standard adenosine kinase assay mixture. The concentration of EHNA and coformycin in assays containing them was 40 micromolar. Radioactivity measurements represent the amount of each compound present in the assay mixture after four minutes incubation time.

(8^{14}C)adenosine to (8^{14}C)AMP. However, *Drosophila* extracts also contain a high level of adenosine deaminase activity which is insensitive to EHNA inhibition at a concentration of 40 micromolar in the assay (Table 10). In order to circumvent this problem in the assay of *Drosophila* adenosine kinase activity, the adenosine analogue coformycin (Dr. R. Engles, NCI, Silver Spring, Md.) was included in the reaction mixture in place of EHNA at the same concentration. The results in Table 10 indicate that coformycin is a potent inhibitor of *Drosophila* adenosine deaminase activity which has a much smaller inhibitory effect on adenosine kinase activity. Despite this improvement, the assay of adenosine kinase activity in *Drosophila* is an unrefined procedure which requires further development to establish the optimal conditions for assay of the *Drosophila* enzyme. The reaction mixture used for the assay of *Drosophila* adenosine kinase activity contains: 50 mM pipes, 0.2 mM magnesium sulfate, 0.1 mM ATP, 5.85×10^{-5} M (8^{14}C)adenosine (50 millicuries/millimole, Schwarz/Mann), 3 mM dithiothreitol, 40 micromolar coformycin pH 6.8 and 5 microliters of *Drosophila* extract in a total volume of 30 microliters. Homogenates of adult *Drosophila* were prepared as described previously. The enzyme reaction was initiated by the addition of *Drosophila* extract to the appropriately concentrated assay mixture which was immediately incubated at 25°C . Aliquots of the reaction mixture were spotted on DEAE filters or PEI plates at 2 and 4 minutes of incubation time. The TLC plate and the DEAE filters were processed in the same fashion as for the assay of *Drosophila* APRT activity.

Protein Assays

Crude extracts of *Drosophila* in distilled water were assayed for protein content using the Lowry Method (69) with bovine serum albumin as the protein standard. Protein assays of purified or partially purified fractions of *Drosophila* APRT (Appendix 6) were performed using the Bradford method (70) with dye reagent from BioRad. Unlike the Lowry method the Bradford assay is insensitive to tris, magnesium ion, ammonium sulfate and sodium dodecylsulfate present in the protein sample.

Appendix 2: Sources and Culture of *Drosophila*

Sources of *Drosophila* Stocks

The purine-resistant mutant *Aprt*¹ was obtained from Dr. V. Finnerty, Emory University. The purine-resistant mutants *Aprt*² and *Aprt*³ were obtained from Dr. A. Chovnick, University of Connecticut. The *Dp(T(Y;3)H141)* was obtained from Dr. D. Roberts, Genetics Laboratory, Oxford. The deletion *Df(3L)R*^{+E} was provided by Dr. J. J. Bonner, Indiana University. All other *Drosophila* stocks were obtained from Dr. R. Woodruff, National Drosophila Stock Center, Bowling Green, Ohio.

Culture of *Drosophila*

Drosophila were cultured at 25°C in a constant temperature incubator. The standard media for *Drosophila* culture is made by the following recipe:

1. Combine 188 gm sucrose, 30 gm brewer's yeast, 185 gm cornmeal, 24 gm carrageenan and 2.2 liters distilled water at room temperature in a large pot and stir until the lumps have broken up.

2. Heat to boiling with the pot covered and stir the mixture frequently until the cornmeal is well cooked and the media consistent.

3. Just prior to dispensing the media, stir in 15 ml of propionic acid to discourage mold.

4. When the media has jelled in the culture bottles, seed the media with a fresh solution of live baker's yeast.

When *Drosophila* were selected for survival on purine containing media, the amount of cornmeal in the standard recipe was reduced by half. This produces a media that does not dry out as rapidly as the standard media which is important for flies growing on purine containing media since the purine slows down their growth. The purine selection media is accurately dispensed in 10 and 30 ml aliquots using a calibrated 50 ml plastic syringe.

Appendix 3: Selection of Purine Resistant Mutants

The complementation analysis of *Aprt*¹, *Aprt*² and *Aprt*³ indicated that all three mutations conferred resistance to purine killing in trans-heterozygotes. This result suggested that the most efficient approach to isolating additional purine-resistant mutations in *Aprt* would involve the isolation of non-complementing mutations of an existing mutant allele. The *Aprt*¹ mutation was arbitrarily chosen for this purpose.

The selection scheme used is presented in Figure 21. The concentration of purine used in this protocol was determined by comparing the sensitivity of *Aprt*¹ / *Aprt*^B heterozygotes to *Aprt*¹ homozygotes (Figure 22). Mutations were induced in the *ru h* marked chromosome to allow the rapid genetic mapping of newly isolated mutations. The *ru* and *h* markers were chosen because mutant alleles of these genes are contained within the *In(3LR)CxF ru h D* balancer chromosome which allows the easy identification of the mutagen treated *ru h* marker chromosome in trans to this balancer. The *In(3LR)CxF ru h D* balancer chromosome was chosen because it carries the easily identified *D* mutation and because this chromosome has a



Figure 21. Selection of Purine Resistant Mutants

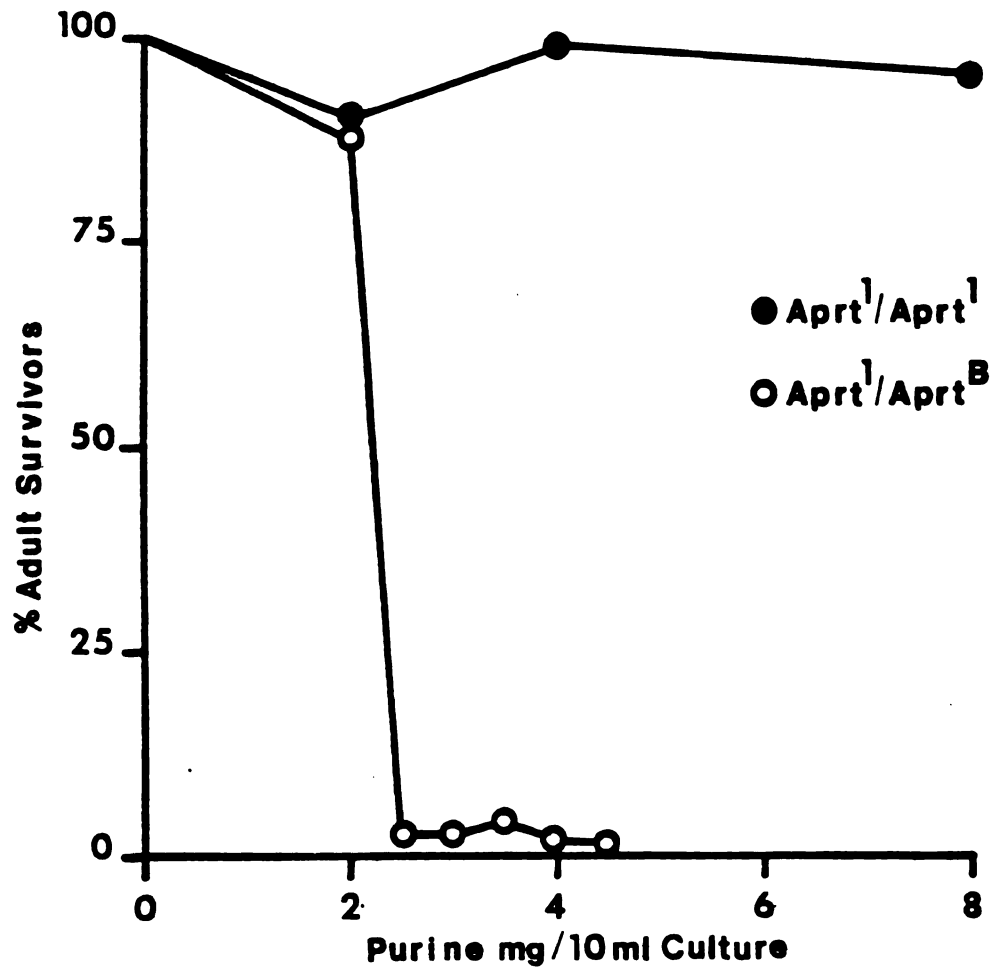


Figure 22. Purine Sensitivity in Drosophila

Figure 22. Legend

First instar larvae of the indicated genotype were placed on shell vials containing 10 ml of culture media (Appendix 2). Twenty larvae were placed in each vial and there were 8 replicate vials for each purine concentration. Purine was added to the vials dissolved in 60 microliters of distilled water at the same time that the larvae were added.

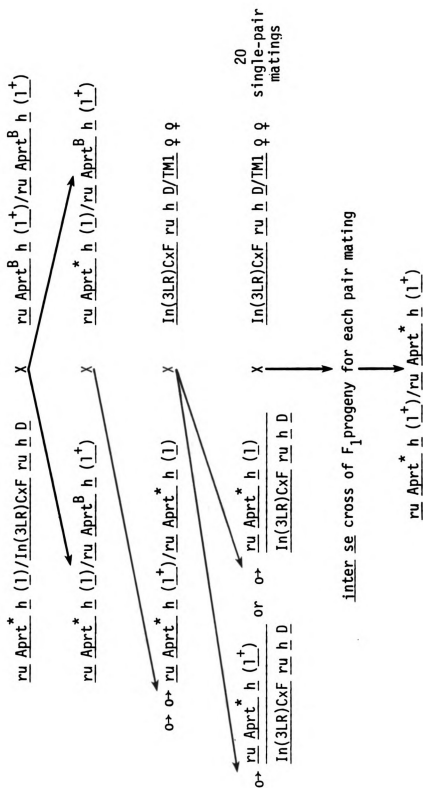
rearrangement breakpoint in region 62 which is close to *Aprt*. Therefore, the likelihood of recovering chromosomes recombinant for *Aprt* in the balanced lethal stock is remote.

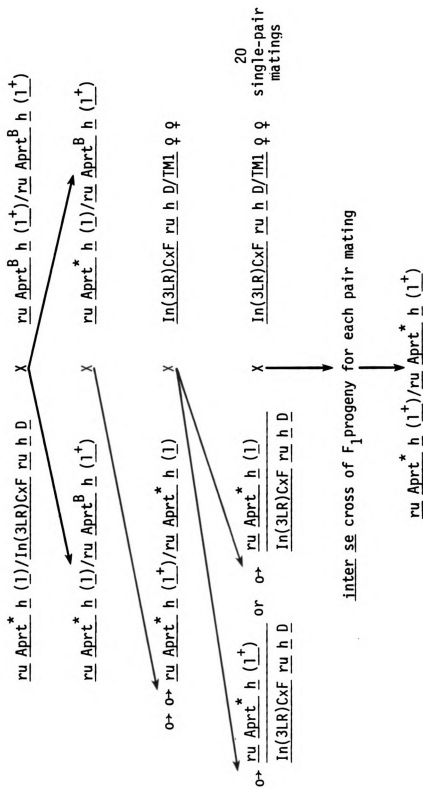
Mutations were induced with ethyl methane sulfonate (EMS, Sigma) according to the procedure of Lewis and Bacher (71). One day old male *ru Aprt^B h* homozygotes were collected and placed in empty bottles at 25°C for 4-6 hours to partially dehydrate them and encourage the uptake of the EMS solution. The EMS solution was made up by adding 72 microliters of EMS to 30 ml of a 1% sucrose solution. The solution was then emulsified by vigorous back-and-forth aspiration in a pasteur pipette and then aliquoted to glass bottles containing several folded kimwipes to hold the solution. The male flies were placed in the bottles and allowed to feed on the EMS solution for 24 hours. EMS is a potent carcinogen and was only handled in a fumehood. Gown, mask and double gloves were worn when handling EMS. Labware in contact with the EMS solution was deactivated by copious rinsing with a solution of 1 N NaOH containing 0.5 % thioglycolic acid (Sigma). The unused portion of the 0.24 % EMS solution is deactivated by two-fold dilution with the alkaline thioglycolic acid solution. Flies being treated with EMS were maintained in the fume hood with constant exhaust.

EMS treated male *ru Aprt^B h* homozygotes were mated to *ru⁺ Aprt¹ h⁺* virgins, 15 females with 5 males per 30 ml culture. After 48 hours the adults were transferred to fresh culture media for an additional 48 hours and then discarded. The 48 hour old cultures were treated with purine (Sigma) 10.5 mg in 0.3 ml of distilled water and

incubated at 25°C. Surviving male flies were mated to virgins carrying *In(3LR)CxF ru h D* to establish a balanced lethal stock of the mutagen treated third chromosome. Males of the balanced lethal stocks were backcrossed to *Appt*¹ homozygotes for 48 hours in shell vials containing 10 ml of media. Adults were then discarded and 4.0 mg purine in 60 microliters of distilled water was added to each culture. Balanced lethal stocks yielding purine-resistant progeny in these F₂ tests were maintained and those which failed to produce purine survivors were discarded. Males which failed to produce purine-resistant progeny probably result from purine-sensitive escapers in the original purine selection. Alternatively, they might result from mosaic purine-resistant male survivors of the initial purine selection which failed to transmit the the mutation in the germ line.

The purine-resistant mutants *Appt*⁴ and *Appt*⁵ isolated by the procedure above, as well as the *Appt*² and *Appt*³ mutants, contained recessive lethal mutations in chromosome three. Homozygous stocks of these purine-resistant mutations were constructed by purine selection of the recessive-lethal-free chromosome carrying the *Appt* mutation (Figure 23).

Figure 23. Removal of Aprt Linked Recessive-Lethal Mutations

Figure 23. Removal of Aprt Linked Recessive-Lethal Mutations

Appendix 4: Gel Electrophoresis and Isoelectric Focusing

SDS Polyacrylamide Gel Electrophoresis

One-dimensional polyacrylamide gel electrophoresis was conducted using the denaturing system of Laemmli (72). Gels were run in order to estimate the subunit weight of *Drosophila* APRT and to monitor the purification of this protein. Electrophoresis was conducted in vertical 11 cm x 14 cm x 0.7 mm slab gels. The recipe for the gel is as follows.

Components

30% Acrylamide Stock Solution : 29.2 gm acrylamide monomer + 0.8 gm bis-acrylamide + distilled water to 100 ml.

Lower Gel Buffer : 4.54 gm tris-base + 0.1 gm SDS + distilled water to 50 ml pH = 8.8 with HCl.

Upper Gel Buffer : 1.51 gm tris-base + 0.1 gm SDS + distilled water to 50 ml pH = 6.8 with HCl.

Tank Buffer : 4.54 gm tris-base + 21.62 gm glycine + 1.5 gm SDS + distilled water to 1.5 liter pH = 8.5.

Ammonium persulfate : 0.5 gm + distilled water to 100 ml.

Sample Buffer (5x concentrate) : 0.756 gm tris-base + 10 gm glycerol +

2 gm SDS + 1.54 gm dithiothreitol + distilled water to 20 ml pH = 6.8 with HCl.

Lower Gel (13% acrylamide)

Mix thoroughly 10 ml lower gel buffer, 1 ml ammonium persulfate, 8.3 ml acrylamide stock solution and 0.655 ml distilled water in a side-arm flask. Degas the solution and add 45 microliters of Temed. Mix the solution thoroughly and pour the gel. Overlay the gel with water. Allow one hour for polymerization.

Upper Gel (4.6% acrylamide)

Decant the water from the polymerized lower gel. Mix thoroughly 5 ml upper gel buffer, 0.5 ml ammonium persulfate, 1.5 ml acrylamide stock solution and 2.95 ml distilled water in a side-arm flask. Degas the solution, add 50 microliters of Temed, mix thoroughly and pour the gel. Insert the gel comb and leave 2.0 cm between the top of the lower gel and the ends of the comb teeth. Allow the gel to polymerized for 30 minutes.

Sample Preparation

Protein samples must be free of salt and are diluted 4 parts sample to 1 part 5x sample buffer. The sample is then boiled immediately for 10 minutes. Boiled samples are loaded on the gel

under tank buffer. Electrophoresis is performed with constant voltage at 9 milliamperes until the schlieren line marking the front reaches the bottom of the gel (approximately 5 hours). Gels were stained with silver according to modifications (R. Switzer, personal communication) of the original Switzer method (73).

Calculation of Protein Molecular Weights

The subunit molecular weight of proteins in the SDS gel is determined by comparing the R_f of a protein whose molecular weight is unknown to the R_f values of standard proteins of known molecular weight which were run in the same gel. The R_f values for the proteins are compared in a semi-log plot where R_f is the arithmetic scale and molecular weight is the logarithmic scale (Figure 9). The R_f of a protein is given by: $R_f = s_p/s_o$ where s_p is the distance between a protein and some reference point (such as the bottom of the lower gel) and s_o is the distance between the same reference point and the top of the lower gel.

Non-denaturing Isoelectric Focusing

Non-denaturing isoelectric focusing was performed in horizontal polyacrylamide slab gels essentially as described by Chasin and Urlaub for HGPRT (33) with modifications for APRT activity in *Drosophila*. Gels of various dimensions contained: 4.85 % acrylamide monomer, 0.15% bis-acrylamide, 5% glycerol, 0.05% riboflavin-5-phosphate, 0.4%

ampholyte pH 3-10 and 1.6% ampholyte pH 4-6. Gels were cast using a BioRad CTL casting system and polymerized by placing a 20 Watt fluorescent lamp 4 inches above the gel for 1 hour. *Drosophila* APRT was prepared by homogenizing 10 adults in 30 microliters of distilled water in a micro glass grinder (Kontes) followed by centrifugation in a Brinkman micro-centrifuge (12,000 rpm) for 2 minutes at 2°C. Aliquots (4-10 microliters) of the resulting supernatant were applied directly to the middle of pre-cooled gels. The anolyte was 1 N phosphoric acid and the catholyte was 1 M sodium hydroxide. Gels were focused in a BioRad 1405 cell at 1°C under a constant power of 4 Watts per 8.8 cm³ of gel for 2 hours using a 2000 volt power supply. APRT activity was visualized by fluorography. Focused gels were covered with the standard APRT reaction mixture (Appendix 1) by gentle spreading with a bent glass rod (0.2 ml of reaction mixture per 10 cm x 12.5 cm of gel surface area). The gel was incubated at 25°C for 10-15 minutes and then blotted with a gel-size PEI 300 TLC plate moistened with distilled water. The gel was left in contact with the PEI plate for 10 minutes after which the gel was peeled from the plate with a metal spatula while submerged in distilled water. The PEI blot was soaked in one liter of distilled water for 10 minutes with three changes. This procedure elutes the (8¹⁴C)adenine from the plate while the (8¹⁴C)AMP formed by APRT activity in the gel remains bound to the plate. The dried PEI sheet is sprayed with a 1% solution of 2,5-diphenyloxazole (PPO) in anhydrous diethylether until the plate is visibly coated with a thin layer of PPO. The dried sheet is then fluorographed using Kodak X-Omat AR X-ray film in a standard

autoradiographic holder at -80°C for 24 to 48 hours. The X-ray film was developed with Kodak X-ray film developer.

Two-dimensional Gel Analysis

Two-dimensional analysis of APRT was conducted using the O'Farrell procedure (39) with minor modifications. This procedure incorporates denaturing conditions in both dimensions. The first dimension separates proteins by isoelectric focusing in a thin tube gel. In the second dimension, the proteins in the focused tube gel are electrophoresed into a Laemmli SDS slab gel which separates the proteins based on their molecular weight.

First Dimension Gel

Components

Acrylamide Stock Solution : 28.3% acrylamide monomer + 1.6% bis-acrylamide.

Gel Overlay : 8 M urea (make fresh before use).

Sample Overlay : 9 M urea + 0.8% ampholyte pH 4-6 + 0.2% ampholyte pH 3-10 (make fresh before use).

Anode Buffer : 0.01 M phosphoric acid.

Cathode Buffer : 0.02 M sodium hydroxide.

Lysis Buffer : 9.5 M urea + 1.6% ampholyte pH 4-6 + 0.4% ampholyte pH 3-10 + 5% 2-mercaptoethanol (make fresh before use).

Cap Gel : 1% agarose in 1x Laemmli SDS sample buffer (see

one-dimensional SDS gel procedure in this Appendix).

Combine 5.5 gm urea, 1.33 ml stock acrylamide solution, 2 ml 10% NP-40 Nonidet, 1.97 ml distilled water, 10 microliters of 25% ammonium persulfate, 0.4 ml ampholyte pH 4-6 and 0.1 ml ampholyte pH 3-10 in a 50 ml side-arm flask. Swirl the mixture in a hot water bath to dissolve the urea. Degas the solution and add 7 microliters of Temed. Mix thoroughly and cast 10 cm long gels in 3 mm (i.d.) glass tubes sealed at the bottom with 2 layers of parafilm. Overlay the gels with gel overlay solution and allow the gels to polymerize for 1 hour. Remove the gel overlay solution and close off the lower end of the gel with dialysis membrane held in place with a short section of silicone tubing. Insert gels into the electrophoresis apparatus with the membrane end submerged in the anode buffer (lower buffer chamber). Rinse the top of the gels with lysis buffer then load the protein samples which are prepared in lysis buffer. Overlay each sample with 40 microliters of sample overlay solution and then carefully fill each tube with cathode buffer without disturbing the sample and overlay. Fill the cathode chamber to a few centimeters above the gel tubes and focus the gels for 3.5 hours at room temperature under a constant power of 4 Watts per 8.8 cm^3 of gel using a 2000 volt power supply. Extrude the focused gels into test tubes containing 3 ml of 1x Laemmli SDS sample buffer and freeze the gels at -80°C . Prepare a one-dimensional Laemmli SDS slab gel as previously described using a notched glass plate to accomodate the tube gel. Pour the lower gel so that the top of the gel is 3 cm below the bottom of the notch. Cast

an upper gel by inserting the flat end of the gel comb to produce a level gel surface 2 mm below the bottom of the notch. Thaw the first-dimension gel and allow the gel to equilibrate in the sample buffer for 15 minutes. Fill the notch space with melted cap gel and rapidly transfer the equilibrated first-dimension gel to the notch. Cover the first-dimension gel with additional cap gel and allow the gel to cool. Transfer the gel sandwich to the electrophoresis apparatus and perform electrophoresis as previously described for the single-dimension SDS gel.

For these procedures ampholyte was obtained from BioRad. BioRad ampholyte does not stain significantly with the modified silver stain procedure unless bromophenol blue is used as a tracking dye. Therefore, the dye was omitted. In experiments where Pharmalyte (Pharmacia) was substituted for the BioRad ampholyte, the Pharmalyte stained very darkly with the silver stain. Thus, Pharmalyte cannot be used when the first-dimension tube gel is to be stained with silver, but may be used when the second-dimension gel is stained since the ampholyte migrates at the front and does not obscure the proteins in the gel. Acrylamide, SDS and Temed were obtained from BioRad. Ultrapure urea was purchased from Swarz/Mann. All other reagents were obtained from Sigma or U.S. Biochemicals and were of reagent grade.

The power requirements for equilibrium isoelectric focusing reported here are estimates since the BioRad 2000 volt power supply (Model 1420A) used in these experiments is known to have an unreliable volt meter (BioRad, personal communication). In the isoelectric focusing experiments described, the beginning voltage was adjusted to

350 volts and the final voltage ranged from 1200 to 1500 volts.

Determination of Isoelectric Points of APRT

The isoelectric points of APRT in isoelectric focusing gels were determined by slicing a portion of the slab gel or a separate tube gel into 2.5 mm gel slices. The slices were incubated in 0.5 ml of distilled water for 12 hours in a refrigerator and the pH determined with a pH meter. The plot of the pH range for a first-dimension gel is linear from pH 3.9 to pH 6.8 (Figure 24).

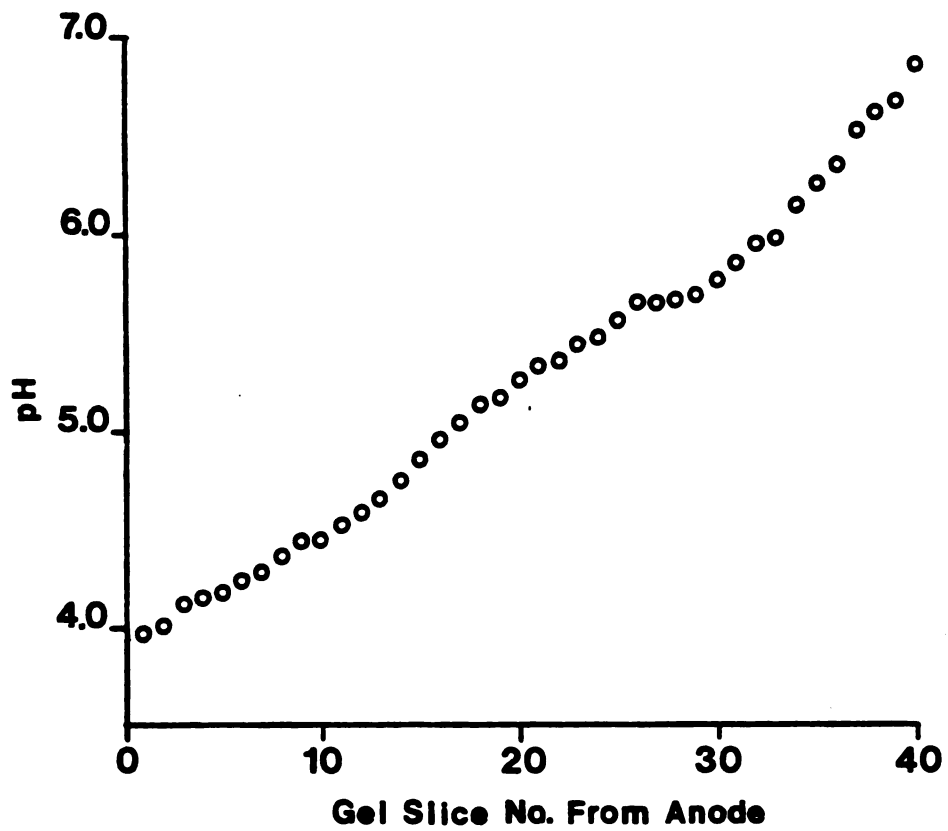


Figure 24. pH Gradient in an Isoelectric Focusing Gel

Appendix 5: Gel Filtration

Gel filtration of *Drosophila* APRT was performed on a 59 cm x 0.9 cm column of S 200 SF Sephacryl (Pharmacia).

Preparation of the Column

1. Remove 125% of the anticipated column volume of preswollen gel from the container and allow the slurry to settle in a side-arm flask.
2. Remove the buffer and replace it with column buffer [50 mM tris, 10 mM magnesium sulfate, 0.3 M ammonium sulfate and 0.1 mM Na-EDTA pH 7.5].
3. Adjust the buffer volume to equal the volume of the settled gel and degas the slurry.
4. Mount the column vertically in a cold room with a gel reservoir at the top. Pour the degassed gel slurry down a glass rod into the column and allow the gel to settle for 5 minutes to permit bubbles to escape from the slurry.
5. Fill the reservoir with column buffer and pack the column at a flow rate of 40 ml/cm^2 until no further packing of the gel can be detected.

The void volume of the column was determined by chromatography of blue dextran 2000 (Pharmacia) which does not permeate this gel. A 0.2 ml aliquot of blue dextran (1 mg/ml in column buffer + 10% sucrose) was loaded onto the packed column and eluted at a flow rate of 0.2 ml per minute. Column eluant was monitored with a UV flow monitor at 280 nm. The recorder was set at 1 AUFS [10 mV] at a recording speed of 10 cm per hour. eluant volumes were determined by mass measurement of the eluant collected in a tared flask at the mid-point of peak elution. The void volume of the packed column was 38% of the gel bed volume.

Calibration of the Column

The column was calibrated in two separate runs which were repeated once. Elution volumes were determined as described for blue dextran 2000 except that the sensitivity of the monitor was adjusted to 0.2 AUFS [10 mV]. The first run contained a mixture of bovine serum albumin (75 microliters) and chymotrypsinogen A (25 microliters). The mixture was made from 10 mg/ml stock solutions of each protein in column buffer containing 10% sucrose. The second run consisted of a mixture of ovalbumin (50 microliters) and ribonuclease A (50 microliters) made up as described for proteins in run 1.

Molecular Weight Determination for APRT

The elution volume for *Drosophila* APRT was determined by mass measurement of the column eluant in tared test tubes. Approximately 1 ml fractions were collected. The APRT activity in each fraction was determined using the DEAE filter assay (Appendix 1) of 10 microliter aliquots from each eluant fraction. *Drosophila* APRT used in this molecular weight determination was prepared by ammonium sulfate precipitation (Appendix 6). Ammonium sulfate purified APRT was resuspended in column buffer containing 10% sucrose. Protein concentration was 14 mg/ml and 0.2 ml of this APRT preparation was used in each of two runs to estimate the native molecular weight of this enzyme. The K_{av} of APRT and standard proteins is given by: $K_{av} = (v_e - v_o) / (v_t - v_o)$ where v_e is the eluant volume at the peak of protein elution, v_o is the void volume as determined with blue dextran 2000 and v_t is the column volume. The molecular weight of APRT was determined from a semi-log plot of the elution data where R_f is the arithmetic scale and molecular weight is the logarithmic scale (Figure 8). Blue dextran and protein standards were obtained from Pharmacia.

Appendix 6: Purification of *Drosophila* APRT

APRT has been purified from humans (36,66) rodents (38,74) and bacteria (67) using a variety of different techniques. Affinity chromatography has been the most successful. Therefore, the approach taken here was to adapt the simplest purification protocol which yields an apparently homogenous APRT in highest yield. The AMP affinity chromatography method of Hershey and Taylor results in the purification of rat liver APRT to homogeneity in two steps (74). The first step is a 100,000 xg centrifugation of a rat liver crude homogenate. The supernatant from this step is loaded directly onto an agarose column to which AMP is covalently linked. APRT binds to its product while most other proteins in the homogenate pass through the column. The column is washed extensively until no additional protein can be detected in the eluant. APRT is then eluted from the column in buffer containing its substrate, PrPP. This eluant specifically removes APRT from the column while other proteins which also bind AMP remain bound to the column (74).

This affinity chromatography procedure was attempted for the purification of *Drosophila* APRT using mouse liver APRT as a positive control for the steps. Although the Hershey and Taylor procedure (74) was effective in purifying APRT activity from mouse liver, it was

totally ineffective in purifying APRT activity from crude extracts of *Drosophila*. Since most of the APRT activity from *Drosophila* extracts is present in the void protein fractions of the AMP column, it appears that *Drosophila* APRT fails to bind the AMP ligand when crude homogenates of *Drosophila* are subjected to chromatography on AMP agarose. It was postulated that this might result for either of two reasons: (i) saturation of the available AMP ligands on the gel by an AMP binding protein(s) more abundant or having a higher affinity for AMP than APRT or (ii) the inhibition of APRT binding to the AMP ligand either because the enzyme was already bound to AMP present in the crude extract or to its AMP competitor PrPP.

The latter hypothesis was tested by dialyzing the crude *Drosophila* extract prior to AMP agarose chromatography. Dialysis was unsuccessful in producing a fraction of crude APRT activity which bound the AMP column. APRT activity is unstable to dialysis in buffer A, all of the enzyme activity was lost during a 12 hour dialysis. The APRT activity remaining from a four hour dialysis was unable to bind the AMP affinity column. However, 4 hours of dialysis is probably inadequate to remove an inhibitor from APRT which has a low K_d for the enzyme at 0°C. It is also possible that the presence of an inhibitor such as AMP or PrPP in the crude *Drosophila* extract is not the explanation for the failure of *Drosophila* APRT to bind the AMP affinity column.

Since dialysis alone did not yield a fraction of crude APRT activity capable of binding to AMP agarose, it was decided that partial purification of the enzyme might be required in order to allow

APRT to bind to AMP. Many of the protocols for the purification of APRT from other organisms incorporate ammonium sulfate precipitation of the enzyme as an early step. Ammonium sulfate precipitation is rapid and generally results in a considerable purification of the enzyme with good yield (36,38,66,74). Therefore, ammonium sulfate precipitation was performed with *Drosophila* APRT. The results in Table 11) show that this step produces an approximate 2 fold increase in specific activity of APRT with a recovery ranging from 40 to 60 percent. Since this procedure results in a fraction of APRT with a high residual concentration of ammonium sulfate, the salt was removed by gel filtration on a column of Sephacryl S 200 SF (Appendix 5). AMP affinity chromatography was then performed with the desalted fraction of ammonium sulfate purified APRT. This fraction of *Drosophila* APRT does bind to AMP agarose. However, there are several proteins which elute from the AMP column in the PrPP eluant containing APRT activity (Figure 25 A, lane 1). Since AMP affinity chromatography alone does not produce a homogeneously pure preparation of *Drosophila* APRT it was necessary to develop an additional step in the purification protocol.

In the last few years a variety of synthetic dyes have been developed whose chemical structure is similar to the purine moiety of nucleotides. One of these dyes, Cibacron blue, has been used to replace AMP as a ligand for the purification of adenine nucleotide binding proteins by affinity chromatography. Therefore, blue agarose (Cibicron blue covalently linked to agarose) chromatography was

Table 11. Summary of APRT Purification Procedure

Purification Step	Specific Activity*	Fold Purification	% Yield
Crude Extract	689.3	-	100
Ammonium Sulfate	1079.2	1.6	40
Octyl Agarose	4174.8	6.1	53
AMP/Blue Agarose	624402.8	905.9	14

* APRT Activity is expressed as DPM(8^{14}C)AMP per minute per microgram of protein.

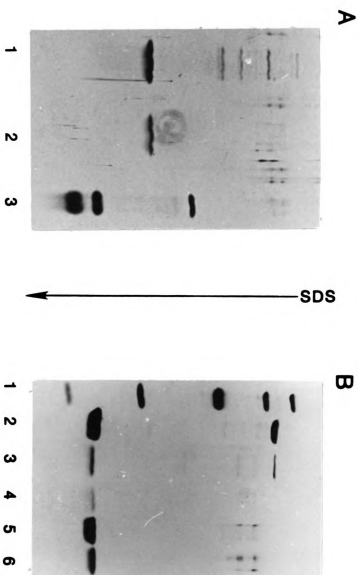


Figure 25. SDS Polyacrylamide Gel Electrophoresis of Purified and Partially Purified Drosophila APRT

Figure 25. Legend

SDS polyacrylamide gel electrophoresis and silver staining of proteins were performed as described in Appendix 4. Gel A contained: (1) APRT purified from *Appt*^A homozygotes by ammonium sulfate precipitation and AMP affinity chromatography, (2) same as lane 1 except the APRT was further purified by Octyl agarose and Blue agarose chromatography, (3) molecular weight markers (Sigma) in descending order, carbonic anhydrase (30,000 daltons), myoglobin (17,200 daltons), cytochrome c (14,600 daltons). Gel B contained: (1) molecular weight markers (Pharmacia) in descending order, phosphorylase b (94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons) and trypsin inhibitor (20,100 daltons), (2) APRT purified from *Appt*^A homozygotes by ammonium sulfate precipitation, AMP agarose and Blue agarose affinity chromatography, (3) same as lane 2 except that APRT was purified from *Appt*^B homozygotes, (4) APRT purified from *Appt*^A as in lane 2 with the addition of the Octyl agarose step (the purified protein was lyophilized and eluted from the flask in 1 ml of distilled water), (5) same fraction of APRT as in lane 4 except that the flask was eluted again with 1 ml of 0.1% SDS in distilled water and (6) APRT purified from *Appt*^B homozygotes by ammonium sulfate precipitation, Octyl agarose, AMP agarose and Blue agarose chromatography.

investigated as a potential step in the purification of *Drosophila* APRT. The ammonium sulfate purified fraction of APRT was found to bind to blue agarose as well as AMP agarose. However, APRT activity could not be eluted from blue agarose with PrPP under the same conditions used in AMP agarose chromatography. Since APRT activity is displaced from AMP agarose by PrPP it was postulated that if APRT was saturated with PrPP prior to blue agarose chromatography, the enzyme might no longer bind to the Cibacron blue ligand since its active site would already be occupied by PrPP. This turns out to be the case as APRT in the PrPP containing eluant of the AMP affinity column is not retained by blue agarose (Figure 26). Therefore, it was possible that the contaminating proteins in the AMP affinity column purified fraction of APRT might be retained by blue agarose while APRT would simply be eluted from this gel. Chromatography of ammonium sulfate purified APRT on AMP agarose followed by blue agarose chromatography yields a protein fraction containing two different proteins (Figure 25 B, lanes 2 and 3) in approximately equal abundance. The larger of these proteins has a molecular weight of approximately 70,000 daltons. Gel filtration of *Drosophila* APRT indicates that APRT has a molecular weight of only 38,000 daltons (Figure 8). Therefore, this larger protein appeared to be a contaminant. The smaller of the two proteins has a molecular weight of 23,000 daltons and assuming that *Drosophila* APRT is a dimer under the non-denaturing conditions of gel filtration, this smaller protein must be the APRT subunit.

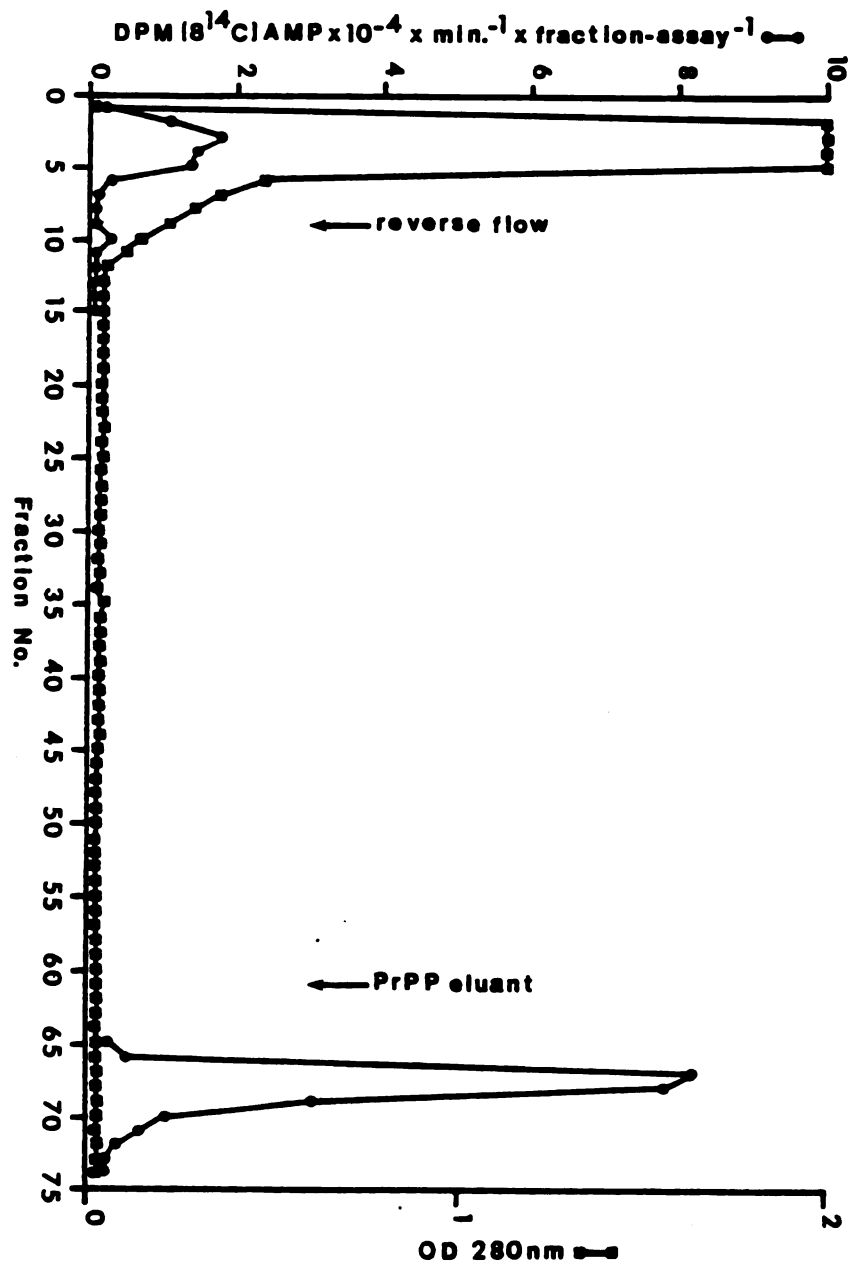


Figure 26. AMP Agarose and Blue Agarose Chromatography of *Drosophila* APRT

Figure 26. Legend

Ammonium sulfate and Octyl agarose purified APRT from *Appt^A* homozygotes was loaded onto an AMP affinity column and the column eluted as described (page 133). The void fractions (1-5) of the AMP column contain approximately 10% of the total APRT activity present in the loaded sample. Fractions 1-60 were approximately 8 ml each. Fractions 61-74, containing the PrPP eluant were approximately 2 ml each. Just prior to elution of the AMP column with PrPP, the outlet of the AMP column was connected to the inlet of a Blue agarose column. The APRT yield in the PrPP eluant ranged from 10-20% of the total APRT activity present in the original crude homogenate.

The two affinity column steps in the purification of *Drosophila* APRT failed to distinguish between APRT and a single remaining protein contaminant. Thus, another purification step compatible with the previous steps, but which separated proteins based on some criterion other than their biological affinity properties was required.

Ammonium sulfate precipitation produces a fraction of APRT which is stable and contains a high ammonium sulfate concentration. Hydrophobic interaction chromatography is generally performed as a step in protein purification immediately following ammonium sulfate precipitation because of the presence of high salt in these fractions of protein. The procedure is conducted by passing proteins through an agarose gel to which saturated carbon chains of particular lengths or benzene molecules are covalently linked. The presence of a chaotropic salt (ammonium sulfate in high concentration) in the protein solution and chromatography buffer causes a partial denaturation of proteins exposing hydrophobic core amino acid residues which can interact with the hydrophobic ligand attached to the gel. At sufficiently high salt concentration this hydrophobic interaction results in the binding of some proteins to the gel while less hydrophobic proteins are eluted from the gel. Octyl agarose (Pharmacia) was chosen for initial trial of this technique with *Drosophila* APRT since this gel promotes strong hydrophobic binding to proteins. *Drosophila* APRT was found to bind with octyl agarose in buffer which was 55% saturated with ammonium sulfate but is eluted from the gel in 30% ammonium sulfate saturated buffer (Figure 27). Octyl agarose chromatography produces a 6 fold

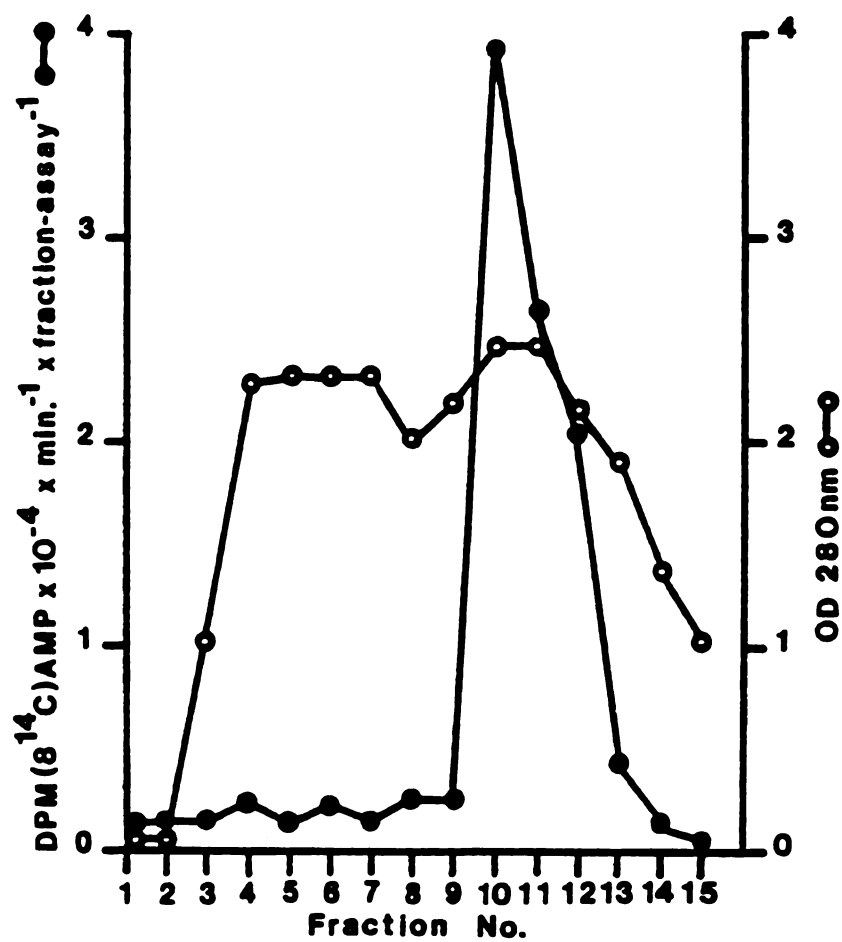


Figure 27. Octyl Agarose Chromatography of *Drosophila* APRT

Figure 27. Legend

Octyl agarose chromatography was conducted as described (page 132). Ammonium sulfate precipitated APRT was loaded onto the gel in 55% ammonium sulfate saturated buffer A and the column was eluted with this buffer (fractions 1-8). The column was then eluted with 30% ammonium sulfate saturated buffer A (fractions 9-15).

purification of APRT (Table 11) and results in the removal of the 70,000 dalton contaminating protein (Figure 25 A, lanes 5 and 6) remaining in the affinity chromatography purified fraction of APRT (Figure 25 B, lanes 2 and 3). A detailed description of each of the steps in the purification of *Drosophila* APRT is presented in the remainder of this appendix in the order in which they are performed.

Purification Steps

All of the steps in the procedure were carried out at 0-2°C. APRT activity was measured using the DEAE filter assay described in Appendix 1. *Drosophila* adults were collected and stored at -80°C until sufficient flies were accumulated for purification (50 grams).

Homogenization

1. 50 grams of frozen flies + 1 gram of norite are homogenized in 300 ml of buffer A [25 mM tris, 10 mM magnesium sulfate and 0.1 mM Na-EDTA pH 7.2] using a Waring blender equipped with a polytron head. The homogenate is then filtered through fine nytex nylon screen and aliquoted to centrifuge tubes.

2. The homogenate is centrifuged at 48,000 xg (g max.) for 30 minutes.

3. The resulting supernatant is poured through nytex (to trap the surface lipid film) into a 500 ml graduate and the volume measured. The hard and soft pellets are discarded.

Ammonium Sulfate Precipitation

4. In order to determine the amount of cold ammonium sulfate to produce 70% saturation of the measured volume of supernatant (weight/volume; ignoring second order volume changes) the following consideration is made. A 100% saturated ammonium sulfate solution in water at 0°C is 3.9 M (75). Therefore, the addition of 0.515 grams of ammonium sulfate to a milliliter of water produces a saturated solution (ignoring the second order volume change) and $0.515 \text{ grams ammonium sulfate/ml} \times \text{volume of supernatant in mls} \times 0.7 = \text{the weight in grams of ammonium sulfate necessary to produce 70\% saturation of the supernatant with ammonium sulfate}$. The supernatant was placed in a 1 liter beaker with a large stir-bar and mounted in an ice-water bath on a magnetic stirring plate. The ammonium sulfate necessary to produced 70% saturation of the supernatant was added with constant stirring and the sample was allowed to stir for 30 minutes.

5. The resulting precipitate was removed by repeating the centrifugation (step 2).

6. The resulting pellets were discarded and the supernatant was returned to a clean beaker on the stirring plate. The difference in grams of ammonium sulfate from the previous calculation was added to produce complete saturation of the supernatant with constant stirring. Additional ammonium sulfate was added until actual saturation of the sample with ammonium sulfate was reached (approximately 20 additional grams of ammonium sulfate for a step 4 volume of 300 ml of supernatant). The sample was allowed to stir for 45 minutes.

7. The centrifugation was repeated (step 2).

8. The resulting supernatant was discarded and the pellets resuspended in the minimum volume of buffer A necessary to dissolve the protein. The sample was placed into a 10,000 dalton exclusion limit dialysis bag and dialyzed for 2 hours against 500 ml of 55% ammonium sulfate saturated (actual degree of saturation) buffer A in a stirred beaker submerged in an ice-water bath.

Octyl Agarose Chromatography

9. The dialysed sample was loaded onto a 1.5 cm x 20 cm column of octyl agarose equilibrated in the dialysis buffer. The column was eluted with 15 ml of dialysis buffer (one column volume) collecting 4 ml fractions of eluant. The column was then developed with 30% ammonium sulfate saturated (actual degree of saturation) buffer A and the APRT collected in 2 ml fractions of this eluant. The eluant

fractions were diluted for the APRT assay, 1 part sample to 9 parts buffer A, prior to assay. The fractions containing APRT activity were pooled and dialyzed for 2 hours against 2 liters of buffer A containing 10% glycerol.

AMP and Blue Agarose Affinity Chromatography

10. The dialyzed protein was loaded onto a 10 ml column of AMP Agarose (P&L Biochemicals, Agarose-Hexane-AMP Type 3, 5.7 micromoles AMP/ml of gel) equilibrated in the dialysis buffer. The column was eluted with 20 ml (2 column volumes) of dialysis buffer. Column buffer flow was then reversed and the column eluted with 10 mM ammonium bicarbonate pH 6.9 until no additional protein could be detected in the eluant (0.005 O.D. max.).

11. The outlet of the AMP column was connected to the bottom inlet of a 1.5 cm x 20 cm column of Blue Agarose (Sigma) equilibrated in the ammonium bicarbonate buffer. Elution of the AMP column was continued through the Blue agarose column with the ammonium bicarbonate buffer containing 0.1 mM Na-PrPP (Sigma). The purified APRT was collected in 2 ml fractions of this eluant which were assayed for APRT activity as described for Octyl agarose chromatography (step 9).

Concentration of APRT

Drosophila APRT is highly sensitive to surface denaturation in the dilute solutions obtained from the affinity column. Where the enzyme is to be used for assay of enzyme activity, glycerol should be added to the sample to a concentration of 10%. The enzyme is then

concentrated by ultrafiltration in an Amacon stirred cell equipped with a PM-10 membrane. The concentrated APRT is stored at -80°C . APRT was also concentrated by lyophilization. However, the lyophilized enzyme has no detectable enzymic activity. The enzyme must be eluted from the lyophilization flask in a detergent solution since the protein adheres tightly to the glass surface and could not be eluted in water alone (Figure 25 B , lanes 4 and 5).

Regeneration of Agarose Gels

The AMP and Blue agarose gels were regenerated with 0.5 M NaCl in buffer A followed by 6 M urea in distilled water. The columns were then stored in buffer A containing 10% glycerol. The blue agarose column was equilibrated in the ammonium bicarbonate buffer just prior to affinity chromatography of APRT since this buffer liberates carbon dioxide which can block the flow of buffer in the column. The octyl agarose gel was regenerated by washing in a fritted glass filter funnel. The gel is first washed with distilled water followed by absolute ethanol and finally with water again. The gel was stored in 55 % ammonium sulfate saturated buffer A.

Appendix 7: Preparation and Partial Characterization of Anti-APRT IgG

An antibody directed against *Drosophila* APRT was generated by inoculation of a New Zealand white rabbit with apparently homogenous *Drosophila* APRT purified from *Aprt*^A homozygotes (Figure 9). The inoculation schedule and temporal response of the rabbit to immunization with *Drosophila* APRT is presented in Table 12. IgG was purified from rabbit serum by protein A chromatography as described in Figure 28.

In order to test whether the anti-APRT IgG obtained from the rabbit immunized with *Drosophila* APRT is specific for *Drosophila* APRT, experiments were conducted to test the ability of this IgG to precipitate hamster APRT activity. In addition, goat anti-hamster APRT serum (gift of M. Taylor, Indiana University) was used as a control for the precipitation assay and to test the ability of this serum to precipitate *Drosophila* APRT activity. The results (Table 13) demonstrate that each antiserum is species-specific for the APRT activity with which it interacts. Therefore, the anti-*Drosophila*-APRT antibody is a specific inactivator of *Drosophila* APRT activity. The dependence of APRT precipitation on the concentration of the *Drosophila*-anti-APRT antiserum is shown in Figure 29.

Table 12 Immunization of a Rabbit with Drosophila APRT

Week	Treatment ¹	% APRT Activity Precipitated ²
0	inject 30 µg APRT*	-
16	inject 30 µg APRT*	-
18	serum collected	0
18	inject 30 µg APRT [#]	-
21	serum collected	20
21	inject 30 µg APRT [#]	-
22	serum collected	66
24	serum collected	37
24	inject 6 µg APRT [#]	-
25	serum collected	78
25	serum collected	54
26	inject 20 µg APRT [#]	-
27	serum collected	84

¹The New Zealand White Rabbit was injected with an emulsion of Drosophila APRT in Freund's Complete Adjuvant* (Difco, 1 part adjuvant to 1 part APRT, v/v) or APRT was suspended in Freund's Incomplete Adjuvant# (same dilution).

²The precipitation assay is described in the legend to Figure 29.

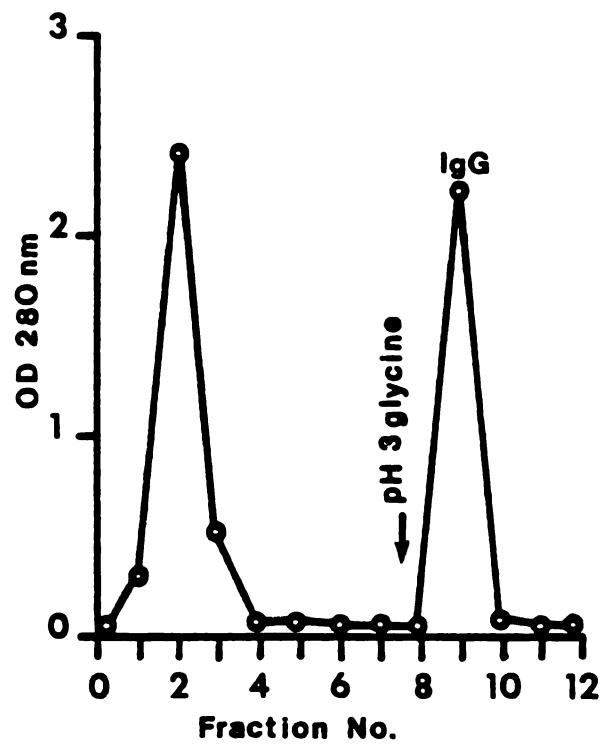


Figure 28. Purification of IgG by Protein A Agarose Chromatography

Figure 28. Legend

Rabbit serum (2 ml) was loaded onto a 1 ml column of Protein A agarose (Pharmacia). The column was washed with 0.1 M sodium phosphate buffer (pH 7.2) until most of the unbound protein was eluted from the gel. The IgG was then eluted from the column with 0.1 M glycine in distilled water (pH 3). The purified IgG was dialyzed against 1 liter of 0.1 M sodium phosphate buffer (pH 7.2) for four hours. This purified fraction of IgG has maintained approximately 80% of its original capacity to precipitate *Drosophila* APRT activity during six months of storage at 2°C.

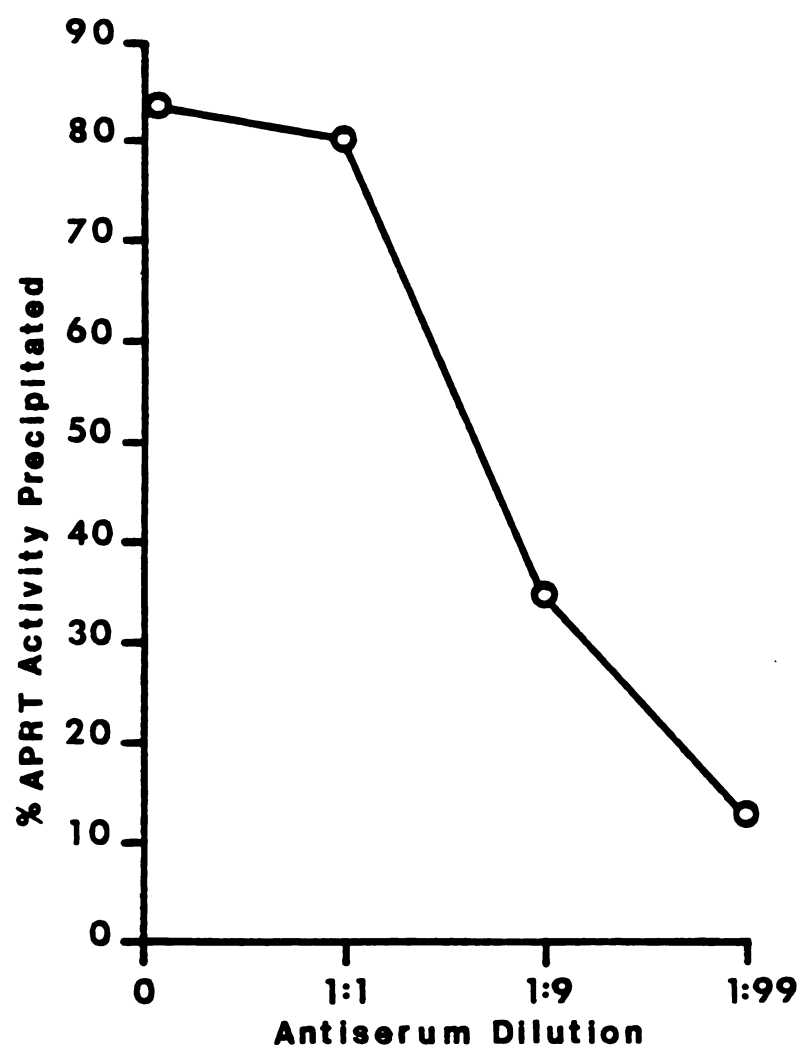


Figure 29. Dilution of *Drosophila* APRT Antiserum

Figure 29. Legend

The precipitation reaction contained 20 microliters of serum and 10 microliters of *Drosophila* extract. *Drosophila* extract was prepared by homogenizing 0.3 gm of adult *Ap^{rt}^A* homozygotes in 1 ml of distilled water. The homogenate was then centrifuged for 3 minutes in a Brinkman micro-centrifuge. The resulting supernatant was used in the precipitation assay. *Drosophila* extract was incubated with the anti-serum for 2 hours and then 5 microliters of fixed *Staphylococcus* cells (Pansorbin, CalBiochem) was added. The mixture was incubated for an additional 15 minutes and the samples were centrifuged as described above. All steps were performed at 2°C. APRT activity was measured in the supernatants of the antiserum and control serum treated samples using the DEAE filter assay (Appendix 1). Antiserum was diluted with control (preimmune) serum from the rabbit.

Table 13. Immunoprecipitation of Hamster and Drosophila APRT

APRT Source	Antiserum ¹	% APRT Activity Precipitated ²
Hamster Liver	Anti-Hamster-APRT	86
Hamster Liver	Anti-Drosophila-APRT	10
Drosophila (Aprt ^A)	Anti-Hamster-APRT	0
Drosophila (Aprt ^A)	Anti-Drosophila-APRT	61
Drosophila (Aprt ^B)	Anti-Drosophila-APRT	40

¹Anti-Drosophila-APRT serum came from the week 22 collection (Table 12).

²The immunoprecipitation assay is described in the Figure 29 legend.

One of the primary reasons for making the antibody to *Drosophila* APRT was to test the presence of APRT cross-reacting material (CRM) in the purine-resistant mutants deficient in APRT activity. Although this analysis has not yet been conducted, experiments were performed to test the ability of the antibody directed against APRT purified from *Appt*^A homozygotes to recognize APRT from the *Appt*^B variant. The results in Table 13 indicate that this antibody is capable of precipitating APRT activity from extracts of *Appt*^B homozygotes. The western blot analysis (Figure 30) shows that the anti-*Drosophila*-APRT IgG is capable of binding with *Drosophila* APRT from both variant strains (B, lane 1, 2 and 3) whereas an identical blot of these proteins treated with pre-immune serum from the APRT immunized rabbit does not interact with these proteins (A). This analysis shows that the anti-*Drosophila* APRT antibody is capable of binding with *Drosophila* APRT. Further analysis of the specificity of this antibody will be conducted in order to develop a test for APRT CRM in purine resistant mutants deficient in APRT activity.

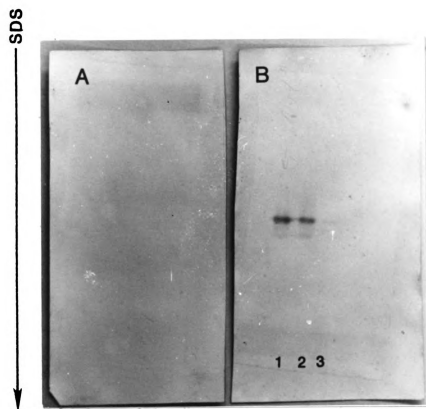


Figure 30. Western Blot of Drosophila APRT

Figure 30. Legend

Western blots were made from SDS gels as described in this Appendix. APRT was electroblotted onto nitrocellulose filters which were treated with rabbit anti-*Drosophila*-APRT serum and with preimmune serum. The blots were then treated with goat anti-rabbit-IgG IgG to which horseradish peroxidase is covalently linked. The blots were stained for peroxidase activity. Blot A and B were identical except that blot A was treated with pre-immune rabbit serum. Lane 1 contained purified APRT from *Appt*^A homozygotes. Lane 2 contained APRT purified from *Appt*^B homozygotes. Lane 3 contained 1.4 micrograms of total *Drosophila* proteins extracted from *Appt*^A homozygotes.

Collection of Serum

Blood was drawn from the marginal ear vein of the rabbit using a vacuum bleeding flask (Bellco). The blood was allowed to clot at room temperature for one hour and then refrigerated overnight. The blood was centrifuged for 30 minutes at 12,000 xg (g max.) at 4°C. The serum was removed from the sample with a pasteur pipette and stored at -80°C.

Western Blot Procedure

APRT was subjected to electrophoresis in a Laemmli SDS slab gel (Appendix 4). Protein in the gel was electrophoretically transferred to a nitrocellulose filter essentially as described by Towbin and co-workers (76) and Krall and co-workers (77) using a Hoefer electroblot apparatus. The gel holder was placed in a dish filled with the electroblot buffer [9.09 gm tris-base, 43.23 gm glycine, 600 ml methanol and distilled water to a final volume of 3 liters pH 8.3]. A quarter inch thick plastic sponge was placed in the holder and squeezed to eject the trapped air. Two sheets of Whatman No. 3 filter paper were laid on top of the sponge and the nitrocellulose sheets (HAHY 00010, Millipore) were laid on top of the filter paper. The gels were laid on top of the nitrocellulose sheets and were covered with two additional sheets of filter paper being careful not to trap air. The loaded plastic holder was then transferred to the electroblot apparatus with the sponge side of the sandwich facing the

anode. Electrophoretic transfer was performed at 17.5 V with constant current for 1.5 hours. The nitrocellulose sheets were then placed in seal-a-meal envelopes containing PBS saturated at room temperature with gelatin (Difco) for one hour to block the remaining protein binding sites on the filters. The blocking solution was discarded and the nitrocellulose sheet was incubated with anti-APRT antiserum diluted 1:1 with fresh blocking solution for 2 hours at room temperature. The filters were then washed in PBS [1.4 gm monobasic sodium phosphate + 8 gm NaCl per liter in distilled water pH 7.2], 5 changes of one liter each for a total of 30 minutes. The washed filters were incubated for one hour at room temperature with goat anti-rabbit-IgG IgG (Miles) to which horseradish peroxidase is covalently linked. The peroxidase-goat-IgG was diluted 2000 fold with blocking solution prior to use. The filters were washed as described above and then incubated with the peroxidase stain solution [0.4 gm tris-base, 15 mg diaminobenzidine (dissolved in 1 ml of 1 M HCl), 49 ml distilled water and 7.5 microliters 30% hydrogen peroxide pH 7.5] which was made up just before use. Diaminobenzidine is a carcinogen and was weighed out and put into solution in a fume hood. The filters stained in approximately five minutes. The stained filters were washed in PBS to terminate the staining reaction. The filters were then dried and stored in the dark in seal-a-meal envelopes. The intensity of stain on the filters stored in this fashion is stable for at least two months.

LIST OF REFERENCES

REFERENCES

1. Glassman, E. (1965) *Fed. Proc.* 24:1243-1251.
2. Duck, P. D. (1973) Doctoral Dissertation, University of Connecticut.
3. Brockman, R. W., Kelly, G. G., Stutts, P. and Copeland, V. (1961) *Nature* 191:469-471.
4. Albertini, R. J. and DeMars, R. (1970) *Science* 169:482-485.
5. Astrin, K. H. and Caskey, C. T. (1976) *Arch. Biochem. Biophys.* 176:397-410.
6. Epstein, J., Leyva, A., Kelly, W. N. and Littlefield, J. W. (1977) *Somat. Cell Genet.* 3:135-148.
7. Reuser, A. J. J. and Mintz, B. (1979) *Somat. Cell Genet.* 5:781-792.
8. Fenwich, R. G., Sawyer, T. H., Kruh, G. D., Astrin, K. H. and Caskey, C. T. (1977) *Cell* 12:383-391.
9. Taylor, M. W., Pipkorn, J. H., Tokito, M. K. and Pozzatti, R. O. (1977) *Somat. Cell Genet.* 3:195-206.
10. Chu, E. H. Y. and Powell, S. S. (1976) in *Advances in Human Genetics*, Harris, H. and Hirshhorn, K. eds., 7:189-258.
11. Caskey, C. T. and Kruh, G. D. (1979) *Cell* 16:1-9.
12. Taylor, M. W., Hershey, H. V. and Simon, A. E. (1979) in *Banbury Report 2*, Hsie, A. W., O'Neill, J. P. and McElheny, V. K. eds., Cold Spring Harbor Laboratory, pgs. 211-221.
13. Lindsley, D. L. and Grell, E. H. (1968) *Genetic Variations of Drosophila melanogaster*, Carnegie Inst. of Washington Publication No. 627.
14. Szybalski, W. (1958) *Microbiol. Genet. Bull.* 16:30.
15. Brockman, R. W., Bennett, L. L., Simpson, M. S., Wilson, A. R., Thompson, J. R. and Skipper, H. E. (1958) *Cancer Res.* 19:856.
16. Brockman, R. W. (1960) *Cancer Res.* 20:643.

17. Brockman, R. W. Dehavadi, C. S., Stutts, P. and Hutchison, B. (1961) *J. Biol. Chem.* 236:1471.
18. Tomizawa, S. and Aranow, L. (1960) *J. Pharmacol. Exp. Ther.* 128:107-128.
19. Simon, A. E., Taylor, M. W., Bradley, N. E. C. and Thompson, L. H. *Genetics* (in press).
20. Merril, C. R., Leavitt, J., Van Keuren, M. L., Ebert, M. H. and Caine, E. D. (1979) *Neurology* 29:131-134.
21. Johnson, D. H. and Friedman, T. B. (1981) *Science* 212:1035-1036.
22. Liu, P., Johnson, D. H., Friedman, T. B. and Trosko, J. manuscript in preparation.
23. Henderson, F. J. and Gadd, R. E. A. (1968) *Cancer Chemother. Rep.* 1:363-373.
24. Becker, J.-L. (1974) *Biochimie* 56:779-781.
25. Johnson, D. H. and Friedman, T. B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
26. Roberts, D. B. and Evans-Roberts, S. (1979) *Genetics* :663-679.
27. Grell, E. H. (1962) *Z. Vererb.* 93:371-377.
28. Schwartz, M. and Sofer, W. (1976) *Genetics* 83:125-136.
29. O'Brien, S. J. and Gethman, R. C. (1973) *Genetics* 75:155-157.
30. Hall, J. C. and Kankel, D. R. (1976) *Genetics* 83:517-535.
31. Wright, T. R. F., Bewley, G. C. and Sberald, A. F. (1976) *Genetics* 84:287-310.
32. Rawls, J. M. and Fristrom, J. W. (1975) *Nature* 255:738-740.
33. Chasin, L. A. and Urlaub, G. (1976) *Somat. Cell Genet.* 2:453-467.
34. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Chasin, L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76:1371-1376.
35. Righetti, P. G. and Drysdale, J. W. (1976) *Isoelectric Focusing*, North-Holland Pub. Co., Amsterdam.

36. Holden, J. A., Meredith, G. S. and Kelley, W. N. (1979) *J. Biol. Chem.* 254:6951-6955.
37. Holden, J. A., Meredith, G. S. and Kelly, W. N. (1979) *Adv. Exp. Med. Biol.* 122:123-129.
38. Kenimer, J. G., Young, C. G. and Groth, D. P. (1975) *Biochim. Biophys. Acta* 384:87-101.
39. O'Farrell, P. H. (1975) *J. Biol. Chem.* 250:4007-4021.
40. Levine, R. A. and Taylor, M. W. (1979) *Adv. Exp. Med. Biol.* 122: 57-60.
41. Hodge, L. D. and Glassman, E. (1967) *Genetics* 57:571-577.
42. Johnson, M. M., Nash, D. and Henderson, J. F. (1980) *Comp. Biochem. Physiol.* 66:555-561.
43. Wyss, C. (1977) *J. Insect Physiol.* 23:739-747.
44. Friedkin, M. and Kalchar, H. (1961) in *The Enzymes*, second edition, Boyer, P. D., Landy, H. and Myrback, K. eds., Academic Press, New York, 5:237.
45. Moiseenko, E. V. and Kakpakov, V. T. (1975) *Genetika* 11:160-162.
46. Becker, J.-L. (1978) *Biochimie* 60:619-625.
47. Friedman, T. B. unpublished observations.
48. Bennett, L. L., Schnebli, H. P., Vail, M. H., Allan, P. W. and Montgomery, J. A. *Mol. Pharmacol.* 2:432-443.
49. Warnick, C. T., Muzik, H. and Patterson, A. R. P. (1972) *Cancer Res.* 32:2017-2022.
50. Patterson, A. R. P., Paran, J. H., Yang, S. and Lynch, T. P. (1979) *Cancer Res.* 39:3607-3611.
51. Johnson, G., Finnerty, V., Hartyl, D. (1981) *Genetics* 98:817-831.
52. Warner, C. K. and Finnerty, V. (1981) *Mol. Gen. Genet.* 184:92-96.
53. Glassman, E., Karan, J. D. and Keller, E. C. (1962) *Z. Vererb.* 93: 399.
54. Yen, T. T. T. and Glassman, E. (1965) *Genetics* 52:977-981.

55. Chovnick, A., Gelbart, W. and McCarron, M. (1979) *Cell* 11:1-10.
56. McDonald, J. F. and Ayala, F. J. (1978) *Genetics* 89:371-388.
57. Grell, E., Jacobson, K. and Murphy, J. (1965) *Science* 149:80-82.
58. Benyajati, C., Wang, N., Reddy, A. Weinberg, E. and Sofer, W. (1980) *Nuc. Acid Res.* 8:5649-5667.
59. Wyss, C. (1979) *Experientia* 37:665.
60. Lowy, I., Pellicer, A., Jackson, J. F., Sim, G.-K., Silverstein, S. and Axel, R. (1980) *Cell* 22:817-823.
61. McCarron, M., Gelbart, W. and Chovnick, A. (1974) *Genetics* 76:289-299.
62. Spradling, A. C. and Rubin, G. M. (1982) *Science* 218:341-347.
63. Rubin, G. M. and Spradling, A. C. (1982) *Science* 218:348-353.
64. Casadaban, J. M. (1976) *J. Mol. Biol.* 104:541-555.
65. Yang, H. and Zubay, G. (1974) *Biochem. Biophys. Res. Commun.* 56:725-731.
66. Srivastava, S. K. and Beutler, E. (1971) *Arch. Biochem. Biophys.* 142:426-434.
67. Hochstadt-Ozer, J. and Stadtmann, E. R. (1971) *J. Biol. Chem.* 246:5294-5303.
68. Herschfield, M. S. and Kredich, N. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77:4292-4296.
69. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193:265-275.
70. Bradford, M. M. (1976) *Anal. Biochem.* 72:248-254.
71. Lewis, E. B. and Bacher, F. (1968) *Drosophila Inform. Serv.* 43:193.
72. Laemmli, U. K. (1970) *Nature* 227:680-685.
73. Switzer, R. C., Merrill, C. R. and Shifrin, D. (1979) *Anal. Biochem.* 98:231-237.

74. Hershey, H. V. and Taylor, M. W. (1978) *Prep. Biochem.* 8:453-462.
75. Green, A. A. and Hughes, W. L. (1955) *Methods in Enzymology* 1:76.
76. Towbin, H., Staehlin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76:4350-4354.
77. Kral, L. G., Johnson, D. H., Wing, M. and Friedman, T. B. (1982) *Dev. Genetics* 3:215-233.

MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 03047 0599