A GENETIC AND BIOCHEMICAL STUDY OF A TEMPERATURE-SENSITIVE VERMILION MUTATION IN *DROSOPHILA MELANOGASTER*

by

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ABSTRACT

The sex-linked vermilion (v) locus is probably the structural gene for the enzyme tryptophan pyrrolase. Mutations at the locus invariably are recessive and result in a bright-red eye colour phenotype accompanied by a loss of tryptophan pyrrolase activity. Extensive genetic, biochemical and developmental studies of v mutations have shown that the gene is a relatively small cistron controlling the catalytic activity of tryptophan pyrrolase which gives rise to kynurenine, a brown eye pigment precursor, in the larval fat body during a defined developmental period. Alleles of the locus can be broadly grouped into two classes: 1) spontaneous v mutations, the majority of which are suppressible by mutation at the non-allelic suppressor of sable [su(s)] locus, 2) induced v mutations which are all unsuppressible by su(s) alleles. Alleles of both classes behave nonautonomously during development and all map within the definable limits of the v cistron.

This investigation was initiated to recover conditional (temperature-sensitive) v alleles which could be used to study further the regulation of the activity of the v gene during development, and to extend our knowledge of the genetic functioning of the locus. A temperature-sensitive (ts) allele of a known structural gene, affecting the catalytic activity of an

assayable enzyme, could also enable a determination of the factors responsible for temperature-sensitivity in Drosophila in terms of changes in the gene product. The temperature-sensitive period (TSP) of a ts mutant in Drosophila is defined as that period during development when exposure to the restrictive temperature commits the organism to a mutant phenotype. With a ts v allele, a correlation can be made between the TSP determined phenotypically and the variation in tryptophan pyrrolase activity during development, and thus contribute to a molecular understanding of the TSP.

This study has consisted mainly of the following approaches: 1) mutagenesis and genetic screening to recover ts v alleles, 2) an examination of the phenogenetics of one ts v allele, including fine structure mapping, complementation properties, nonautonomous expression in gynandromorphs, and suppressibility, and a comparison of these properties with those exhibited by some non-ts v mutations, 3) a biochemical analysis of the effect of a ts v mutation on the properties of tryptophan pyrrolase, 4) a determination of the TSP of a ts v allele based on the eye phenotype.

Both ts v alleles, v^{ts1} and v^{ts2}, recovered in this investigation cause a vermilion phenotype if v^{ts} flies are raised at the restrictive temperature (29°C), whereas v^{ts} flies raised at the permissive temperature (17° or 22°C) have almost normal eye colour. The activity of tryptophan pyrrolase, extracted from v^{ts1} flies raised at 29°C and 22°C respectively, parallels the

temperature-dependent phenotypic properties; enzyme activity is markedly reduced in $\underline{v^{ts1}}$ flies raised at 29°C but is almost normal in flies raised at the permissive temperature. The $\underline{v^{ts1}}$ mutation behaves like non-ts, induced $\underline{v}$ alleles at 29°C in its complementation, suppressibility and nonautonomy. Thus, it fails to complement any other $\underline{v}$ point mutant, is unsuppressible by $\underline{su(s)^2}$ and is developmentally nonautonomous when present with $\underline{v^+}$ tissue in gynandromorphs raised at 29°C. Since the $\underline{v^{ts1}}$ allele is viable when heterozygous with deletions removing the $\underline{v}$ locus and maps within the $\underline{v}$ cistron as a point, it is assumed to be a point mutation in the $\underline{v}$ structural gene. Furthermore, the tryptophan pyrrolase controlled by the $\underline{v^{ts1}}$ mutant has different *in vitro* kinetic and temperature-dependent properties when $\underline{v^{ts1}}$ flies are raised at 29°C compared to either wild type or tryptophan pyrrolase extracted from $\underline{v^{ts1}}$ flies raised at 22°C.

The $\underline{v^{ts1}}$ mutant demonstrates different phenotypic and enzyme properties between males and females raised at 29°C; hemizygous males are more mutant in phenotype and have lower tryptophan pyrrolase activity than their homozygous sibs. This result apparently is the reverse of the dosage compensation normally demonstrated by wild type tryptophan pyrrolase in which males with one dose of the $\underline{v^+}$ gene have at least the enzyme activity obtained from females with two doses of the $\underline{v^+}$ gene. However, the TSP for the $\underline{v^{ts1}}$ mutant is the same for males and fem-

ales and falls between the early third instar larva and early pupa stages of development. This period corresponds to the maximum pre-adult activity of tryptophan pyrrolase and also correlates with the formation of kynurenine in the cells of the fat body.

These results are discussed in relation to a molecular model explaining the genetic and molecular functioning of the v locus during development. The results are consistent with the hypothesis that v^{ts} and nonconditional v mutations affect different aspects of active tryptophan pyrrolase structure rather than regulation of the rate of synthesis of the enzyme. Thus, suppressible v mutations affect allosteric or regulatory sites of the enzyme which interact with metabolic and developmental cofactors, whereas the nonconditional, unsuppressible, induced v mutations probably affect the catalytic sites of tryptophan pyrrolase. The ts v mutation, v^{ts1}, has genetic and biochemical properties which are compatible with an effect on the aggregation of enzyme subunits due to conformational changes during enzyme synthesis at the restrictive temperature.

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REVIEW

In recent years much attention has been devoted to the elucidation of the fundamental structure and function of genetic units in eukaryotes (GEORGIEV 1969, 1972; CRICK 1971; JUDD, SHEN AND KAUFMAN 1972; SORSA, GREEN AND BEERMANN 1972; FRISTROM AND YUND 1973). Much of this analysis has been performed in Drosophila because of the availability of a detailed cytogenetic map of the giant salivary gland chromosomes and the sophisticated genetic contrivances possible in this organism. More recently, these approaches have been joined by a rapidly developing application of biochemical techniques.

Central to these studies has been the identification and description of the genetic unit(s) corresponding to the classical complementation unit and what relationship(s) this unit has to the cytologically defined band and interband in the salivary chromosomes. Elegant genetic experiments indicate a one to one relationship between chromomeres (bands) and functional groups (JUDD et al. 1972) thereby suggesting about 5,000 complementation groups in Drosophila. This contrasts with DNA hybridization studies which suggest sufficient unique sequences for approximately 100,000 genes (LAIRD 1971). This disparity in results remains a central issue for resolution.

JUDD et al. (1972) have interpreted this excess but relatively unique DNA in each band as comprising cis-dominant

regulatory elements which function in such a way that if several different functions were in fact present in each complementation group they would not be recognized by standard complementation tests since a mutation in any one of the regulatory elements would act to shut off the entire array of functional units. An alternative explanation is that a chromomere is a complex unit consisting of interspersed unique and repetitive sequences (TURNER AND LAIRD 1973; WU, HURN AND BONNER 1972). In this model, only one, or at most a few structural genes are translated into functional protein from each chromomere, although the precursor transcript to the functional mRNA is a larger molecule of heterogeneous nuclear RNA representing both the unique and repeated sequences of the DNA in the chromomere (DANEHOLT 1972; WILLIAMSON, DREWENKIEWICZ AND PAUL 1973). However, if unique sequences correspond to structural information and a number of these are interspersed with repetitive sequences in each chromomere, there should be more than one complementation group per chromomere. In none of the studies on number of complementation groups per band in Drosophila has this been observed (LIFSCHYTZ AND FALK 1969; HOCHMAN 1971; JUDD et al. 1972).

The most direct approach to the twin problems of genetic organization and regulation of structural gene activity in Drosophila is to select loci whose protein products, preferably enzymes, are amenable to precise assays of activity and relative amount of protein, and to amino acid sequencing. The changes in

these parameters directed by mutations in the structural gene and in control elements mapping outside the structural cistron, should then be related to fine structure mapping of these mutations. Such studies are beginning in several laboratories, (SOFER; MULLER-HILL; ASHBURNER, CLARK AND AMBLER with ADH; MacINTYRE with acid phosphatase). Added resolution is gained if the genetic locus is clearly localized cytologically to a band or region of a band in the salivary gland chromosomes and, additionally, the enzyme specified by the locus interacts with developmentally important systems.

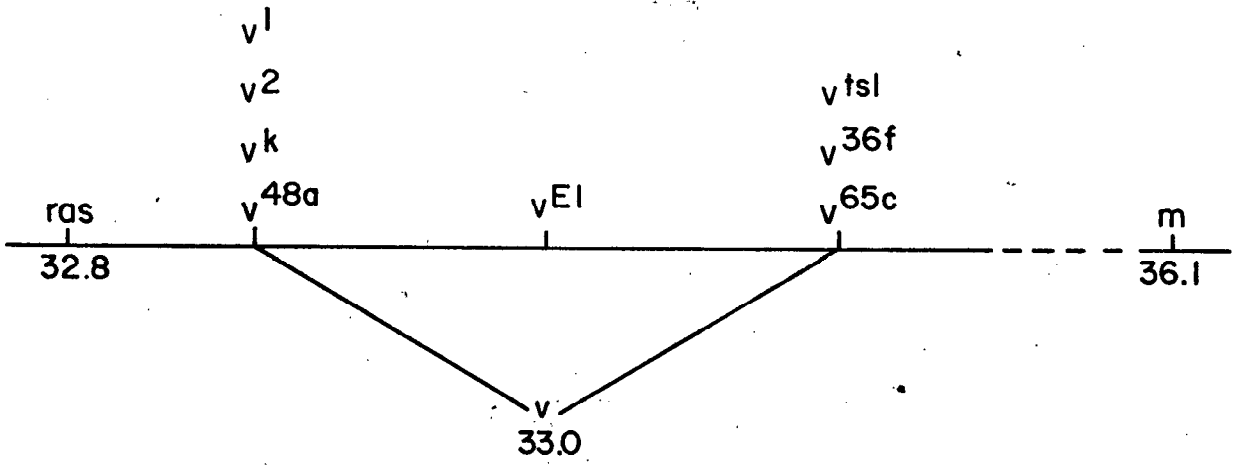
The vermilion (v, 1-33.0) gene satisfies most of these requirements. While no electrophoretic variants of tryptophan pyrrolase (TP) have, as yet, been mapped to the locus, by all other criteria the v gene specifies the structure of this enzyme. Thus, TARTOF (1969) has shown that in suppressed v^k flies, a TP is synthesized which is kinetically different to wild type suggesting that mutation in the v locus causes an alteration in TP structure, since the suppressor mutation, su(s), alone does not cause any change in the activity or kinetics of TP. BAILLIE AND CHOVNICK (1971) have clearly demonstrated that a linear increase in TP activity is a direct function of increase in the dosage of v^+ alleles, supporting the contention that each v^+ allele codes the information required for a unit of TP activity. Finally, CAMFIELD AND SUZUKI (1973) have recovered two temperature-sensitive v mutations, one of which has been shown to produce changes in the activity and kinetics of TP in in vitro assays.

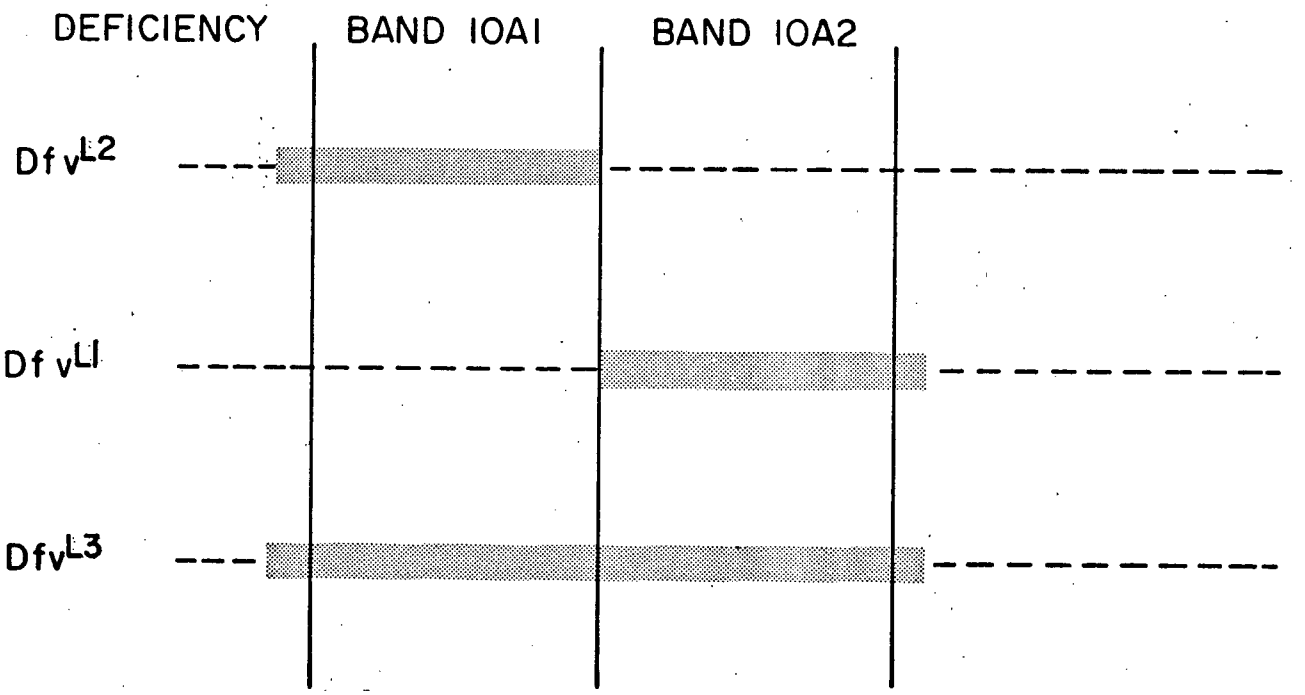
1. Cytogenetics of the v locus.

The v gene has been unambiguously assigned to band 10A1-2 (LEFEVRE 1969). In the extensive sample of deletions examined, LEFEVRE found that females heterozygous for different deletions interrupting the integrity of the v locus are, without exception, lethal. In at least one heterozygous combination of deletions there is minimal or no overlap of 10A1-2 deleted material and yet the heterozygous female does not survive although apparently containing one complete copy of the 10A1-2 genetic information in *trans* configuration (for example, Dfv^{L1}/Dfv^{L2}, Figure 1). This implies that contiguous genetic material in *cis* arrangement is necessary for the essential function performed by 10A1-2, an observation consistent with the interpretation of JUDD *et al.* concerning the functional organization of the typical band. This model would predict an impairment of v^+ function even in a heterozygote for 2 nonoverlapping deletions of 10A1-2 because of the *cis*-dominant nature of the control of the functional unit.

In spite of extensive searches for lethal point mutations in the v locus, none has been found (LEFEVRE 1967, 1969, 1971; SCHALET 1971; CAMFIELD - unpublished). Furthermore, females heterozygous for any v deletion and any v point mutant are invariably v in phenotype and are devoid of TP activity. Thus, paradoxically, by all cytological criteria, deletions only for the locus of v are lethal, yet every point mutant detected in the region is

FIGURE 1 Genetic map of the vermilion cistron in relation to outside markers and deficiencies of the v locus. The maps are not drawn to scale but represent the relationships of the v alleles to each other and the parts of the v locus deleted by the three v deficiencies.


v1
v2
vk
v48a
vEI
vtsl
v36f
v65c
ras
32.8
m
36.1
v
33.0


DEFICIENCY
BAND 10A1
BAND 10A2
Df vL2
Df vL1
Df vL3

viable despite the complete absence of TP. One solution to this apparent paradox may be the hypothesis of BRITTEN AND DAVIDSON (1969) that certain bands contain control elements which exert regulatory direction over more than one structural gene. Therefore, loss of the structural part of the v locus is not lethal but loss of some or all of the regulatory elements, which might also be present in 10A1-2 but are not manifested by complementation or mutational analysis of the v locus, may be lethal because they control the function of a separate but unidentified, indispensable locus(i). This loss of necessary function therefore is obvious only when 10A1-2 is deleted. It follows that point mutations in this control element would not be seen unless the function of the separate, indispensable locus is identified, although even then it is possible that they would never be detected owing to genetic redundancy, so that a sizable deletion is required before the regulatory function is lost and recognizable.

Some support for this explanation is offered by the preliminary analysis of the distribution of the v point mutations through the 10A1-2 band. They appear to be clustered in a tightly linked group (total map distance about 0.007 map units) in the left hand region of 10A1-2, restricted to a short interval about 0.10 - 0.15 map units from the left edge but nearly 0.5 map units from the right edge of 10A1-2 (LEFEVRE 1971). Thus, representatives of the visible v class are not extensively distributed throughout the 10A1-2 band. Moreover, the 3,000 base pair size

of v estimated by recombination values (FRISTROM AND YUND 1973) is reasonable judged by the molecular weight of the subunits which probably comprise the active TP enzyme (TARTOF 1969; BAILLIE AND CHVONICK 1971). This is in striking contrast to the conservative estimate of 2 - 2.4 x 10^5 base pairs determined cytophotometrically (RUDKIN 1965; LEFEVRE 1971). Thus, most of the DNA in band 10A1-2 does not appear to be concerned with structural information for tryptophan pyrrolase synthesis. LEFEVRE (1971) has also estimated that band 10A1-2 alone is responsible for about 0.60 - 0.65% of the crossing over in the ras - fw region, yet the v cistron itself comprises only about 0.007 map units.

The regulatory element(s) that appear to be present in at least part of the 10A1-2 band must play some role in controlling the activity of the v^+ structural gene as well since lethality has not been separated from an effect on the v locus in any of the deletions affecting 10A1-2. Deletions which appear to remove just the right hand part of 10A1-2 (Dfv^{L1} for example), still produce a v phenotype when heterozygous with any v point mutation (Figure 1). Since there is virtually no TP activity in such combinations (BAILLIE AND CHOVNICK 1971; CAMFIELD - unpublished), this could be explained by assuming that the deletion chromosome does not contribute any TP product to the flies because regulation of synthesis is lacking, and the chromosome containing the v point mutation codes for a catalytically deficient TP; hence their combination gives rise to no net TP activity. Furthermore,

when an insertional translocation involving a small segment of the X chromosome containing the $\underline{v^+}$ locus ($\underline{T(1;2)v^{65b}}$) is inserted as a duplication into the centric heterochromatin of the second chromosome, position effect depression of $\underline{v^+}$ activity results, and the male duplication segregant exhibits an eye colour phenotype midway between $\underline{v^+}$ and $\underline{v}$ (LEFEVRE 1969). This demonstrates that there is X-chromosomal control of $\underline{v^+}$ structural gene activity although it does not, of course, define its nature.

In summary, the $\underline{v}$ locus is associated with a particularly large band containing, by any reasonable criterion, a remarkable excess of DNA for which a definite function, other than viability and some control of $\underline{v^+}$ structural gene activity cannot at present be ascribed. Most of this excess DNA is not delineated by mutational change resulting in a $\underline{v}$ phenotype and yet its deletion results in a $\underline{v}$ phenotype. No known mutant with a $\underline{v}$ phenotype is lethal and yet deletion of the band containing the locus is. Hence, this apparently "excess" DNA may be concerned with regulating the function of another indispensable locus, as well as having a $\underline{cis}$ - dominant regulatory control of $\underline{v^+}$ structural gene activity. It is also possible that the essential function performed by part of 10A1-2 is associated with structural information of a necessary but unidentified locus.

In the discussion to follow, the interactions of $\underline{v}$ mutations with the $\underline{su(s)}$ locus and the fine structure mapping of $\underline{v}$ point mutants will be assessed in detail since these two aspects of $\underline{v}$

function contribute information which enables a testable molecular model to be advanced to explain the genetic regulation and biochemical properties of the v locus.

2. Suppression of v mutations.

BRIDGES (1915) originally observed that homozygosity for a mutation at the non-allelic su(s) locus suppressed the mutant phenotype of v^1 thereby resulting in a wild type eye colour. GREEN (1952, 1954) systematically tested the suppressibility of 6 spontaneous and 16 induced v mutations by 4 different su(s) alleles and showed that the only suppressible v alleles are of spontaneous origin, whereas some spontaneous and all induced v alleles are unsuppressible. The allele specificity indicated by the su(s) - v interactions appears to depend entirely upon the v locus since a v allele, if suppressible by one su(s) allele, is suppressible by all other su(s) mutations tested (GREEN 1954; SHAPARD 1960; TARTOF 1969). This rule holds irrespective of the mode of origin of the su(s) mutation. Spontaneous, X-ray and chemically induced su(s) mutants are available and all of the suppressible v mutants (v^s) tested with them show an identical response with respect to the particular amount of restoration of wild type phenotype and TP activity in each v^s - su(s) interaction (GREEN 1954; SHAPARD 1960; TARTOF 1969; SCHALET 1971).

Following BAGLIONI'S (1960) demonstration that homozygous

su(s) muations partially restore TP activity to some v mutants, MARZLUF (1965 a, b) and TARTOF (1969) extended the biochemical analysis of the su(s) - v^s relationship by examining the effects of independently derived su(s) mutations on the kinetic properties of TP produced by different v mutations. MARZLUF (1965,a) showed that suppressed v^1 and v^+ TP have indistinguishable Kms, pH optima, thermal and inhibition properties, even though $su(s)^2$ - v^1 flies have only about 10-20% of v^+ TP activity. This suggests that mutation of the su(s) locus permits the synthesis of a small amount of normal enzyme by v^1. TARTOF (1969) found for 3 different su(s) alleles and 3 different v^s mutations that the kinetics of the suppressed v^s TPs were similar to wild type, except for v^k (which varied from wild type and the other suppressed v^s TPs in its pH optimum and Km). The extent of restoration of TP activity varied among the different v^s alleles but for any one was constant with any of the su(s) alleles used. The v^s alleles tested by TARTOF in this study (v^k, v^1 and v^{36f}) could be ranked in order of their suppressibility with $v^k > v^1 > v^{36f}$. This gradation in suppressibility represents quite marked differences in the amount of TP activity restored. Thus, $su(s)^2\ v^k$ has about 21% of wild type TP activity, $su(s)^2\ v^1$, 9% and $su(s)^2\ v^{36f}$, 5%. These differences in the amount of TP activity restored in the various v^s alleles with the same su(s) mutation and the different kinetic properties of suppressed v^k TP indicate that the probable mechanism of suppression is post-translational; that is, a restoration of

activity to $\underline{v^s}$ alleles which differentially affect the structure of TP, rather than an increase in the amount of enzyme synthesized.

Elegant experiments by JACOBSON and coworkers (1971) and WHITE and his colleagues (1973) have clarified the mechanism by which su(s) mutations suppress $\underline{v^s}$ alleles. TWARDZIK, GRELL AND JACOBSON (1971) treated a homogenate of adult $\underline{v^1}$ flies with ribonuclease T1 and obtained activation of TP, whereas wild type TP was unaffected by this treatment. The activated $\underline{v^1}$ TP demonstrated normal, linear kinetics and was inactivated by unfractionated tRNA prepared from wild type flies. By chromatographing wild type tRNA on a reverse-phase column and testing each fraction for its ability to inhibit activated TP from $\underline{v^1}$, this inhibition was shown to be due to a specific isoacceptor of tyrosyl-tRNA. The major inhibitory fraction contained 3 peaks, 2 of which contained tyrosyl-tRNA as shown by their specific labelled-tyrosine accepting ability. Only one of the purified tyrosyl-tRNA peaks inhibited the activated TP of $\underline{v^1}$ in the *in vitro* assay system.

The key finding linking inhibition of activated $\underline{v^1}$ TP by an isoaccepting form of tyrosyl-tRNA to mutation at the su(s) locus was that *in vivo* suppression of $\underline{v^1}$ by su(s) is accompanied by the disappearance of the isoaccepting form of tyrosyl-tRNA which produces the inhibition of activated TP *in vitro*. In the $\underline{su(s)^2}$ mutation there is an absence of this species of tyrosyl-tRNA but a proportional increase in the other major fraction. Genetic identification located the control of this change in tyrosyl-tRNA

profile to su(s) and indicated that the biochemical change was affected by a recessive mutation as are su(s) alleles. JACOBSON et al. therefore suggested that the change in distribution of the two major isoaccepting forms of tyrosyl-tRNA is due to a change in an enzyme controlled by the $su(s)^+$ locus which allows the production of the v^1 TP inhibiting fraction of tyrosyl-tRNA by modifying the structure of the primary tyrosyl-tRNA gene product. Thus, the wild type fly can synthesize this modifying enzyme but the homozygous su(s)/su(s) cannot. The mechanism of suppression could then involve wild type and v^s TP complexing with the inhibiting form of tyrosyl-tRNA but, whereas this association is reversible in the case of v^+ TP, v^s mutations result in an alteration of the enzyme structure such that the associated tyrosyl-tRNA then causes inhibition, possibly by forming an irreversible complex. This inhibition is removed by digesting the tyrosyl-tRNA with RNase T1. Similarly, su(s) prevents the formation of the inhibiting form of tyrosyl-tRNA and consequently allows the TP of v^s to function as an enzyme.

At present, the reasons why v^s alleles vary in the degree to which TP activity is restored in the presence of mutation at the su(s) locus can only be speculation without more direct information about how they effectively abolish TP activity. However, since the enzymes synthesized by v^s alleles are capable of TP activity under suppressed conditions (sufficient to permit a v^+ eye colour in all cases except v^{36f}), and the molecular weight of unsuppressed v^k

mutant TP is similar to wild type (BAILLIE AND CHOVNICK 1971), it is highly probable that they are missense mutants which affect TP in different positions. This would account for the variation in their degree of suppressibility. Moreover, the interactions of the TP enzymes with the inhibiting form of tyrosyl-tRNA, possibly reversible with $\underline{v}^{+}$ TP but irreversible with $\underline{v}^{s}$ TP, indicates that this association probably involves regulatory or allosteric mechanisms. Therefore, $\underline{v}^{s}$ mutations probably cause changes in these regulatory sites of the TP enzyme, rather than in indispensable catalytic sites such as the active centre.

Under *in vivo* conditions, wild type TP reversibly complexes with the inhibiting form of tyrosyl-tRNA but can be dissociated to perform its catalytic function at the appropriate time in development probably by an *su(s)*-controlled change in distribution of the forms of tyrosyl-tRNA during development. Changes in the distribution of the two major isoaccepting forms of tyrosyl-tRNA during wild type development have recently been shown by WHITE, HOLDEN, TENER AND SUZUKI (1973). These changes appear to reduce the amount of the TP inhibiting form of tyrosyl-tRNA markedly at about the developmental time at which the catalytic function of wild type TP occurs.

The lack of suppressibility of other *v* alleles ($\underline{v}^{u}$) could be due to the possible direct effect of these mutations on the important catalytic sites of the enzyme rather than on the regulatory or allosteric regions which complex with the inhibiting form of

tyrosyl-tRNA. Therefore, no enzyme activity is recovered from the TP specified by $\underline{v^u}$ alleles whether this tyrosyl-tRNA is present (as in $\underline{su(s)^+}$) or not (as in $\underline{su(s)}/\underline{su(s)}$).

Clear evidence that $\underline{su(s)^+}$ is responsible for the production of the inhibiting form of tyrosyl-tRNA recently has come from the work of WHITE *et al.* (1973). These workers examined the chromatographic elution profiles of labelled tyrosyl-tRNAs (as well as the other 19 amino acid tRNAs) from different developmental stages of $\underline{v^+}$ and $\underline{su(s)^2\ v^1}$ flies. They found that the relative proportions of chromatographically distinct forms of the tyrosyl-tRNA from $\underline{v^+}$ and $\underline{su(s)^2\ v^1}$ are altered in a quantitatively different manner during the life cycle. The separable forms of tRNAs, not only of tyrosyl-tRNA but also of asparaginyl-, aspartyl- and histidyl-tRNAs, all vary in the same way from wild type in their relative distribution at different developmental stages of the $\underline{su(s)^2}$ strain. Presumably, this is because of the lack of the conversion enzyme specified by $\underline{su(s)^+}$ which converts one chromatographic form of these tRNAs into another by post-transcriptional modification. The modification results in a change in distribution of homogeneic tRNAs which have the same sequences and are products of the same gene but are chromatographically distinct because of a conversion enzyme - mediated change in a minor nucleoside analogous to Q of *E. coli*.

Of interest to the mechanism of suppression of $\underline{v^s}$ TP by *su(s)* are the developmental fluxes in the two major homogeneic

forms of tyrosyl-tRNA. During the development of wild type flies from eggs to late third instar larvae, the tyrosyl-tRNA form designated δ by WHITE et al. decreases, while the γ form increases. This γ form is equivalent to the v^1 TP inhibitory tyrosyl-tRNA fraction of TWARDZIK et al. (1971). At a prepupal stage in wild type development, the δ form begins to increase at the expense of the γ form. This trend continues until in 2 week-old adults the δ and γ forms are approximately equal. In the $su(s)^2\ v^1$ strain, the δ and γ forms are approximately equal in late third instar larvae in contrast to wild type in which a great excess of the γ form over the δ form is present at this stage. The period in development from about the middle of the third instar larva to the early pupa appears to be the time during which TP is catalytically active, based on the temperature-sensitive period of the v^{ts1} mutation (CAMFIELD AND SUZUKI 1973), and the accumulation of kynurenine, the product of the TP catalyzed reaction, in the fat body (RIZKI AND RIZKI 1968). This time therefore correlates well with the period during which there is a preponderance of the γ form of tyrosyl-tRNA (the form which binds to and inhibits v^1 TP). The $su(s)^2\ v^1$ strain has a greatly reduced proportion of the γ form, hence inhibition of v^1 TP is relieved. Although binding of γ tyrosyl-tRNA to v^+ TP has yet to be demonstrated, it seems likely that a reversible complex is formed between them at some earlier time in development. Then, preparatory to the catalytic action of v^+ TP, the γ form is modified to the δ form by the $su(s)^+$ conversion enzyme which enables

the complex to be disassociated thereby releasing enzymatically active, free v^+ TP. This mechanism implicates the unmodified nucleotide G Υ p of the Υ form as the key part of the tyrosyl-tRNA responsible for binding mutant TP and possibly also v^+ TP, since this nucleotide is the only difference between the γ and δ forms of tyrosyl-tRNA. As WHITE et al. (1973) point out, there must be a mechanism by which v^1 TP distinguishes the G Υ p nucleotide in the γ tyrosyl-tRNA from the same nucleotide in the γ forms of 5 other amino acid-tRNAs since these do not inhibit v^1 TP. A variety of studies (cf. MARZLUF 1965 a, b; GHOSH AND FORREST 1967; BAILLIE AND CHOVNICK 1971; TOBLER, BOWMAN AND SIMMONS 1971) have shown that both v^+ and mutant TP probably have allosteric regulatory sites to which both negative and positive effectors bind. WHITE et al. suggest that the binding of a specific in vivo inhibitor, such as a pteridine or allopurinol, to an allosteric site of mutant TP may enable the specific recognition and interaction of the enzyme with G δ p which may be located in the anticodon loop of γ tyrosyl-tRNA.

These mechanisms of interaction between mutations at the su(s) locus and v^s TP do not explain why only some spontaneous v mutants are suppressible, whereas other spontaneous and all induced v mutations tested are not. In fact, the generalization that only spontaneous mutants are suppressible extends, with very few exceptions, to every other locus in Drosophila for which a suppressor of mutations in the locus as well as spontaneous and induced mutations are known. KAUFMAN, TASAKA AND SUZUKI (1973) have noted that of the

85 separate mutations listed by LINDSLEY AND GRELL(1968) as being suppressible, only four are induced and the rest are of spontaneous origin. At present it is difficult to postulate just what this distinction may imply. However, it does seem obvious that suppressible mutants cannot have such a deleterious effect on the activity of the gene product as the unsuppressible mutants have if, as seems clear from the analysis of the su(s)-v relationship, the normal method of suppression in Drosophila involves post-translational metabolic modification which allows a potentially functional gene product to become active. Thus, unsuppressible mutations might result in a kind of alteration in the gene product that renders it inactive under all metabolic conditions. This inactivity could result from a failure of the gene product to be formed at all such as in deletions or mutations in regulator genes, failure to form a complete gene product as in nonsense mutations, or missense mutation in a part of the product essential to activity. Except that induced mutations result in chromosomal aberrations such as deletions more often than spontaneous ones, there is no a priori basis for expecting induced mutations to be more drastically altered than spontaneous ones.

3. Genetic fine structure of the v cistron.

If, as has been suggested here, the primary difference between v^s and v^u alleles is in the position that is mutated in the

enzyme, then since colinearity presumably occurs between a gene and its product in Drosophila, a study of the genetic fine structure of the v cistron should show that v^s and v^u occupy different sites in the cistron.

A representative sample of v alleles has been subjected to fine structure recombination studies (GREEN 1952, 1954; BARISH AND FOX 1956; LEFEVRE 1971; SCHALET 1971; CAMFIELD AND SUZUKI 1973) and a collated summary of the map of the locus is presented in Figure 1. As shown in the Figure, at least three sites have been separated by crossing over, with the majority of the mutants localized thus far falling into two distinct sites. The spontaneous, suppressible v mutations v^1, v^2 and v^k, are located at the left end of the map but have not been separated from the induced, unsuppressible mutation, v^{48a}. The induced, unsuppressible mutations v^{ts1} and v^{65c} map to the right site of the cistron with the spontaneous, suppressible v^{36f}.

Recombination studies within the v cistron reveal very tight linkage between the alleles. SCHALET (1971) used a system of balanced lethals to enrich for crossovers in the v region and found no recombinants between v^1 and v^2 in an estimated sample of 890,000 progeny of v^1/v^2 heterozygotes. GREEN (1954) separated the spontaneous, suppressible v^{36f} mutation from v^1 in an attached-X chromosome so that he was able to recover and demonstrate the $v^1\ v^{36f}$ double mutant. The frequency of recombination was reported at about 1/30,000 with v^1 mapping to the left of v^{36f}. SCHALET (1971) also separated v^{36f} from v^2 and v^k, v^{36f} mapping to the right of v^2

and $\underline{v}^{k}$. BARISH AND FOX (1956) localized the X-ray induced, unsuppressible $\underline{v}^{48a}$ mutation to the left of $\underline{v}^{36f}$ (2 recombinants in approximately 80,000 progeny) but were unable to resolve it with respect to $\underline{v}^{1}$ in a sample of 40,000 zygotes. The X-ray induced mutation $\underline{v}^{65c}$ was inseparable from $\underline{v}^{36f}$ in an estimated sample of 250,000 zygotes (SCHALET 1971). Two EMS-induced and unsuppressible mutations have been mapped; $\underline{v}^{E1}$ has been localized to the right of $\underline{v}^{1}$ and to the left of $\underline{v}^{36f}$ (SCHALET 1971) and so defines a third site in the cistron situated between $\underline{v}^{1}$, $\underline{v}^{2}$ and $\underline{v}^{k}$ occupying the left hand site and $\underline{v}^{36f}$ and $\underline{v}^{65c}$ occupying the right hand site. The temperature-sensitive mutation $\underline{v}^{ts1}$ maps to the right of $\underline{v}^{1}$ but was not separated from $\underline{v}^{36f}$ (CAMFIELD AND SUZUKI 1973).

The sample sizes were sufficiently large in most cases to permit the recovery of crossovers within the v locus. Therefore, the limited number of sites and their extremely close linkage probably are real reflections of the detectable mutable regions within the cistron and their physical distances apart rather than any artifact of selection or limited resolution. By computing average recombination values between the separable v alleles from these studies, the map distance between the left and right sites of the cistron is about 0.007 map units, a distance corresponding to about 2,400 base pairs, which is sufficient to code for a polypeptide about 800 amino acids long. The best estimate of the molecular weight of active TP is about 150,000 daltons (BAILLIE AND CHOVNICK 1971). Hence the active enzyme may consist of more than one

polypeptide.

Since no complementation at either the genetic or the enzyme activity levels has ever been observed between any two v alleles, a multimer consisting of two, or multiple of two identical polypeptide subunits is the most likely structure of active TP. Therefore, it appears that the v cistron is simple in organization, consisting of just the linear array of nucleotides necessary to code for a TP subunit. In this view, all visible v mutations would represent changes in the nucleotide sequence of the structural gene which give rise to equivalent amino acid changes in the TP subunit.

It is obvious from a consideration of the map of the locus that the distribution of mutable sites is not continuous through the cistron but that marked clustering of alleles occurs. GREEN and FRISTROM (cf. FRISTROM AND YUND 1973) have interpreted such discontinuous intralocus organization of mutable sites as reflecting the presence of spacer DNA between structural cistrons, or the existence of neutral genes, neither of which would normally be recognized by visible mutation. However, this explanation seems unlikely for the v cistron since it is incompatible with complementation, deletion and gene product properties. Alternative explanations for the marked clustering of v mutations could be that mutation at only a limited number of positions in the TP protein leads to a visible v phenotype or that some sites in the v cistron are differentially sensitive to mutation. Moreover, a polypeptide unit of about 750 to 800 amino acids in length coded for by a

locus consisting of about 2,400 to 3,000 bases would seem to preclude any significant portion of DNA not concerned with structural information for TP.

When map position, mode of origin and suppressibility are compared for the various v alleles for which these data are available (TABLE 1) an unambiguous correlation is not obtained. Thus, v^1, v^2 and v^k are all v^s and map to the left hand site of the cistron but these alleles are physically separated from the spontaneous, suppressible v^{36f} which maps in the right hand site identical with the v^u alleles, v^{ts1} and v^{65c}. The v^u allele, v^{48a} maps to the left hand site separated from v^{E1}, another v^u mutation, which occupies the middle site of the cistron. This map distribution of v^s and v^u alleles therefore does not clearly support the suggestion that v^s and v^u represent distinct classes of v mutations because they cause mutation in functionally distinct regions of the TP polypeptide. There does appear to be a general trend for v^s mutations to map to the left hand site (three out of four map there) and for v^u mutations to map to any of the three sites (all three sites have at least one v^u representative). In any case, apparent ambiguity of seriation of alleles in a clustered cistron does not exclude the possibility that mutations which are at different sites in the structural gene (such as v^1 and v^{36f}) and therefore cause changes at different sites in the gene product, may in fact be functionally closely related because of folding of the polypeptide chain into active enzyme. Similarly, although v^u mutations map at

TABLE 1 Map position, origin and suppressibility of some *v* alleles.

MUTATION	MAP POSITION	ORIGIN	SUPPRESSIBILITY
v^{1}	left site	spontaneous	+ *
v^{2}	left site	spontaneous	+
v^{k}	left site	spontaneous	+
v^{48a}	left site	X-ray	-
v^{E1}	middle site	EMS	-
v^{ts1}	right site	EMS	-
v^{36f}	right site	spontaneous	+
v^{65c}	right site	X-ray	?

* + = allele is suppressible

\- = allele is unsuppressible

different sites in the structural gene, they still could alter amino acids which are directly involved in the catalytic region of the enzyme.

The assumption that $\underline{v}^s$ alleles are structural gene mutations is strongly supported by the evidence that they produce a potentially functional enzyme whose molecular weight is the same as wild type TP. The evidence that $\underline{v}^u$ also represent structural gene mutations is not so convincing. Formally, they could be mutations in a control element which regulates $\underline{v}^+$ structural gene activity since they completely abolish TP activity and are unsuppressible. Without a demonstration that $\underline{v}^u$ mutants form no, or very little, cross reacting material for TP which would clearly distinguish between the possibilities, two indirect lines of evidence argue that they are defects in the $\underline{v}$ structural gene: (i) they fail to complement with any $\underline{v}^s$ mutation. It is highly unlikely that a regulator gene mutation could be *trans* as well as *cis* dominant to structural gene mutations; and (ii) they map at all three sites in the $\underline{v}$ cistron, including sites occupied by structural gene mutations.

The model proposed for the genetic organization and regulation of the $\underline{v}$ locus therefore predicts that the visible $\underline{v}$ mutations so far recovered are all mutations in the structural gene which consists of uninterrupted information specifying the structure of TP, and is located in the left part of the 10A1-2 band. Control elements regulating $\underline{v}^+$ structural gene activity are located outside the presently defined limits of the $\underline{v}$ cistron and are probably present

in the apparently excess DNA located to the right of the y cistron but still within the boundaries of the 10A1-2 band. A direct test of this proposed organization would be to clearly define the visible y mutations to the left of a y deficiency which, on cytological evidence, just removes the right hand part of the 10A1-2 band. The necessary deletions are available and, since they produce a y phenotype when heterozygous with y point mutations mapping in the left part of 10A1-2, might therefore remove the hypothetical control element. To identify point mutations in this, and any other y control element, would require comparing the presence or absence of CRM for TP with sensitive assays of the activity of the enzyme for each of the putative regulatory mutants.

INTRODUCTION

It is now becoming apparent that an understanding of the control of differential gene activity during development in higher organisms will depend upon the resolution of the basic features of genetic organization and regulation in these organisms. It is also clear that the elucidation of these problems in Drosophila will necessitate concerted genetic, developmental and molecular approaches to loci amenable to these analyses. Such a gene in Drosophila melanogaster is the sex-linked vermilion (v) locus which comprises a cistron controlling the activity of the enzyme tryptophan pyrrolase and which, as will be outlined below, has been the subject of considerable genetic, cytological, developmental and biochemical studies.

This investigation was initiated to contribute further information concerning the functioning of this locus by the induction and recovery of conditional (temperature-sensitive, ts) mutant v alleles which might provide additional resolving power for the analysis of the means by which v gene expression is regulated during development. Furthermore, the recovery of a ts mutation in a gene controlling a known protein product could permit a determination of the factors responsible for temperature-sensitivity in terms of changes in the gene product and a molecular understanding of the temperature-sensitive period of the v^{ts} mutant.

The spontaneous, vermilion mutation, v^{1} (standard map position

1-33.0, LINDSLEY AND GRELL 1968) was the fourth mutation recovered in Drosophila (MORGAN 1910) and was so-named because of the bright, scarlet-red eye colour exhibited by homozygous females and hemizygous males carrying the mutation. Since 1910, many spontaneous and induced v mutations have been recovered. Despite functional diversity amongst them, all are completely recessive to wild type and their bright-red eye phenotypes are virtually indistinguishable. Mutation at the v locus results in the absence of the brown pigments (ommochromes) from the eye and ocelli of adults and pale yellow larval Malpighian tubules (ZIEGLER 1961).

At this point it may be worthwhile to review briefly the most pertinent information concerning the v locus to demonstrate its appropriateness as a system for studying control of differential gene activity during development. Investigations of v mutations played a major role in the early development of biochemical and developmental genetics in Drosophila. The gynandromorph studies of STURTEVANT (1920) utilizing v^1, the reciprocal transplantation of v and cn eye discs by BEADLE AND EPHRUSSI (1937), and the chemical identification of "v^+ hormone" as kynurenine by BUTENANDT, WEIDEL AND BECKER (1940), stand as classical experiments pioneering the studies on gene-controlled biochemical processes and the epigenetic control of development.

STURTEVANT (1920) demonstrated that gynandromorphs containing v and v^+ tissue always exhibit v^+ eye colour and BEADLE AND EPHRUSSI (1937) showed that implants of v optic discs into wild

type host larvae result in the discs developing into v^+ eyes. Thus, v was defined as a "nonautonomous" mutant in that the phenotype of the eyes does not depend on their own genotype but rather on that of the surrounding tissue which can produce a substance (called "v^+ hormone" or "v^+ substance") enabling the v eyes to form normal brown pigment. This was one of the first indications that not all genes act in all tissues during development. In the present study, the nonautonomy of a ts v mutation, v^{ts1}, was tested in gynandromorphs at both the permissive and restrictive temperatures and was shown to be developmentally nonautonomous with respect to v^+ but was autonomous in v^1 : v^{ts1} gynanders raised at the permissive temperature.

In reciprocal transplants between cn and v optic discs, it was found that the "lymph" of cn host larvae could supply implanted v optic discs with v^+ substance, whereas the v host could not supply the implanted cn optic disc with the substance necessary to form brown pigment (BEADLE AND EPHRUSSI 1936). It was therefore concluded that v and cn control different steps in the reaction chain leading to brown pigment formation and further, that the block caused by the cn mutation is distal to the one caused by v. Following chemical identification of the v^+ and cn^+ substances as kynurenine and 3 -hydroxykynurenine respectively, the first gene-controlled reaction chain in Drosophila was elucidated (BUTENANDT, WEIDEL AND BECKER 1940; KIKKAWA 1941; TATUM AND HAAGEN-SMIT 1941; BUTENANDT, WEIDEL AND BIEKERT 1949).

Kynurenine is derived from tryptophan in the metabolism of mammals (BUTENENDT et al. 1940; KNOX AND MEHLER 1955), bacteria (POILLON, MAENO, KOIKE AND FEIGELSON 1969) and Neurospora (BEADLE AND MITCHELL 1947) as well as in insect ommochrome synthesis and so is an ancient and universal pathway in which the key enzymes, tryptophan pyrrolase and kynurenine formamidase appear to have similar kinetic and metabolic properties in these diverse organisms (MARZLUF 1965).

GREEN (1949) definitively showed that the blocks in the ommochrome metabolic pathway caused by the v and cn mutations were between tryptophan and formyl kynurenine and kynurenine and 3-hydroxykynurenine respectively, since, (a) adult v mutants accumulate free non-protein tryptophan, whereas in the cn mutant more kynurenine is found than in wild type; and (b) feeding or injection of formyl kynurenine or kynurenine results in the restoration of brown pigment to the eyes of adults, whereas it is necessary to feed cn larvae 3-hydroxykynurenine before brown pigment is deposited in the developing eyes. GLASSMAN (1956) showed that the enzyme kynurenine formamidase, which converts formyl kynurenine to kynurenine, is present in normal quantities in v and cn mutants. Hence v controls the proximal reaction, tryptophan to formyl kynurenine, catalyzed by tryptophan pyrrolase, and cn controls the distal step, kynurenine to 3-hydroxykynurenine, catalyzed by kynurenine hydroxylase. Finally, (BAGLIONI 1959, 1960) succeeded in demonstrating that v mutations specifically block tryptophan pyrrolase activity, whereas cn has elevated tryptophan pyrrolase activity but lacks

kynurenine hydroxylase activity.

The biochemical basis of the phenotypic effects of v mutations was therefore focussed on changes in properties of tryptophan pyrrolase (KAUFMAN 1962; MARZLUF 1965; TARTOF 1969) which led to the conclusion that the v locus constitutes a cistron controlling the formation and activity of this enzyme (BAILLIE AND CHOVNICK 1971; TOBLER, BOWMAN AND SIMMONS 1971). In the present study, extensive assays of tryptophan pyrrolase activity were conducted in fly extracts derived from wild type, v^{ts} and various nonconditional v mutants and rearrangements, to monitor the activity of the locus under different mutational, dosage and environmental conditions.

Concomitant genetic and cytological studies of the v locus by a number of workers have yielded information about the organization and functioning of this gene. Thus, GREEN (1954) showed that the mutations, v^{1} and v^{36f} are resolvable by crossing over. The two mutations were recovered in *cis* arrangement by the use of an attached-X chromosome and therefore could be phenotypically compared with the *trans* form of the mutations. The *cis* configuration ($v^{1}\ v^{36f}$ / + +) produced a wild type phenotype, whereas the *trans* form of this pair (v^{1} + / + v^{36f}), and any other pair of v mutations, is mutant. Therefore, the v mutations comprise a single complementation group and, as defined by the classical *cis-trans* test (LEWIS 1950), constitute a cistron. Complementation studies of the two v^{ts} mutations recovered in this investigation and fine

structure mapping of one of them, confirms the single complementation group and the presence of only three mutable sites, defined by crossing over, at the v locus.

GREEN (1949, 1952, 1954) demonstrated that by a number of criteria, 22 different v mutations could be broadly classified into two categories. Thus, many spontaneous mutations are suppressible by homozygous suppressor of sable (su(s)) mutations and are collectively termed suppressible v alleles (v^s), whereas all induced and some spontaneous v mutations are unsuppressible (v^u). When v^s larvae are placed on a partial starvation diet, a certain amount of brown pigment is restored to the adult eye (BEADLE, TATUM AND CLANCY 1938, 1939), which is accompanied by a proportional increase in tryptophan pyrrolase activity and kynurenine synthesis (GREEN 1954; TOBLER, BOWMAN AND SIMMONS 1968). Partial larval starvation has no effect on the expression of v^u alleles. Since both v^s and v^u accumulate non-protein tryptophan to about the same extent and both produce v^+ eye colour when larvae are fed formyl kynurenine or kynurenine (GREEN 1954), it is probable that v^s alleles allow the synthesis of a potentially active tryptophan pyrrolase but v^u either do not permit enzyme synthesis or result in an enzyme whose activity cannot be restored by changing *in vivo* metabolic conditions (MARZLUF 1965; TARTOF 1969). The EMS-induced v^{ts1} allele has been tested for its suppressibility by a su(s) mutation and, as expected, is not suppressed.

GREEN (1954) and LEFEVRE (1969) used X-chromosome deficiencies to delimit the cytological boundaries of the v locus to the X chromosome salivary band doublet 10A1-2. They also demonstrated that homo- or hemizygous deletion of the v locus is lethal. The v^{ts1} allele was tested with several v deletions and, in all cases, the heterozygous females survived and were clearly vermilion in phenotype at the restrictive temperature for v^{ts1}. Therefore, the v^{ts1} mutation is most probably a point mutation within the v cistron.

The major site of action of the v^{+} gene in the larval body is also known. BEADLE (1937) showed that the larval fat body and Malpighian tubules are the probable sources of kynurenine, since transplanting these specific tissues from v^{+} larvae into v larvae restored brown pigment to the eyes of v adults. In an elegant series of experiments, RIZKI (1963, 1964, 1968) firmly established that the fat body is the primary source of kynurenine in the developing third instar larva and pupa and that kynurenine synthesis is an autonomous property of the fat body cells. In wild type as well as in a number of mutants (for example, cn, ca, w, bw) which affect the formation of red and brown pigments in the eye, light-blue fluorescent globules begin to accumulate in the cytoplasm of the cells of the anterior region of the fat body of the third instar larva prior to puparium formation (RIZKI 1963). The fluorescence of these globules increases in intensity and size as development proceeds through the third larval instar into the

white puparium stage. The notable exception to the presence of blue autofluorescence globules is in v larvae (RIZKI 1964). In $su(s)^2$ $v^s/su(s)^2$ v^s larvae, the characteristic autofluorescence of kynurenine is returned to the cells of the anterior region of the fat body, demonstrating that the absence of a physiological process in a differentiated cell does not necessarily represent a permanent loss of genetic potential for that process (RIZKI 1968). By transplanting various regions of fat bodies from v^+ third instar larvae into v adult hosts, RIZKI showed that the ability of the fat body to synthesize kynurenine is an autonomous property of specific areas of that tissue. Thus, when the anterior region of v^+ larval fat body, of developmental age at which kynurenine first starts to appear, is implanted into a v adult host for 16 hours and then removed, kynurenine autofluorescence is present, whereas neither the posterior region of v^+ fat body, or v^{36f} whole fat body when similarly implanted, develop any fluorescence (RIZKI 1968). Feeding additional tryptophan (substrate) to v^+ larvae prior to the time when kynurenine autofluorescence starts to appear in the anterior regions of the fat body, induces additional fluorescence in the posterior regions. Therefore, kynurenine synthesis in the fat body is at least partly substrate inducible, and is correlated with increased levels of tryptophan pyrrolase activity in v^+ and v^s flies fed, as larvae, on a tryptophan supplemented medium (MARZLUF 1965; RIZKI 1964, 1968; TOBLER, BOWMAN AND SIMMONS 1968).

Thus, the detailed genetic, biochemical and developmental information about the functioning of the v locus makes it a likely candidate for investigations dealing with the control of differential gene activity during Drosophila development.

Temperature-sensitive (ts) mutations have formed a widespread class of conditional mutations in Drosophila which have been useful in the analysis of a variety of genetic, developmental and behavioural problems (SUZUKI 1970). Their usefulness for developmental studies derives from the ability to determine a critical period in development during which exposure of a developing fly, carrying a specific ts mutation, to a restrictive temperature commits the organism to a mutant phenotype (TARASOFF AND SUZUKI 1970; SUZUKI 1970).

Although some information regarding the regulation of gene activity during development has been obtained using ts mutations (GRIGLIATTI AND SUZUKI 1970; FOSTER AND SUZUKI 1970; POODRY, HALL AND SUZUKI 1973), direct approaches have been limited because of the difficulty in identifying the gene products controlled by these mutants. This study was therefore initiated in the hope that recovering a ts mutation in a structural gene for a known and assayable enzyme might provide a biochemical marker relating known phenotypic and developmental fluxes in gene activity with their possible molecular correlations.

Accordingly, the initial part of this investigation is concerned with recovering ts mutations of the v gene. Genetic char-

acterization of one ts v mutation (v^{ts1}) was then undertaken. This involved comparing the complementation properties, intralocus location, suppressibility and autonomy properties of this conditional v mutation with equivalent properties exhibited by some nonconditional v mutations. Subsequently, the temperature-dependent phenotypic expression of v^{ts1} was studied and compared with a detailed analysis of the biochemical properties of tryptophan pyrrolase controlled by v^{ts1} at the permissive and restrictive temperatures. In all cases, comparable studies on wild type and some mutant tryptophan pyrrolases controlled by nonconditional v mutations were performed. Finally, developmental studies were made on the phenotypic expression of v^{ts1} by determining the TSP and correlating this with the known variation in activity of TP during development and the accumulation of kynurenine in the fat body. The results are then interpreted according to a molecular model which seeks to explain the diverse and sometimes seemingly conradictory genetic, biochemical and cytological observations of the functioning of the v locus.

MATERIALS AND METHODS

I. Induction of v mutations.

The potent DNA alkylating agent, ethyl methanesulfonate (EMS) was used to induce v mutations in the following manner. Males of genotype +/Y;bw/bw (a detailed description of the mutations and chromosomes used can be found in LINDSLEY AND GRELL 1968) were collected within 48 hours of eclosion and fed 0.025M EMS in a 1% sucrose solution for 24 hours (LEWIS AND BACHER 1968). About 1 ml of EMS solution was placed on a filter paper in an empty half-pint milk bottle. Approximately 100 males were placed in each bottle.

In each such mutagenesis treatment, about 10 bottles of males were treated at a time. After the 24 hour EMS - treatment, the males were transferred to bottles containing standard Drosophila medium for a 12 hour recovery period. The males were then mated with 2-3 day old virgin females of genotype In(1)dl-49, sc v B^{M1}/ In(1)dl-49,sc v B^{M1};bw/bw at 29°C, each culture bottle containing about 15-20 treated males and 20-30 females. After 3 days, the parents were transferred to fresh bottles for a second 3 day culture at 29°C and then discarded. Cultures were left at 29°C so that all F_1 progeny were grown throughout their lives at 29°C. The mating procedure can be seen in Figure 2. Note that only F_1 females receive a mutagenized X chromosome which might carry a newly-induced mutation. Therefore, all F_1 females were examined for the

FIGURE 2 Protocol for the recovery of temperature-sensitive *vermilion* mutations.

+/Y ; bw/bw ♂♂ (0.025M EMS) X In(1)dl-49, sc v B^{M1}/In(1)dl-49, sc v B^{M1}; bw/bw ♀♀

29°

F_1 +*/In(1)dl-49, sc v B^{M1}; bw/bw ♀♀ X In(1)dl-49, sc v B^{M1}/Y ; bw/bw ♂♂

22° 29°

+*/In(1)dl-49, sc v B^{M1}; bw/bw ♀♀ (brown - eyed)

+*/In(1)dl-49, sc v B^{M1}; bw/bw ♀♀ (white - eyed)

and: +*/Y ; bw/bw ♂♂ (brown - eyed)

+*/Y ; bw/bw ♂♂ (white - eyed)

Retest at 22° and 29°C several times.

presence of white eyes, since flies homozygous for v and bw have no eye pigment.

Most F_1 females were +*/In(1)dl-49,sc v B^{M1};bw/bw (where +* indicates a mutagenized X chromosome) and therefore exhibited only the brown eye-colour phenotype. A newly-induced v mutation results in homozygosity for v thereby yielding the interaction with bw to give a white eye. Each newly-induced putative v mutation was then tested for the heritability of the v phenotype and the effect of temperature on its expression. Each white-eyed F_1 female was mated individually to 3-5 In(1)dl-49,sc v B^{M1}/Y;bw/bw males in vials at 22°C. After 3 days, the parents were transferred to fresh vials at 29°C. Note that the females retained were not necessarily virgin. However, their male sibs were the desired genotype.

The F_2 progeny could be separated into different genotypic groups. Males and females, hemi- and homozygous for the dl-49 chromosome could be recognized by the sc and B^{M1} phenotypes. The dl-49 inversion prevents any crossing over in the v region with the homologs so that an induced v allele cannot be exchanged by crossing over with v^1 in the dl-49 chromosome.

The recovery of +*/Y;bw/bw males and +*/In(1)dl-49,sc v B^{M1}; bw/bw females as white-eyed flies at 29°C confirms the induction of a stable v mutation. The recovery of brown-eyed +*/Y;bw/bw males and +*/In(1)dl-49,sc v B^{M1};bw/bw females from the 22°C culture of the same F_1 female suggests a ts expression of the new v allele. Any v mutation with a non-white eye colour at 22°C was

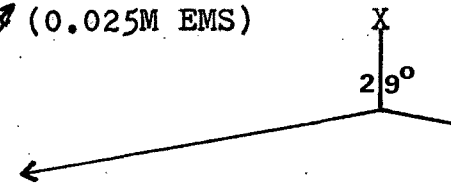
retained and tested again several times at 22°C and 29°C to recover confirmed $\underline{v^{ts}}$ mutations.

A second screening method (Figure 3) for obtaining $\underline{v^{ts}}$ mutations was initiated when deficiencies for the v locus were made available (LEFEVRE 1969). This method had some advantages over the first screen: (a) the number of flies handled at the F_1 stage was reduced by a quarter as males hemizygous for the deficiencies do not survive. This is not an inconsiderable advantage when thousands of flies are to be scored as F_1s. Since only F_1 females carry a mutagenized X chromosome, the frequency of recovery of potential v mutations is not affected, (b) the possibility that some $\underline{v^{ts}}$ alleles might complement (i.e., express a wild type phenotype) with $\underline{v^1}$ was obviated by removal of the v locus. Thus, a comparison between the mutations recovered by the two screening systems could be of interest.

The chromosome used in this second screen was $\underline{Df(1)v^{L3}}$, which is missing salivary gland chromosome bands 9F5 through 10A5 (LEFEVRE 1969). The crosses made to recover putative v and confirmed $\underline{v^{ts}}$ mutations are seen in Figure 3. All F_1 females recovered at 29°C were examined for eye colour. Again, a putative v mutation is detected by the occurrence of white-eyed females. These white-eyed females were retested by crossing them individually to 3-5 $\underline{In(1)dl\text{-}49,sc\ v\ B^{M1}}$/Y;$\underline{bw/bw}$ males in vials at 22°C. Any v mutation with a non-white eye colour at 29°C was retained and retested several times to ensure that the inherited temperature-sensitivity was stable.

FIGURE 3 Protocol for the recovery of temperature-sensitive *vermilion* alleles using a deficiency for the locus.

+/Y ; bw/bw ♂ ♂ (0.025M EMS)



F_1 females: +*/Dfv^{L3}; bw/bw

(white - eyed)

Cross to In(1)dl-49, sc v B^{M1}/Y ;

22° and 29°C.

$\underline{Dfv}^{L3}/\underline{In(1)dl\text{-}49}$, $\underline{sc}$ $\underline{v}$ $\underline{B}^{M1}$; $\underline{bw}/\underline{bw}$ ♀♀

$+^{*}/\underline{In(1)dl\text{-}49}$, $\underline{sc}$ $\underline{v}$ $\underline{B}^{M1}$; $\underline{bw}/\underline{bw}$

$\underline{bw}/\underline{bw}$ ♂♂ and retest several times at

II. Procedures for temperature-shift experiments.

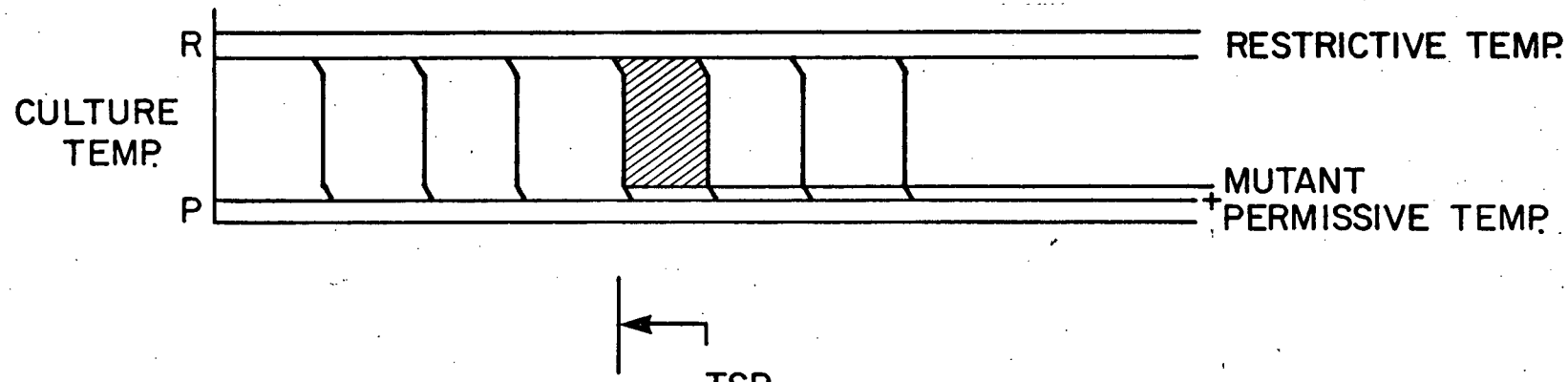
An important feature of temperature-sensitive mutants is that the critical time during development when temperature induces the mutant phenotype can be determined by shifting cultures from one temperature to another at different successive developmental intervals. This critical period in development during which a restrictive temperature commits the organism to a mutant phenotype has been defined the temperature-sensitive period or TSP (SUZUKI 1970).

For any particular gene, the TSP may be thought of as that period when the gene's active biological product is necessary for normal development of the organism. A ts allele of v permits a correlation to be made between the TSP and the activity of tryptophan pyrrolase which is known to be affected by v alleles. The important question of what the TSP means in molecular terms therefore may be asked using this particular mutation.

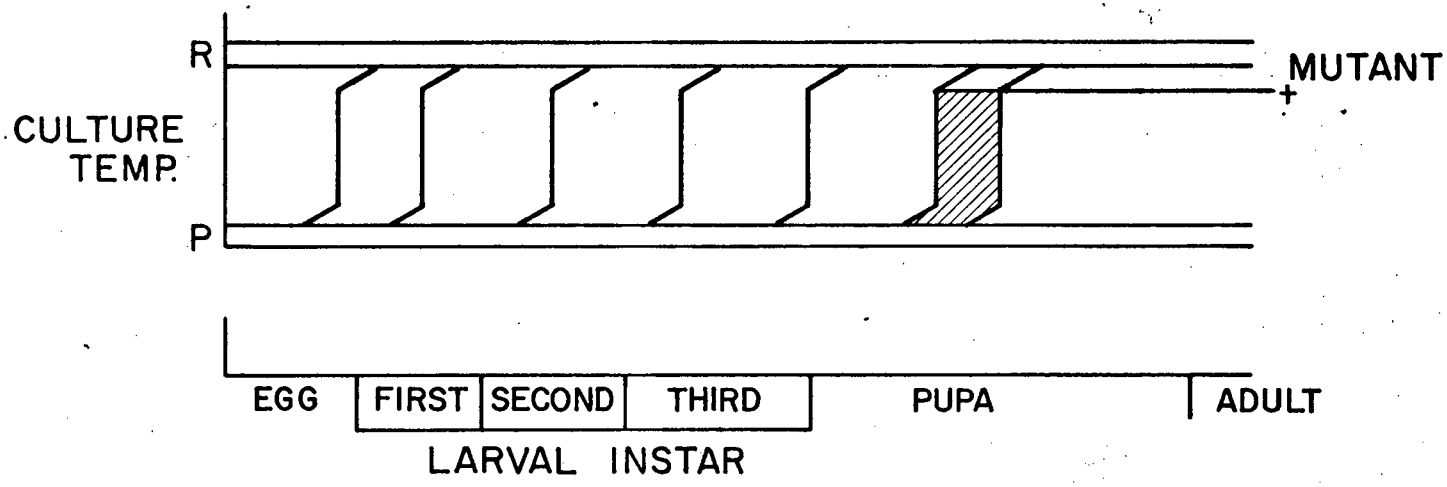
The v^{ts} alleles recovered express a mutant phenotype at 29°C (restrictive condition) and are wild type at 22°C (permissive). Shifts made from 22°C to 29°C and vice versa are defined as "shift-ups" and "shift-downs:, respectively. An illustrative example of a shift experiment can be seen in Figure 4. It can be seen that the first shift-down to yield mutant flies defines the beginning of the TSP and the first shift-up to give wild type flies marks the end.

FIGURE 4 Shift experiments to delineate the temperature-sensitive period (TSP) of a temperature-sensitive mutation.

(a) SHIFT-DOWN



(b) SHIFT-UP



DEVELOPMENTAL STAGE AT TIME OF SHIFT

For the shift studies, a homozygous $\underline{ras^{2}\ v^{ts1}}$ stock was used because the eye-colour phenotype resulting from the $\underline{ras^{2}\text{-}v^{ts1}}$ interaction at 29°C (orange in males and dilute raspberry in females) is independent of age, whereas the eye-colour of $\underline{v^{ts1}}$ flies tends to darken with age.

Sufficient numbers of eggs for experiments involving temperature shifts were obtained from bottle stocks of $\underline{ras^{2}\text{-}v^{ts1}}$. About 100-200 aged parents were placed in each empty half-pint milk bottle which was kept on its side. The bottles were capped with petri plates containing fresh, standard Drosophila medium. Eggs were collected at 22°C and 29°C. Maximal egg recovery was obtained under the following conditions:

(i) The surface of the medium on which the eggs were deposited was held vertically;

(ii) The surface of the medium was scoured with a needle as Drosophila females prefer irregular surfaces for oviposition;

(iii) A few drops of vinegar added to the surface of the medium of each petri plate stimulated egg-laying;

(iv) The cultures were maintained in darkness.

For most shift experiments, eggs were collected within a two-hour period so that cultures were to some degree homogeneous in development. Cultures were shifted at successive intervals after egg collection, and the developmental stage of the individuals at the time of each shift was noted. Adult males and females

emerging from these cultures were scored for eye colour.

The intervals between the initial shifts were about 12 hours. Once an approximate TSP had been defined, the intervals between shifts at developmental times approaching the start and finish of the TSP were reduced to delineate the onset and end of the TSP more precisely. In all cases, the exact time elapsed from the end of the two-hour egg collection period to the shift-time was noted.

The larval instars present in the cultures at the time of each shift were identified according to the morphology of their mouth parts. The number of teeth present on the mandibular hooks is different for each of the 3 larval instars and readily allows recognition of each stage (BODENSTEIN 1950).

Considerable asynchrony in the developmental times of each stage of Drosophila development was noted in the cultures shifted. Consequently the determination of the start and finish of the TSP was not precise. To ameliorate this problem as far as possible, the following procedures were adopted:

(i) From each culture, individual larvae to be shifted were selected on the basis of the synchrony of their developmental stage. Thus, recently hatched (within 5 hours) first instar larvae were selected from individual cultures initiated from the same two-hour egg lay and transferred to fresh vials and placed at the new temperature. For shifts involving second or third instar larvae, larvae reaching the same

approximate stage of development were selected according to size and transferred to fresh vials at the new temperature.

(ii) In performing rough determinations of the TSP for the $\underline{v^{ts1}}$ mutant using 12-hour shifts, the third instar period was the critical time in development when change of temperature induced change in mutant expression in the adults. Therefore, third instar larvae were partially synchronized by selecting recently-moulted (within 5 hours) individuals on the basis of eversion of the anterior spiracles, an event characteristic of recently-moulted thirds (BODENSTEIN 1950). However, none of these procedures produced particularly well-synchronized cultures as shown by the variation in eclosion times of adults emerging from the same selected cultures.

III. Tryptophan pyrrolase assays

Various strains of *Drosophila melanogaster* to be assayed for enzyme activity were raised in half-pint milk bottles containing standard medium at 17± 0.5°C, 22± 1°C and 29± 0.5°C. Approximately 30 pairs of adult flies were allowed to mate and deposit eggs in each bottle for 3-5 days, then removed. Each enzyme assay required 20-40 bottles of flies.

KAUFMAN (1962) has shown that levels of tryptophan pyrrolase activity in wild type flies vary significantly with adult age with maximum activity occurring about two days after eclosion. The effect of age was minimized in all tests by assaying flies collected within 24±24 hours of eclosion. The flies were collected in 1-10 g quantities (1 fly weighs about 1 mg) by lightly etherizing, separating males from females when necessary, and quickly freezing in a dry ice: 95% ethyl alcohol mixture maintained at -20°C. After being weighed, the flies were homogenized for 3 minutes at top speed in an Omnimix homogenizer with 5 volumes (weight/volume) of a homogenizing medium containing 0.1M potassium phosphate buffer at pH 7.4, 5% glycerol, 20 mg/ml neutral Norit and 0.3 mM 2-mercaptoethanol. This step was carried out in an ice-bucket at 0-4°C to minimize denaturation of the enzyme by the heat developed in the blending.

After standing for 1-2 hours at 0-4°C, the homogenate was centrifuged at 48,000 x g for 30 minutes in a refrigerated Sorvall centrifuge. The resulting clear, straw-coloured supernatant was

decanted and filtered through a Whatman No. 1 filter paper to remove the lipid layer and cell debris. The filtered supernatant, containing a crude preparation of tryptophan pyrrolase, was either stored at -20°C (where it remained stable for 1-2 months), used immediately as a crude source of the enzyme, or subjected to further purification.

As the activity of tryptophan pyrrolase in the crude supernatant is usually quite low, procedures which concentrate this activity have been employed by other workers (KAUFMAN 1962; MARZLUF 1965; TARTOF 1969; BAILLIE AND CHOVNICK 1971). These procedures, with modifications, were repeated in the present study. The crude supernatant was recentrifuged for an additional 30-60 minutes at 48,000 x g and refiltered through a Whatman No. 1 filter paper. Cold, saturated ammonium sulfate was then added dropwise with stirring to the crude enzyme preparation until it was brought to 42% saturation. The mixture was maintained at pH 7.4 by the addition of 2N ammonium hydroxide. After standing for 30-60 minutes at 0-4°C, the solution was centrifuged at 30,000 x g for 10 minutes. The pellet, which contains no detectable tryptophan pyrrolase activity (BAILLIE AND CHOVNICK 1971), was discarded. The supernatant was then brought to 59% ammonium sulfate saturation by further dropwise additions. The precipitate, representing the fraction between 43 and 59% saturation, was recovered by centrifugation at 30,000 x g for 30 minutes. The pellet was rinsed lightly with distilled water and redissolved in 1/10

the original homogenizing volume of 0.1 M potassium phosphate buffer at pH 7.4 containing 0.3 mM 2-mercaptoethanol and 5% glycerol. This fraction was either used immediately or stored at -20°C where it retained 70-90% activity for several months.

Since tryptophan pyrrolase (TP) activity differed between some strains and between sexes within strains, TP activity was estimated relative to the total protein content of the flies. Additionally, protein determination is necessary to measure the recovery and purification of TP resulting from precedures adopted for extraction of the enzyme.

Fractions from various strains were assayed for their protein contents by the LOWRY method (LOWRY, ROSEBROUGH, FARR AND RANDALL 1951). In this method, the reagent labelled C consists of 50 parts of reagent A (20 g Na_2CO_3;4 g NaOH;0.2 g Na tartrate/litre) plus one part of reagent B (5 g $CuSO_4$-5 H_2O/litre) and is mixed on the day of use. Five parts of FOLIN-CIOCALTEAU reagent were diluted in 7 parts of water. This method of protein estimation depends upon the specific reaction, under alkaline conditions, between the FOLIN-CIOCALTEAU reagent and the tyrosine and tryptophan moieties in the proteins.

A standard protein curve was obtained using 25 to 250 µg of crystalline bovine serum albumin. The Drosophila fractions whose protein contents were estimated were diluted until their protein contents fell within the range of the standards employed.

TP assays described by MARZLUF (1965), TARTOF (1969) and

BAILLIE AND CHOVNICK (1971) were tried. Slight modifications of BAILLIE AND CHOVNICK'S (1971) assay yielded consistently higher and reproducible TP activities in all strains tested.

Owing to the low levels of TP activity in the fly extracts (compared to rat liver [SCHIMKE, SWEENEY AND BERLIN 1965] and Pseudomonas preparations [POILLON, MAENO, KOIKE AND FEIGELSON 1969]) it is necessary to assay for the enzyme under conditions which maximize the recovered activity and reproducibility of the results. Consequently, an effort was made to determine precisely the optimal assay conditions for TP extracted from wild type flies and from various v mutants and strain combinations. Unless otherwise stated in the Results, the assays were performed under the predetermined optimal conditions. To minimize the inherent variability in results of enzyme determinations, more than one test was usually performed for any given experiment using separate enzyme preparations and reaction systems.

The reaction mixture for TP assays contained the following ingredients:

(i) Substrate. Unless otherwise indicated, a final concentration of l-tryptophan of 5 mM was used routinely. Aqueous solutions of l-tryptophan require the addition of a few drops of concentrated (6N)NaOH before dissolution is achieved. The pH is returned to about 7.4 by the dropwise addition of dilute (1 or 2N) HCl. Stock solutions of either 20 or 25 M l-tryptophan were routinely prepared and renewed weekly for use in the reaction

mixture (KNOX 1955).

(ii) Buffer. A final reaction mixture concentration of 40 mM potassium phosphate buffer at pH 7.4 was used to maintain the pH of the reaction at 7.4, the optimal pH for TP catalytic activity. A stock solution of 200 mM potassium phosphate buffer at pH 7.4 was renewed monthly for this purpose.

(iii) Cofactors. (a) 2-mercaptoethanol is a potent activator of TP (MARZLUF 1965; BAILLIE AND CHOVNICK 1971) and a final reaction mixture concentration of 2 mM provides maximum activation of v^+ TP. Activation with 2 mM 2-mercaptoethanol results in about a five-fold increase in activity of v^+ TP. A solution of 10 mM 2-mercaptoethanol was prepared on the day of use for maximum effect.

(b) Met-hemoglobin. Some uncertainty surrounds the role of met-hemoglobin or hematin as cofactors for Drosophila TP activity. TP from other sources (notably rat liver and Pseudomonas) possess a heme prosthetic group and it is assumed (MARZLUF 1965; BAILLIE AND CHOVNICK 1971) that Drosophila TP also has such a group. Thus, the addition of a compound containing a heme group could activate a prosthetic group-dependent enzyme which may have lost the heme during the extraction procedures.

Conflicting results have been reported by MARZLUF (1965) using hematin and BAILLIE AND CHOVNICK (1971) using met-hemoglobin respectively as the source of the heme group. MARZLUF reported negligible increase in activity with the addition of hematin to

the reaction mixture. On the other hand, BAILLIE AND CHOVNICK report that met-hemoglobin at a final reaction mixture concentration of 0.5 mg/ml enhances the activity of $\underline{v}^+$ TP twofold in a crude preparation and fourfold after ammonium sulfate precipitation. In the present study, met-hemoglobin did appear to stimulate TP activity in both crude and ammonium sulfate fractionated extracts but not to the extent reported by BAILLIE AND CHOVNICK. Therefore, met-hemoglobin was routinely used as a cofactor for the enzyme reaction at a final reaction mixture concentration of 0.5 mg/ml.

(iv) In all assays, a sufficient amount of enzyme was added to the reaction mixture to ensure that the kynurenine released could be estimated by the TARTOF adaptation (1969) of the BRATTON AND MARSHALL (1939) diazotization procedure for aromatic amine determination.

Low levels of kynurenine are released in the assay mixture. Thus, long periods of incubation are necessary for the liberation of sufficient kynurenine to be conveniently and reproducibly determined by the diazotization procedure. Normally a period of at least 2 hours was allowed for incubation of the reaction. A reaction temperature of 41°C was found to be optimal for maximal release of kynurenine over a two hour period without significant effect on $\underline{v}^+$ TP stability. Specific tests of the catalytic properties of TP in wild type and $\underline{v}$ mutants were conducted at different incubation temperatures as will be indicated in the Results.

The routine reaction mixture contained 40 mM potassium

phosphate buffer pH 7.4, 5-7 mM l-tryptophan, 2 mM 2-mercaptoethanol, 0.5 mg/ml methemoglobin, 0.1 to 0.8 mls of enzyme extract and distilled water to a total assay volume of 2.0 mls. The tubes were preincubated for 15 minutes at the assay temperature before the reaction was started by the addition of enzyme. All tubes were set up in duplicate and the reaction in one of the tubes was stopped immediately after addition of enzyme by the addition of 2.0 mls of 5% TCA to provide a control measure of background kynurenine formation. Another occasionally used control was the omission of tryptophan from the reaction vessel during incubation and its addition after the enzyme was precipitated with 5% TCA. Both types of controls gave approximately the same blank values for non-enzymatic formation of kynurenine which were then subtracted from the appropriate experimental tube values.

After a designated period of incubation, the reaction was stopped in the experimental tubes by the addition of 2.0 mls of 5% TCA and all tubes were then heated to 90°C for 10 minutes to ensure that the conversion of formyl kynurenine to kynurenine was complete. All tubes were then assayed for kynurenine content according to the following precedure.

The technique for the detection and quantitative estimation of kynurenine, produced by the combined actions of TP and kynurenine formamidase (GLASSMAN 1956), was originally designed by BRATTON AND MARSHALL (1939) for the estimation of aromatic amines. After centrifugation of the TCA-stopped reaction tubes at 2000 RPM for

5 minutes, the supernatants were filtered through a Whatman No. 5 filter paper and 0.8 ml of 3-3.2 mls of supernatant were assayed for kynurenine content. A 0.2 ml aliquot of 0.1% sodium nitrite was added to 0.8 ml of the supernatant. The mixture was allowed to stand for 2 minutes, then 0.2 ml of 0.5% ammonium sulfamate was added and the mix allowed to stand for another 3 minutes after which 0.2 ml of 0.1% N-1-napthylethylenediamine dihydrochloride was added. The samples (now 1.4 ml in volume) were left in the dark for 2 hours at 25°C and the absorbancies were then read in a Gilford spectrophotometer at 560 mμ against a reagent blank. Kynurenine content of the samples was found by comparing the absorbancies with similarly treated standards of l-kynurenine.

The BRATTON AND MARSHALL diazotization procedure is a sensitive assay and for good reproducibility, the following attention to detail is required: (a) sodium nitrite solution should be prepared on the day of the assay: (b) the dye, N-1-napthylethylenediamine dihydrochloride (obtainable from the Eastman Kodak Company) was found to yield the most reliably reproducible results if prepared fresh every 30 days and stored in a brown stoppered bottle at about 2°C.

For most enzyme assays, the activity of TP was measured as μM kynurenine formed per 2 hours of incubation per g wt. of flies. In the cases where protein content of the fraction was measured, specific activity of TP was measured as μM kynurenine per 2 hours per mg of protein. Other units used are defined where appropriate.

Wild type TP catalyzes the transformation of l-tryptophan to formyl kynurenine. The kinetics of this reaction were examined with respect to the amount of kynurenine released with increasing periods of incubation time. The crude preparation of the enzyme was used and in each case 0.4 ml of enzyme extract was added to the optimal reaction mixture to start the reaction. All tubes were set up in duplicate with the reaction in one tube immediately stopped with 2.0 ml of 5% TCA and the other tube of the pair stopped after the designated incubation time which ranged from 30 to 300 minutes.

The optimal substrate concentration and the Km for the wild type enzyme were measured by determining the rate of kynurenine released with increasing l-tryptophan concentrations under standard conditions.

The optimal temperature for the release of product catalyzed by wild type TP was assessed by subjecting the reaction mixture to a given temperature of incubation over a 2 hour period and assaying the resulting rate of formation of kynurenine in the standard manner.

For each of these kinetic criteria, TP derived from the $\underline{v^{ts1}}$ mutant strain grown at 22° and 29°C was similarly prepared, assayed and compared with wild type enzyme tested under the same conditions. In the case of determining the optimal temperature for the release of product by TP derived from $\underline{v^{ts1}}$ grown at 29°C, a 40-60% ammonium sulphate fraction was assayed.

RESULTS

A. INDUCTION OF v AND v^{ts} MUTATIONS

The numbers of v mutations recovered by the two screening procedures are shown in Table 2. Among F_1 females classed as "white-eyed" were several whose eyes were intermediate in colour between white and brown (yellow-brown) and they also exhibited intermediate colouration of the ocelli. On retesting, these females regularly gave a darker eye colour in both males and females in 22°C cultures. The females were B^{M1};bw in phenotype so their genotype was +*/In(1)dl-49,sc v B^{M1};bw/bw and the males were $sc^{+}B^{M1+}$;bw, indicating a +*/Y;bw/bw genotype. These were included in the number which failed to reconfirm as v or v^{ts} mutations and were discarded.

Of the 33 mutations which behaved as v at 29°C, only two were genuine temperature-sensitives. That is, females heterozygous for the v^{ts} allele and v^{1} or a deficiency, interacted with the homozygous brown condition to yield white eyes at 29°C and brown eyes at 22°C. The two new v^{ts} alleles were distinguishable from each other in their properties. Of the two mutants, v^{ts2} which was recovered in screen 2, appeared to have more brown pigment at 29°C than v^{ts1} in v^{ts}/In(1)dl-49,sc v B^{M1};bw/bw flies.

Studies on the interaction of other vermilion mutants with brown, show that the amount of brown pigment in the white eye is

TABLE 2 Results of screening for *v* and v^{ts} mutations by two different methods (see text for details of experiments 1 and 2).

Experiment	Number of mutagenized males	Number of Progeny: Putative *v* mutations at 29°C	Sterile or not confirmed *v* at 29°C	Non-ts *v* mutations	v^{ts} mutations
1	10,000	75	48	26	1
2	3,000	21	15	5	1
Totals	13,000	96	63	31	2

directly correlated with detectable levels of tryptophan pyrrolase activity. The presence of detectable brown pigment and associated TP activity is confined to those v mutants which are suppressible by mutations at the su(s) locus (GREEN 1952; SHAPARD 1960; TARTOF 1969).

Males carrying v^{ts2} were viable and fertile so that homozygous v^{ts2} females were generated, whereas males bearing v^{ts1} did not survive at 22° or 29°C. Since LEFEVRE (1969) has found that only deletions spanning the v locus appear to be lethal, lethality of the v^{ts1} chromosome could be an effect of v^{ts1} itself or to a lethal mutation induced elsewhere on the chromosome. The latter possibility was tested by determining the viability of v^{ts1} bearing chromosomes having crossovers on either side of the v^{ts1} locus.

The crosses can be seen in Figure 5. Since v^{ts1} m / Y males were recovered at 29°C (Line 3), a lethal was present to the right of v^{ts1}. Crossovers removing the m marker were then recovered (Line 5) and the v^{ts1} chromosome homozygosed as seen in the Figure. The autosomal marker bw was eliminated in both v^{ts1} and v^{ts2} stocks by the use of the sutosomal balancer SM5.

The phenotype of v^{ts1}- and v^{ts2}- bearing flies can be seen in Table 3. It can be seen that v^{ts1} is a more extreme mutant than v^{ts2} at 29°C. Furthermore, in contrast to v^{ts2}, v^{ts1} males are distinctively more mutant at 29°C than v^{ts1} females. This sexually dimorphic expression of v^{ts1} at 29°C was maintained through subsequent selection procedures and outcrossings which would eliminate sex-linked or autosomal modifiers of v^{ts1} mutant expression in males or females.

FIGURE 5 Removal of lethal from the $\underline{v^{ts1}}$ chromosome and homozygosis of $\underline{v^{ts1}}$.

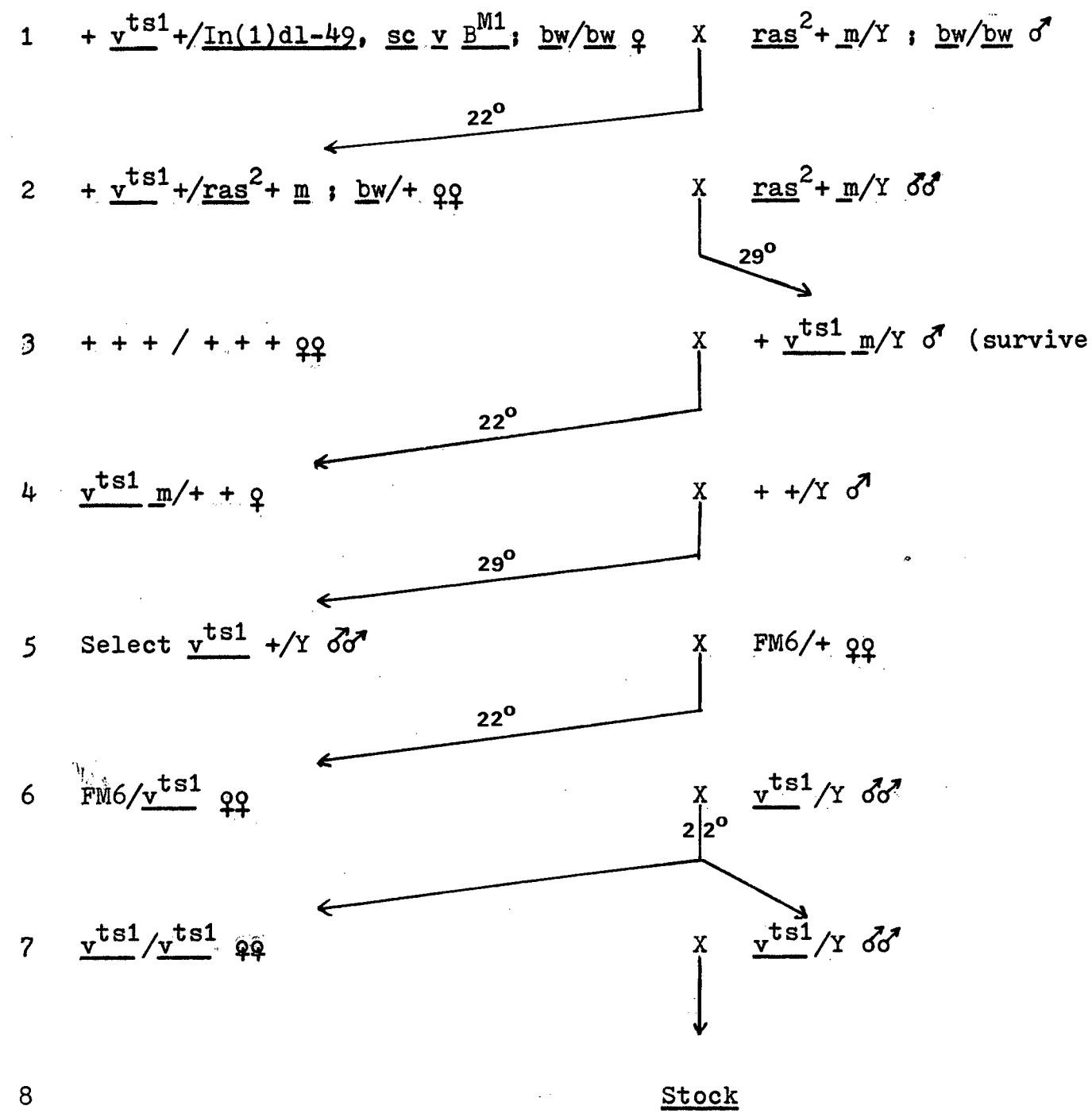

Line
1 + v^ts1 +/In(1)dl-49, sc v B^M1; bw/bw ♀ X ras^2 + m/Y ; bw/bw ♂
22°
2 + v^ts1 +/ras^2 + m ; bw/+ ♀♀ X ras^2 + m/Y ♂♂
29°
3 + + + / + + + ♀♀ X + v^ts1 m/Y ♂ (survive)
22°
4 v^ts1 m/+ + ♀ X + +/Y ♂
29°
5 Select v^ts1 +/Y ♂♂ X FM6/+ ♀♀
22°
6 FM6/v^ts1 ♀♀ X v^ts1/Y ♂♂
22°
7 v^ts1/v^ts1 ♀♀ X v^ts1/Y ♂♂
8 Stock

TABLE 3 Eye phenotypes of males and females carrying v alleles at 22° and 29°C.

	22°C		29°C	
ALLELE	MALES	FEMALES	MALES	FEMALES
v^1	v	v	v	v
v^{ts1}	+	+	v	v →+*
v^{ts2}	+	+	+ →v**	+ →v

* Represents an intermediate phenotype in which the eye colour is closer to vermilion than wild type.

** Represents an eye phenotype intermediate between wild type and vermilion.

Selection of $\underline{v^{ts1}}$ males and females showing the most mutant expression at 29°C and most wild type expression at 22°C was carried out by rearing stocks of $\underline{v^{ts1}}$/Y males crossed to $\underline{C(1)DX,v^{0f}}$ $\underline{f}$ /Y females, and $\underline{v^{ts1}}/\underline{v^{ts1}}$ females crossed to $\underline{v^{ts1}}$/Y males at 22° and 29°C. In the first generation, males from the former and virgin females from the latter stock, which showed the most mutant expression of the $\underline{v^{ts1}}$ allele at 29°C, were selected and individually crossed to their respective sibs at both temperatures. From the cross of $\underline{v^{ts1}}$/Y males to $\underline{C(1)DX,v^{0f}}$ $\underline{f}$ /Y virgin females, males showing the most wild-type expression of the $\underline{v^{ts1}}$ allele at 22°C, whose male sibs showed the most vermilion expression at 29°C, were selected and crossed to $\underline{C(1)DX,v^{0f}}$ $\underline{f}$ /Y virgin females at 22° and 29°C. Similarly, $\underline{v^{ts1}}/\underline{v^{ts1}}$ virgin females from the second series of crosses exhibiting the most wild type phenotype at 22°C whose female sibs showed the most mutant phenotype at 29°C, were selected and crossed to their $\underline{v^{ts1}}$/Y sibs at 22° and 29°C. These procedures were repeated through five generations and the separate male and female $\underline{v^{ts1}}$ lines were then crossed to produce the stock line used in subsequent genetic and biochemical studies. No significant phenotypic changes were detectable during or after this selection procedure indicating that $\underline{v^{ts1}}$ is a highly stable mutation.

Replacement of second and third and reconstitution of the X chromosomes in the $\underline{v^{ts1}}$ stock line were periodically carried out by outcrossing to second and third chromosome balancers and by allowing

free crossing over of the $\underline{v^{ts1}}$-bearing chromosome with wild type X chromosomes. Again, no significant differences in the temperature-sensitive expression of $\underline{v^{ts1}}$ in males or females was observed.

Fertility and viability of the $\underline{v^{ts1}}$ and $\underline{v^{ts2}}$ stocks were excellent at both 17° and 22°C and not different from most other stocks at 29°C.

Complementation tests between $\underline{v^{ts1}}$ and $\underline{v^{ts2}}$ were made by generating heterozygous $\underline{v^{ts1}}/\underline{v^{ts2}}$ females in reciprocal crosses raised at 22° and 29°C, and examining for eye pigmentation. The eye colour of these females was nearly wild type at 22°C and was intermediate between wild type and vermilion at 29°C. The phenotypes of the females from both reciprocal crosses were identical. Thus, while $\underline{v^{ts1}}$ and $\underline{v^{ts2}}$ have distinctive properties, they fail to complement. This further corroborates the single complementation group of all $\underline{v}$ mutations (GREEN 1949, 1952; BARISH AND FOX 1956; SHAPARD 1960; TARTOF 1969; LEFEVRE 1969, 1971).

From this point on, the $\underline{v^{ts1}}$ allele was studied exclusively since it is more mutant in phenotype at 29°C and exhibits the sexually dimorphic phenotype.

B. PHENOGENETICS OF THE v^{ts1} MUTATION

Extensive tests of the phenotypic interactions of different v alleles inter se and with a variety of deficiencies at or near the v locus have been made (BRIDGES 1919; GREEN 1949, 1952, 1954; BARISH AND FOX 1956; LEFEVRE 1969, 1971). These observations were extended by similarly testing the phenotypic interactions of v^{ts1} at different temperatures.

The extent of "vermilioness" resulting from these interactions was based on a semi-quantitative scale from 0.0 (colour of v^{1}) to 5.0 (wild type). Cultures of v^{ts1} were raised at 17°, 22°, 29° and 31°C and the resulting eye pigmentation was estimated visually. Estimates of pigmentation based on visual inspection of eye colour and on quantitation by spectrofluorometry correlate very closely (BAKER AND SPOFFORD 1959). Wild type and other vermilion alleles were also tested at these temperatures as controls. The results are given in Table 4.

Several points arising from the results in Table 4 warrant attention:

(i) In v^{ts1} females and males both, there is an incremental increase in the mutant vermilion phenotype of the flies at progressively higher temperatures.

(ii) At 17° and 22°C, v^{ts1} males and females are phenotypically similar. However, at the higher temperatures, males are distinctly more mutant than females. At 31°C, v^{ts1} males are indis-

TABLE 4 Visually estimated eye pigmentation of different *v* alleles at several temperatures.

GENOTYPE	SEX	PHENOTYPE (VERMILION INDEX) 17°C	22°C	29°C	31°C
v^+/v^+	Females	5.0*	5.0	5.0	5.0
v^+/Y	Males	5.0	5.0	5.0	5.0
v^{ts1}/v^{ts1}	Females	5.0	4.5	2.0	1.5
v^{ts1}/Y	Males	5.0	4.5	1.0	0.5
v^+/v^{ts1}	Females	5.0	5.0	5.0	5.0
v^1/v^1	Females	0.0	0.0	0.0	0.0
v^1/Y	Males	0.0	0.0	0.0	0.0
v^{36f}/v^{36f}	Females	0.0	0.0	0.0	0.0
v^{36f}/Y	Males	0.0	0.0	0.0	0.0
$C(1)DX, v^{0f}f/Y$	Females	0.0	0.0	0.0	0.0

* In the scale, 0.0 = completely vermilion, 5.0 = completely wild type.

tinguishable from $\underline{v^1}/Y$ and $\underline{v^{36f}}/Y$ males raised at this temperature, whereas $\underline{v^{ts1}}$ females are still less mutant than $\underline{v^1}/\underline{v^1}$ and $\underline{v^{36f}}/\underline{v^{36f}}$ females at 31°C.

(iii) Three different v alleles ($\underline{v^1}$, $\underline{v^{36f}}$ and $\underline{v^{0f}}$) which differ in their suppressibility, map position within the v locus and mode of origin, have identical phenotypes at all temperatures. Thus, temperature-sensitivity is not a general property of v alleles and occurs in a low proportion of EMS-induced v mutations.

(iv) The eye colour of Oregon-R males and females is not ts.

(v) $\underline{v^{ts1}}$ is completely recessive to wild type at all temperatures.

I. Complementation properties of selected $\underline{v^{ts1}}$ combinations.

No complementation between v alleles has ever been noted (GREEN 1952; BARISH AND FOX 1956; SHAPARD 1960; TARTOF 1969; LEFEVRE 1971), even though some v alleles can be readily distinguished on other grounds, such as suppressibility by the non-allelic su(s) mutations, map position within the locus, phenotypic response to tryptophan feeding of larvae and their spontaneous or induced origin, all of which will be discussed in detail in other sections. However, temperature-sensitivity of a v allele could show a different pattern of interaction. Consequently, $\underline{v^{ts1}}$ males were crossed to females carrying different v alleles

in order to generate females heterozygous for $\underline{v^{ts1}}$ and another $\underline{v}$ allele. These females were raised at 17°, 22°, 29° (and in some cases, 31°C) and scored for eye colour. Additionally, heterozygotes for other $\underline{v}$ alleles were tested for their eye colour phenotypes at 22° and 29°C. Table 5 provides a summary of the results of these complementation tests.

It can be seen that the eye colour of females heterozygous for $\underline{v^{ts1}}$ and any other $\underline{v}$ allele, is temperature-dependent. Interestingly, even though $\underline{v^{ts1}}/\underline{v^{ts1}}$ females have wild type eyes at 17°C, when the allele is present with a non-ts allele, the eye colour is intermediate. This could be interpreted as a partial dominance of non-ts $\underline{v}$ alleles over $\underline{v^{ts1}}$, and is a demonstration of the altered activity of $\underline{v^{ts1}}$ even at permissive temperatures. As expected, at 29° and 31°C, all eyes are completely $\underline{v}$ in phenotype.

These data extend the observations of the lack of complementation between all $\underline{v}$ alleles tested. The present 17°C data could be explained, if, in a female, each $\underline{v}$ allele functions independently and produces a product which does not interact, at least at the phenotypic level, with the product formed by the other allele. Alternatively, if the polypeptide products of the two alleles do interact, the hybrid polymer produced should be mutant to account for the reduced wild type pigmentation in the 17°C-reared heterozygotes of $\underline{v^{ts1}}$ and non-ts $\underline{v}$ alleles.

Three deficiencies within the X chromosome, $\underline{Df\ v^{L1}}$, $\underline{Df\ v^{L2}}$

TABLE 5 Phenotypes of females heterozygous for v^{ts1} and other v alleles at different temperatures.

GENOTYPE	PHENOTYPE (VERMILION INDEX) 17°C	22°C	29°C	31°C	PROPERTIES OF THE NON-ts v ALLELE SUPPRESSIBILITY	ORIGIN	MAP POSITION
v^{ts1}/v^{1}	*2.5	1.5	0.0	0.0	Suppressible (GREEN, 1952)	Spontaneous (MORGAN, 1910)	Left of v^{36f} (GREEN, 1954
v^{ts1}/v^{36f}	2.5	1.5	0.0	0.0	Unsuppressible (GREEN, 1952) Suppressible (TARTOF, 1969)	Spontaneous (WILLIAMS, 1936)	Right of v^{1} (GREEN, 1954
v^{ts1}/v^{sp}	2.5	1.5	0.0	0.0	Not known	Spontaneous (KAUFMAN, 1972)	Not known
v^{ts1}/v^{0f}	2.5	1.5	0.0	0.0	Not known	X-ray (OFFERMAN, 1935)	Not known
v^{1}/v^{36f}	0.0	0.0	0.0	0.0	Combination is unsuppressible (TARTOF, 1969)		
v^{1}/v^{sp}	0.0	0.0	0.0	0.0	Not known		

* Scale = 0.0 = complete vermilion expression of the v locus.

5.0 = complete wild type expression of the v locus.

and Df v^{L3} lack different parts of the v locus, as was shown in Figure 1. The v locus has been localized to salivary chromosome bands 10A1-2 and Df v^{L1} has lost band 10A2 with a breakpoint close to the right edge of 10A1, whereas Df v^{L2} is missing band 10A1 with its right breakpoint at the junction of 10A1 and 10A2 (LEFEVRE 1969). Hence, the combination of Df v^{L1} and Df v^{L2} has minimal deficiency overlap as shown in the Figure. On the other hand, Df v^{L3} lacks the entire v locus (LEFEVRE 1969).

When crossed inter se at 17°, 22° and 29°C, none of the deficiency heterozygotes survive, even when there is minimal overlap as in the case of Df v^{L1} with Df v^{L2} (LEFEVRE 1969, and some repeated here). Each of the deficiencies was tested in females carrying v^{1} at 17°, 22° and 29°C. In all crosses, the heterozygotes were viable and clearly vermilion in phenotype. All v^{ts1}/Df v females also survived at 17°, 22° and 29°C and their phenotypes are summarized in Table 6.

Since all combinations of deficiencies for the v locus are lethal (LEFEVRE 1969; TABLE 6) it could be argued that a loss of any part of the v locus results in lethality, in which case the survival of v^{ts1}/Df v females would suggest that v^{ts1} is not a deficiency. Indeed, cytological examination of salivary gland chromosomes of v^{ts1} larvae by Dr. T. Kaufman revealed no detectable abnormality, even though very small deficiencies involving the v locus (Df v^{L2}, for example) are readily observable (LEFEVRE 1969).

TABLE 6 Eye phenotype of females heterozygous for different combinations of Df(1)v ; v^1 and v^{ts1} at different temperatures.

GENOTYPE	INDEX OF VERMILIONESS AT DIFFERENT TEMPERATURES 17°C	22°C	29°C
Dfv^{L1}/Dfv^{L2}	-	-	-
Dfv^{L1}/Dfv^{L3}	-	-	-
Dfv^{L2}/Dfv^{L3}	-	-	-
Dfv^{L1}/v^1	* 0.0	0.0	0.0
Dfv^{L2}/v^1	0.0	0.0	0.0
Dfv^{L3}/v^1	0.0	0.0	0.0
Dfv^{L1}/v^{ts1}	1.5	1.0	0.0
Dfv^{L2}/v^{ts1}	1.5	1.0	0.0
Dfv^{L3}/v^{ts1}	1.5	1.0	0.0
Dfv^{L3}/v^+	5.0	5.0	5.0

* Scale : 0.0 = completely vermilion; 5.0 = completely wild type.

It can be seen (TABLE 6) that the phenotype of $\underline{v^{ts1}}/\underline{Df\ v}$ at all temperatures is more mutant than $\underline{v^{ts1}}/\underline{v^{ts1}}$ females raised at the same temperatures (compare TABLES 4 and 6). While a perceptible phenotypic difference exists between $\underline{v^{ts1}}/\underline{Df\ v}$ females raised at 17° and 22°C, the magnitude is small. Thus, in the presence of a deficiency, $\underline{v^{ts1}}$ behaves more like a non-ts $\underline{v}$ mutation, even though $\underline{v^{ts1}}$/Y males are nearly normal. However, $\underline{v^{ts1}}/\underline{Df\ v}$ females are more extreme at 17° and 22°C than $\underline{v^{ts1}}/\underline{v}$ females. At this point, it is worth pointing out that while $\underline{v^{ts1}}/\underline{Df\ v}$ females and $\underline{v^{ts1}}$/Y males have the same number of $\underline{v^{ts1}}$ genes, the females are much more mutant than the males, which is further corroboration of the sex difference in expression of $\underline{v^{ts1}}$.

It could be suggested that the Y chromosome affects the expression of $\underline{v^{ts1}}$. Consequently, $\underline{v^{ts1}}$/O males were generated by the cross: $\underline{v^{ts1}}$/Y ♂ x C(1)RM/O ♀. The $\underline{v^{ts1}}$/O males were identical in phenotypes at 17°, 22° and 29°C to $\underline{v^{ts1}}$/Y males raised at these temperatures, thereby showing that the Y chromosome does not affect the expression of $\underline{v^{ts1}}$ in males.

A Y chromosome bearing a duplication of $\underline{v^+}$, $\underline{y^+}$ $\underline{v^+}$.Y (CHOVNICK 1968), restores wild type eye colour to males carrying a $\underline{v}$ allele on the X chromosome (LEFEVRE 1971; TOBLER, BOWMAN AND SIMMONS 1971). $\underline{y}$ $\underline{v^{ts1}}/\underline{y^+}$ $\underline{v^+}$.Y males were generated in the cross $\underline{y}$ $\underline{v^{ts1}}$/Y ♂ x C(1)RM, $\underline{y}$ $\underline{f}/\underline{y^+}$ $\underline{v^+}$.Y ♀ at 17°, 22° and 29°C. At all three temperatures, the phenotype of the $\underline{y}$ $\underline{v^{ts1}}/\underline{y^+}$ $\underline{v^+}$.Y males was wild type, again indicating that $\underline{v^{ts1}}$ is recessive like all other $\underline{v}$ alleles.

Other eye colour mutants interact with vermilion mutants. Two such mutants, bw and ras^2 were tested with v^{ts1}. In combination with bw, suppressible v alleles have residual brown pigment in an otherwise white eye, whereas unsuppressible v alleles have pure white eyes (GREEN 1952; SHAPARD 1960). A stock of v^{ts1}/v^{ts1}; bw/bw ♀ X v^{ts1}/Y;bw/bw ♂ was generated and grown at 17°, 22° and 29°C. At 17° and 22°C, the eye colour of males and females was nearly bw. At 29°C, the eyes of the males were white with a very small amount of residual brown pigment, whereas the females had considerably more brown pigment, which produced a "yellow-light brown" hue. These results are consistent with the temperature-sensitive expression of v^{ts1}. The presence of some brown in an an otherwise white eye in males and females could suggest that v^{ts1} is suppressible at this temperature. However, as will be shown later, v^{ts1} is unsuppressible by $su(s)^2$. It would thus appear that v alleles which have some residual activity allow a slight expression of bw.

In combination with the sex linked eye colour mutant, ras^2, non-ts v alleles produce an orange eye colour. $ras^2\ v^{ts1}$ and ras^2 flies were grown at 17°, 22° and 29°C. The ras^2 phenotype remained constant at all three temperatures. Males and females of the $ras^2\ v^{ts1}$ stock at 17°C were indistinguishable from ras^2 males and females. At 22°C, the eye colour of $ras^2\ v^{ts1}$ males and females was similar and exhibited a slight orange tinge not present in ras^2. At 29°C, the $ras^2\ v^{ts1}$ males were definitely orange in eye

colour, in contrast to an intermediate (between raspberry and orange) colour of the females. This 29°C eye colour of $\underline{ras^2\ v^{ts1}}$ females will be referred to as "dilute-raspberry".

The interactions of $\underline{v^{ts1}}$ either with bw or $\underline{ras^2}$ show that at the restrictive temperature, $\underline{v^{ts1}}$ does behave like a standard v allele with some residual activity. The temperature-dependent interaction of $\underline{v^{ts1}}$ with $\underline{ras^2}$ and bw show that the phenotypic differences of $\underline{v^{ts1}}$ at different temperatures are indeed valid criteria of the activity of the allele.

II. Suppressibility of $\underline{v^{ts1}}$ by the $\underline{su(s)^2}$ mutation.

One of the most interesting and potentially revealing properties of v alleles (from the standpoint of the nature of the molecular control of gene action in Drosophila) is their relationship with the su(s) locus. The mutant, su(s), was the first suppressor mutation discovered in Drosophila (BRIDGES 1915) and has since been the subject of intensive study at both the phenotypic and molecular levels (SCHULTZ AND BRIDGES 1932; GREEN 1952, 1954; SHAPARD 1960; BAGLIONI 1960; MARZLUF 1965; TARTOF 1969; JACOBSON 1971; TWARDZIK, JACOBSON AND GRELL 1971; WHITE, TENER, HOLDEN AND SUZUKI 1973).

The original su(s) allele has been lost but many others, both spontaneous and induced, have since been recovered and all exhibit similar properties in relation to the v locus. The su(s) mutations are recessive and interact with certain v alleles to produce a wild

FIGURE 6 Crosses to generate and test the effect of $su(s)^2$ on v^{ts1}.

Line

1 $su(s)^2$ v/Y ; bw/bw ♂ X +/+ ; Bl sp/SM5 sp ♀ & $su(s)^2$ v/$su(s)^2$ v ; bw/bw ♀ X +/Y ; Bl sp/SM5 sp

2 $su(s)^2$ v/+ ; SM5, sp/bw ♀ X $su(s)^2$ v/Y ; Bl sp/bw ♂

3 Select : $su(s)^2$ v/$su(s)^2$v ; SM5 sp/Bl sp ♀ X $su(s)^2$ v/Y ; SM5, sp/Bl sp ♂

---

4 ras^2 v^{ts1}/Y ; +/+ ♂ X +/+ ; Bl sp/SM5 sp ♀ & ras^2 v^{ts1}/ras^2 v^{ts1} ; +/+ ♀ X +/Y ; Bl sp/SM5, sp

5 ras^2 v^{ts1}/+ ; SM5, sp/+ ♀ X ras^2 v^{ts1}/Y ; Bl sp/+ ♂

6 Select : ras^2 v^{ts1}/ras^2 v^{ts1} ; Bl sp/SM5, sp ♀ X ras^2 v^{ts1}/Y ; Bl sp/SM5, sp ♂

---

7 $su(s)^2$ v/$su(s)^2$ v ; SM5, sp/Bl sp ♀ X ras^2 v^{ts1}/Y ; Bl sp/SM5, sp ♂

8 $su(s)^2$ + v/+ ras^2 v^{ts1} ; SM5, sp/Bl sp ♀ X ras^2 v^{ts1}/Y ; SM5, sp/Bl sp ♂

9 Select, $su(s)^2$, ras^2, v^{ts1} crossover progeny: $su(s)^2$ ras^2 v^{ts1}/Y ; SM5, sp/Bl sp ♂

10 Homozygose and test at 22° and 29°C.

type eye colour. Those v alleles whose mutant expression is altered by su(s) are classed as suppressible. All of the suppressible v alleles which have been assayed for tryptophan pyrrolase activity show a varying but partial restoration of enzyme activity in combination with homozygous su(s) alleles (BAGLIONI 1960; KAUFMAN 1962; MARZLUF 1965; TARTOF 1969; TWARDZIK, JACOBSON AND GRELL 1971).

A $su(s)^2$ v^{ts1} chromosome was generated to test the suppressibility of v^{ts1}. As an independent check to verify the presence of $su(s)^2$, the suppressible mutation, sp, was introduced on the second chromosome. The crosses used to generate $su(s)^2$ ras^2 v^{ts1}; sp/sp are shown in Figure 6.

At 22°C, flies of this stock were non-sp thereby verifying the presence of $su(s)^2$. The eye colour was raspberry showing that v^{ts1} was acting as a wild type allele. At 29°C, the non-sp phenotype showed that $su(s)^2$ was not temperature-sensitive. The males had an orange eye colour characteristic of v ras flies thereby showing that $su(s)^2$ did not suppress v^{ts1}. This is consistent with the general observation that spontaneous v alleles are suppressible, whereas induced v mutants are not.

III. Studies on the nonautonomous expression of v^{ts1}.

Expression of v alleles in gynandromorphs (STURTEVANT 1932; GREEN 1952; SHAPARD 1960) and of v^1 eye discs transplanted into v^+ larvae (BEADLE AND EPHRUSSI 1936) has been found to be nonautonomous

That is, the eye colour phenotype expressed in eye cells which are genotypically v depends on the genotype of the surrounding tissue. When v^+ tissue is present, the phenotype of the transplant is v^+, whereas the phenotype is v when the host is also v. This nonautonomous expression of v alleles holds for both suppressible (STURTEVANT 1932; GREEN 1952; SHAPARD 1960) and unsuppressible v alleles (GREEN 1952; SHAPARD 1960).

Autonomy of v^{ts1} was studied at 22° and 29°C by the construction of gynandromorphs of v^{ts1} and + cells. This tests the generalization that all types of v alleles are nonautonomous. The fundamental question is whether v^{ts1} can be shown to differ from v^+ at permissive temperatures. Furthermore, combination of v^{ts1} with v in gynanders could show whether v^{ts1} behaves as a genuine v^+ allele at 22°C in producing a diffusible product capable of modifying v expression.

Gynandromorphs could be constructed using the effect of the third chromosome eye colour mutant claret non-disjunctional (ca^{nd}) on chromosome disjunction. Offspring of homozygous ca^{nd} females exhibit a greatly increased frequency of somatic elmination of the maternal X chromosome (LEWIS AND GENCARELLA 1952) shortly after fertilization. This results in the production of mosaics of the general type: X maternal/X paternal : X paternal/0. Cells in which the X chromosome is lost could be recognized by the cuticular expression of the recessive mutation yellow (y) which marked the paternal X chromosome. Where the XO tissue included sexually distinct

structures, male tissue could also be recognised.

In the first crosses, tests of autonomy of $\underline{v^{ts1}}$ in gynanders with $\underline{v^{+}}$ were established to determine whether $\underline{v^{ts1}}$ behaves as a $\underline{v}$ allele at 29°C. Gynanders were synthesized by the crosses shown in Figure 7. Females were scored for the presence of yellow tissue indicative of X/O cells. The location of the yellow tissue of each mosaic was recorded on a master sheet and the colour of both eyes carefully noted.

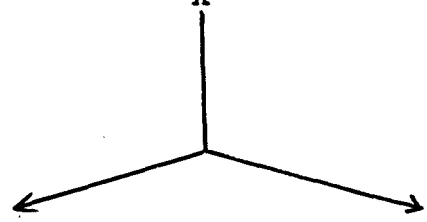
A total of 9 gynanders was recovered, 7 at 22°C and 2 at 29°C. Three of the 22°C gynanders were complete bilaterals and both eyes in all three were wild type. The other four had smaller regions of mutant tissue and all had wild type eyes. These results for the 22°C-raised gynanders show that $\underline{v^{ts1}}$ behaves as a normal $\underline{v^{+}}$ allele at 22°C.

Of the two gynanders recovered at 29°C, one had a catercorner arrangement of mutant tissue (Figure 8). The dorsal and ventral surfaces of the head, thorax, wing and legs of one half of the body were mutant, whereas the ventral and dorsal surfaces of the other half of the abdomen were mutant. Both eyes of this gynander were wild type, even though the genotype of one of the eyes was $\underline{y}\ \underline{v^{ts1}}/0$. The presence of wild type tissue in one half of the body (including the other eye) was apparently sufficient to bestow a wild type eye colour to the genotypically $\underline{v}$ eye, thereby showing that $\underline{v^{ts1}}$ is nonautonomous like all other $\underline{v}$ alleles.

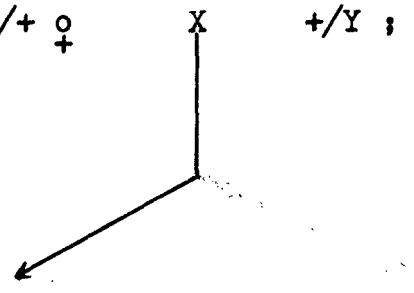
The other gynander recovered at 29°C was also a bilateral

FIGURE 7 Crosses used to generate gynandromorphs of v^+ and v^{ts1}.

1 +/+ ; +/+ ♀♀ X +/Y ; $\underline{e^s}$ $\underline{ca^{nd}}$/$\underline{TM6}$ ♂♂



2 +/+ ; $\underline{e^s}$ $\underline{ca^{nd}}$/+ ♀ X +/Y ; $\underline{e^s}$ $\underline{ca^{nd}}$/+ ♂

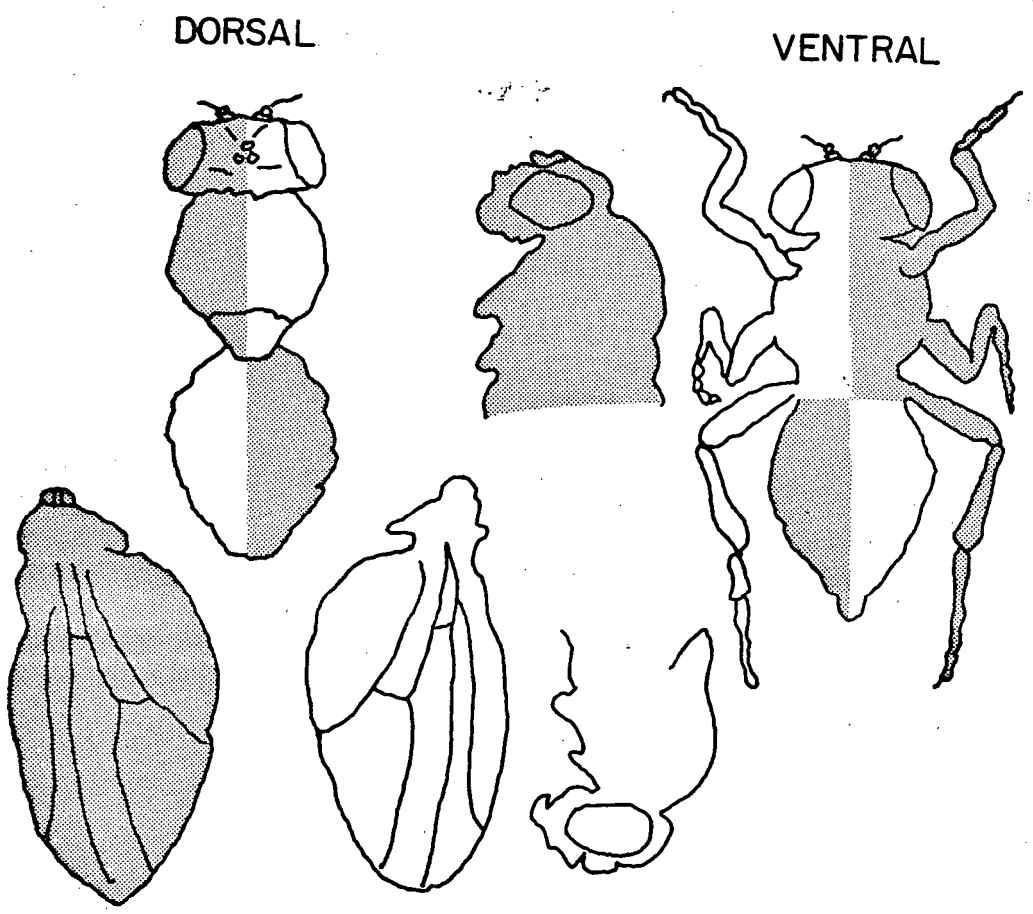


3 Collect $\underline{e^s}$, $\underline{ca^{nd}}$ progeny:

+/+ ; $\underline{e^s}$ $\underline{ca^{nd}}$/$\underline{e^s}$ $\underline{ca^{nd}}$ ♀ X $\underline{y}$ $\underline{v^{ts1}}$/Y ; +/+ ♂

4 Scoring cross: examine all progeny at both temperatures for gynandromorphs resulting from somatic elimination of a maternal X chromosome.

FIGURE 8 An example of a $\underline{v^+}/\underline{y}\ \underline{v^{ts1}}$ ♂ $\underline{y}\ \underline{v^{ts1}}/0$ gynandromorph raised at 29°C.



SHADED REGIONS REPRESENT $\underline{y}\ \underline{v^{tsl}}/O$
TISSUE (BOTH EYES ARE $\underline{v^+}$ IN PHENOTYPE)

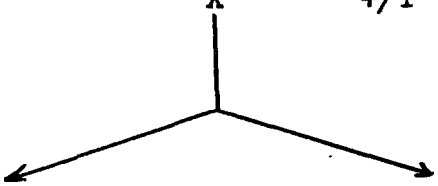
gynander for the head, thorax and legs, but was mutant for the entire abdomen. Again, both eyes were wild type corroborating the result of $\underline{v^{ts1}}$ nonautonomy found with the previous gynander. Interestingly, the abdomen was completely mutant, thereby showing that a mutant genotype of a region in which the fat body of the larvae, pupa and early adult resides, plays no role in determining $\underline{v^{ts1}}$ mutant expression. This is of interest considering RIZKI'S (1963) report that the fat body of the larvae is the probable location of tryptophan pyrrolase activity and the formation of brown pigment precursor (kynurenine) destined for the eye.

The argument outlined here rests on the assumption that in Drosophila mosaics, the genotype of the internal tissues corresponds to the observed genotype of the equivalent external tissues, for which there is some experimental evidence (HOTTA AND BENZER 1972).

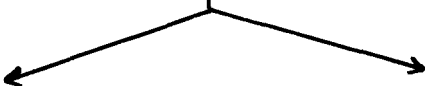
Any interaction of $\underline{v^{ts1}}$ with $\underline{v^{1}}$ could be determined in gynanders of the two alleles constructed as shown in Figure 9. A total of 9 gynanders was recovered; 6 from the 22°C crosses and 3 from the 29°C crosses. The positions of the mutant tissue in an example of one of the 6 gynanders recovered at 22°C can be seen in Figure 10. As one might expect, both eyes were wild type in all 6 gynanders. In view of the vermilion-like phenotype of $\underline{v^{ts1}}/\underline{v^{1}}$ eyes at 22°C (Table 5), this is of interest. These gynanders show that the $\underline{y}$ $\underline{v^{ts1}}/0$ tissue produces enough brown pigment (or brown pigment precursor) at 22°C to allow a genotypically $\underline{y}$ $\underline{v^{ts1}}/\underline{v^{1}}$ eye to become wild type.

FIGURE 9 Crosses used to generate gynandromorphs of $\underline{v^{ts1}}$ and $\underline{v^{1}}$.

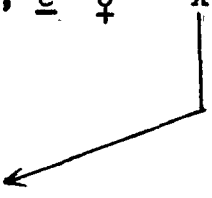
1 $\underline{v^1}/\underline{v^1}$; +/+ ♀ X +/Y ; $\underline{e^s}$ $\underline{ca^{nd}}/\underline{e^s}$ $\underline{ca^{nd}}$ ♂



2 $\underline{v^1}$/Y ; $\underline{e^s}$ $\underline{ca^{nd}}$/+ ♂ X $\underline{v^1}$/+ ; $\underline{TM6}$, $\underline{e^s}$/+ ♀



3 $\underline{v^1}/\underline{v^1}$; $\underline{e^s}$ $\underline{ca^{nd}}/\underline{TM6}$, $\underline{e^s}$ ♀ X $\underline{v^1}$/Y ; $\underline{e^s}$ $\underline{ca^{nd}}/\underline{TM6}$, $\underline{e^s}$ ♂



4 $\underline{v^1}/\underline{v^1}$; $\underline{e^s}$ $\underline{ca^{nd}}/\underline{e^s}$ $\underline{ca^{nd}}$ ♀ * X $\underline{y}$ $\underline{v^{ts1}}$/Y ; +/+ ♂

5 Scoring cross: examine all progeny at both temperatures for gynandromorphs resulting from somatic elimination of a maternal X chromosome.

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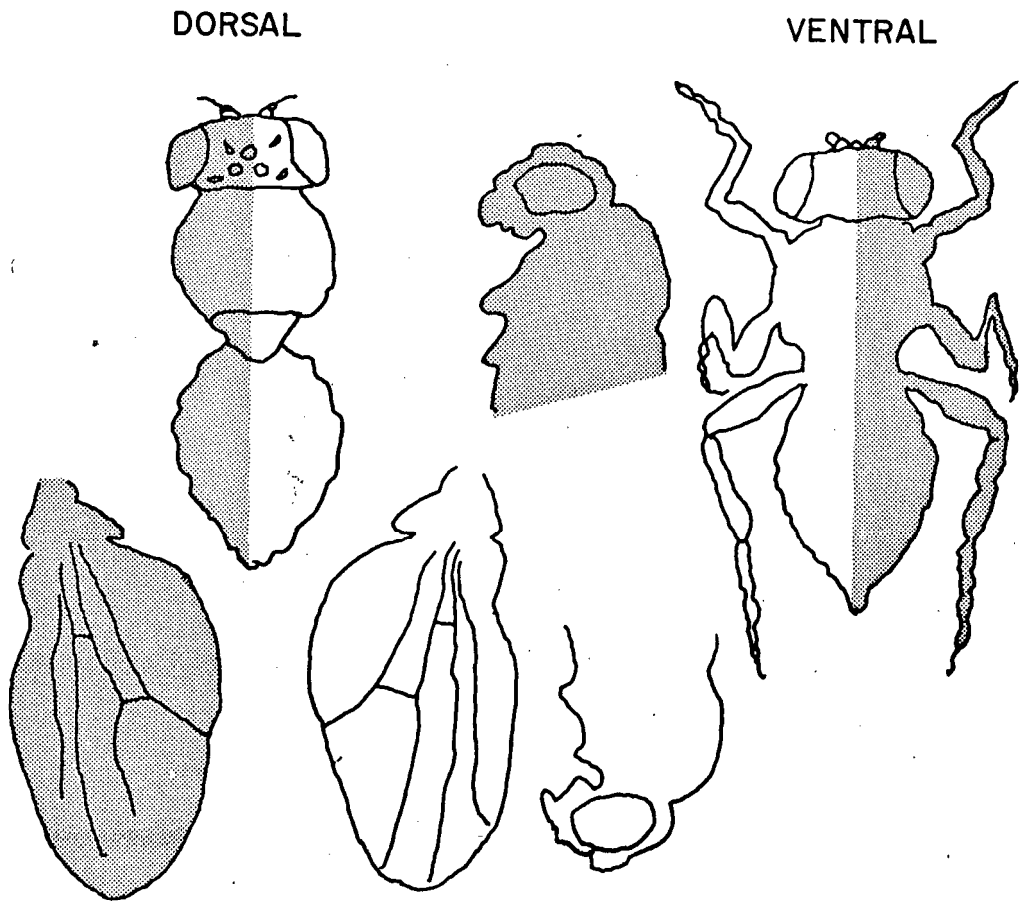
* Homozygous $\underline{v^1}/\underline{v^1}$, $\underline{e^s}$ $\underline{ca^{nd}}/\underline{e^s}$ $\underline{ca^{nd}}$ females are readily identified because $\underline{v}$ and $\underline{ca^{nd}}$ when homozygous, interact to give a characteristic clear orange eye.

FIGURE 10 An example of a $\underline{v^1}/\underline{y}\ \underline{v^{ts1}}$ ♀ $\underline{y}\ \underline{v^{ts1}}/0$ gynandromorph raised at 22°C.



SHADED REGIONS REPRESENT $\underline{y}$ $\underline{v^{ts1}}/O$ TISSUE
(BOTH EYES ARE $\underline{v^{+}}$ IN PHENOTYPE)

Two of the 3 gynanders at 29°C were complete bilaterals. In both gynanders, both eyes were clearly vermilion in phenotype. Thus, the y v^{ts1}/0 genotype retains its temperature-dependent mutant expression when combined with v^{ts1}/v^{1} tissue in the same fly. The third gyndander recovered at 29°C had mutant tissue confined to the head and, as expected, both eyes were clearly vermilion.

IV. Mapping of v^{ts1}.

It was of interest to determine whether a ts allele of v mapped at a distinct site within the v locus. Therefore, the v^{ts1} mutant was tested for recombination with v^{1} which maps in the left site of the v cistron and v^{36f} which maps in the right. The closely linked flanking markers, ras^{2} (32.8) and m (36.1) were crossed on to the v^{ts1} chromosome to permit an unequivocal ordering of the alleles.

Crossing over was studied in ras^{2} v^{ts1} m/+ v^{1} + and ras^{2} v^{ts1} m/+ v^{36f} + females. The second and third chromosome multiple inversions, SM5 and TM2, were introduced into each heterozygous female, since GREEN (1952) has shown that this increases recombination within the v locus.

The crosses employed to construct the stocks used in the recombination studies are shown in Figure 11. In step three, about 10-15 females and 20-30 males were crossed at 29°C and the parents transferred to fresh medium after 3 days. All cultures were maintained at 29°C and all F_1 flies were scored for eye colour

FIGURE 11 Crosses used for recombination tests between $\underline{v^{ts1}}$ and $\underline{v^{1}}$ or $\underline{v^{36f}}$.

1 $\underline{ras^2}\ \underline{v^{ts1}}\ \underline{m}/\underline{ras^2}\ \underline{v^{ts1}}\ \underline{m}\ ♀$ X +/Y ; SM5/Pm

2 $\underline{ras^2}\ \underline{v^{ts1}}\ \underline{m}/Y$; SM5/+ ; TM2/+ ♂

3 $\underline{ras^2}\ \underline{v^{ts1}}\ \underline{m}/+\ \underline{v}^*\ +$; SM5/+ ; TM2/+ ♀

4

$\underline{v}^*$ - refers to either $\underline{v^1}$ or $\underline{v^{36f}}$.

; TM2/Sb ♂ & v*/Y ♂ X +/+ ; SM5/Pm ; TM2/Sb ♀

X v*/+ ; SM5/+ ; TM2/+ ♀

X ras^{2} v^{ts1} m/Y ; +/+ ; +/+ ♂

29°

Score

and wing phenotypes. Since it is known that double mutants for two v alleles are v in phenotype (GREEN 1952), only the wild type recombinant within the v locus would be recognized.

A total of 20,610 progeny of v^{ts1}/v^{1} females was scored and the results are reported in Table 7. It can be seen that v^{+} flies which were not recombinant for flanking markers were recovered and they will be discussed later. If these classes are omitted, the crossover interval between ras^{2} and m is 3.25%, a value which is remarkably close to the standard map distance of 3.3% (LINDSLEY AND GRELL 1968).

Recombinants within the v locus were recovered. The two crossovers were recovered as ras^{2} + + + /ras^{2} + v^{ts1} m females and were test crossed to ras^{2} v^{ts1} m/Y males at 22° and 29°C. In both cases, offspring verified the assumed genotype and showed the absence of a v allele on the ras^{2} + + chromosome. If it is assumed that the reciprocal crossover, + v^{1} v^{ts1} m, was generated with the same frequency, a recombination frequency between v^{1} and v^{ts1} of 4/20,610 is obtained. Thus, v^{1} is about 0.02 map units to the left of v^{ts1}. This recombination frequency between v^{1} and v^{ts1} is higher than the frequency of about 2/30,000 found by GREEN (1954) for recombination between v^{1} and v^{36f}, but at these low frequencies, the numbers are not statistically different.

A surprising result was the recovery of chromosomes carrying v^{+} but noncrossovers for flanking markers (the + + + and ras^{2} + m classes of Table 7. Their generation by normal crossing over would

TABLE 7 Genotypes of progeny resulting from a testcross of $\underline{ras^2}$ $\underline{v^{ts1}}$ $\underline{m}$ / + $\underline{v^1}$ + females at 29°C.

CLASS	GENOTYPE	MALES	FEMALES	TOTAL	% CROSSOVERS IN $\underline{ras^2}$ - $\underline{m}$ INTERVAL
NCO	$\underline{ras^2}$ $\underline{v^*}$ $\underline{m}$	4,880	4,750	9,630	0
	+ $\underline{v^*}$ +	5,219	5,017	10,236	0
CO	$\underline{ras^2}$ $\underline{v^*}$ +	151	154	305	1.48
	+ $\underline{v^*}$ $\underline{m}$	178	182	360	1.75
CO	$\underline{ras^2}$ + +	0	2	2	0.02 **
***	+ + +	23	34	57	[0.028]
***	$\underline{ras^2}$ + $\underline{m}$	3	9	12	[0.06]
TOTALS		10,452	10,158	20,610	3.25

$\underline{v^*}$ - either $\underline{v^{ts1}}$ or $\underline{v^1}$

** - includes two unrecognized + $\underline{v^1}$ $\underline{v^{ts1}}$ $\underline{m}$ reciprocal chromosomes.

*** - classes of uncertain origin.

require double crossovers within a very short genetic interval of 3.3 units. While it could be suggested that interference within short genetic intervals is negative (SUZUKI, BAILLIE AND PARRY 1966) such a high frequency (2.1×10^{-4}) has never been recorded in Drosophila.

The origin of these wild type and $\underline{ras^2}$ + $\underline{m}$ flies by gene conversion is a possible alternative although the high frequency observed here is most unusual. The possibility that the wild type flies were the result of the occasional use of non-virgin $\underline{ras^2}$ $\underline{v^{ts1}}$-$\underline{m}$ /+ $\underline{v^1}$ + females which had mated with a wild type sib (see Figure 11) was minimized by careful collection of the females at 10-12 hour intervals. Moreover, the wild type progeny were not recovered in clusters in certain bottles as might be expected if a female had not been virgin. They were picked up, either singly or in twos or threes, from bottles scattered amongst the 150 or so scored in the test cross, thereby indicating that individual events in independent females gave rise to them.

The recovery of the other unusual class, the $\underline{ras^2}$ + $\underline{m}$ parentals in female offspring, required fertilization by the appropriately marked male. Finally, recovery of + + + and $\underline{ras^2}$ + $\underline{m}$ male offspring regardless of paternal genotype proves the genuine genetic origin of the chromosomes.

Each $\underline{v^+}$ male exception was individually test crossed to 3-5 $\underline{ras^2}$ $\underline{v^{ts1}}$ $\underline{m}$/$\underline{ras^2}$ $\underline{v^{ts1}}$ $\underline{m}$ females at 22° and 29°C. Male and female progeny were scored for eye colour and wing phenotypes. Each wild

type female exception was mated with 3-5 $\underline{ras^2}$ $\underline{v^{ts1}}$ $\underline{m}$/Y males at 22° and 29°C and male and female progeny again scored for eye colour and wing phenotypes. Of the 55 exceptional males and females tested, 7 were sterile and 48 yielded progeny verifying the original classification of genotype.

In the same way, the $\underline{ras^2}$ + $\underline{m}$ exceptional males and females were tested. Of the 3 exceptional males, only 1 was fertile and yielded wild type eye colour, miniature daughters. Thus, the one fertile male did not confirm its original classification. Four of the 6 fertile $\underline{ras^2}$ + $\underline{m}$ female exceptions confirmed that they indeed carried a $\underline{ras^2}$ + $\underline{m}$ exceptional chromosome. Two females proved to be $\underline{ras^2}$ $\underline{v^{ts1}}$ $\underline{m}$ /$\underline{ras^2}$ $\underline{v^{ts1}}$ $\underline{m}$ in genotype. The reasons for misclassification of the two females and the single male can only be conjecture. The important point is that 4 of the $\underline{ras^2}$ + $\underline{m}$ chromosomes were verified and must be reckoned as gene conversion-like events when considered together with the confirmation of 48 of the 57 + + + chromosomes recovered.

Thus, the "parental wilds" appear to be genuine meiotic products which are best explained by conversion events. The asymmetrical recovery of the two classes of parental wilds conforms to observations made of gene conversion in numerous fungi (HOLLIDAY 1964; MURRAY 1969; FOGEL, HURST AND MORTIMER 1970; HOLLIDAY AND WHITEHOUSE 1970), and at the $\underline{ry}$ locus of $\underline{Drosophila\ melanogaster}$ (CHOVNICK, BALLANTYNE AND HOLM 1971).

Crossover tests were made between $\underline{v^{ts1}}$ and $\underline{v^{36f}}$ and the

results are shown in Table 8. The crossover distance between $\underline{ras^2}$ and $\underline{m}$ was calculated as 3.4, a value very close to the standard value of 3.3. Incidentally, the standard crossover values obtained for both recombination tests indicate an absence of the frequently encountered interchromosomal effect on crossing over in the $\underline{ras^2}$ - $\underline{m}$ interval in which heterologous rearrangements increase recombination frequency (LUCCHESI AND SUZUKI 1968).

No crossovers between $\underline{v^{ts1}}$ and $\underline{v^{36f}}$ were recovered. While the relatively low number of flies scored (15,553), does not rule out their possible position at different sites it is clear that $\underline{v^{ts1}}$ maps very close to $\underline{v^{36f}}$ and that both are unambiguously separable from, and to the right of $\underline{v^1}$.

A second point of interest arising out of the data of Table 8 is the absence of the wild type and $\underline{ras^2}$ + $\underline{m}$ exceptions found in the tests of $\underline{v^{ts1}}$ and $\underline{v^1}$. This may imply that the mechanism involved in the production of such exceptions is specific for the $\underline{v^{ts1}}$ - $\underline{v^1}$ combination, or at least is specific for a restricted class of $\underline{v}$ alleles in combination with $\underline{v^{ts1}}$. It is likely that the generation of the exceptions occurs only with alleles at genetically separable sites such as $\underline{v^1}$ and $\underline{v^{ts1}}$.

V. Temperature-sensitive period (TSP) of $\underline{v^{ts1}}$.

The effect of temperature on expression of $\underline{v^{ts1}}$ permits a determination of the developmental interval during which eye

TABLE 8 Genotypes of progeny resulting from a testcross of $\underline{ras^2}\ \underline{v^{ts1}}\ \underline{m}$ / + $\underline{v^{36f}}$ + females at 29°C.

CLASS	GENOTYPE	MALES	FEMALES	TOTAL	% CROSSOVERS IN $\underline{ras^2}$ - $\underline{m}$ INTERVAL
NCO	$\underline{ras^2}\ \underline{v}^*\ \underline{m}$	3,785	3,739	7,524	0
	+ $\underline{v}^*$ +	3,833	3,662	7,495	0
CO	$\underline{ras^2}\ \underline{v}^*$ +	143	161	304	1.95
	+ $\underline{v}^*\ \underline{m}$	117	113	230	1.45
TOTALS		7,878	7,675	15,553	3.40

$\underline{v}^*$ - either $\underline{v^{ts1}}$ or $\underline{v^{36f}}$

colour is affected by temperature. As previously described, at 29°C, $\underline{v^{ts1}}$ males exhibit a vermilion phenotype, whereas females have an intermediate but distinctively mutant phenotype. At 22°C, $\underline{v^{ts1}}$ males and females are phenotypically similar and were considered as wild type in the studies of the TSP.

A similar temperature-sensitivity and difference in male-female phenotype was seen with the $\underline{ras^2\ v^{ts1}}$ stock. At 29°C, such males are orange in eye colour, whereas females are "dilute raspberry". This dilute raspberry phenotype exhibited by 29°C - reared females is quite distinguishable from the uniform raspberry phenotype expressed by both males and females of the $\underline{ras^2\ v^{ts1}}$ stock raised at 22°C. The dilute raspberry eye colour is translucent, and the raspberry is diluted by an orange component. The raspberry eye colour of males and females of $\underline{ras^2\ v^{ts1}}$ grown at 22°C is similar to $\underline{ras^2}$ and is light ruby. This phenotype does not vary if $\underline{ras^2}$ males and females are grown at 29°C, indicating that $\underline{ras^2}$ exhibits no temperature-sensitivity at this temperature.

The eye colour of both male and female $\underline{ras^2\ v^{ts1}}$ flies grown at 29°C is relatively stable with time in contrast to the rapid darkening of eyes of $\underline{v^{ts1}}$ flies. Therefore, $\underline{ras^2\ v^{ts1}}$ flies were used in the shift studies since there was less ambiguity in scoring the eye phenotype. Nevertheless, flies shifted at the beginning or end of the TSP exhibited intermediate eye colours.

A summary of the results derived from three independent shift-up (22° to 29°C) and shift-down (29° to 22°C) experiments is given

in Tables 9 and 10. These data are plotted in Figure 12 together with the approximate lengths of the developmental stages for the $\underline{ras^2\ v^{ts1}}$ stock when raised at 22° and 29°C respectively.

The first shift-downs which yield some completely wild type adults occurred in culture 3 (females) and 4 (males) (Table 10). However, the number of mutant males and females rose sharply in cultures shifted down between 64 and 75 hours. Most flies in these cultures at this time were in the early to middle third larval instar and this represents the start of the TSP. Note that this is long before any visible pigment production occurs in the prospective eye cells (CLANCY 1940).

Thus, exposure of $\underline{ras^2\ v^{ts1}}$ flies to the restrictive temperature during the early to middle third larval instar commits the adults to exhibit defective eye pigmentation, even though synthesis of the coloured compounds occurs in the pupae kept at 22°C.

The transition from cultures yielding wild type or mutant-eyed individuals in the shifts-down is quite sharp. For example, culture number 4 differs in shift time from culture number 6 by only 11 hours yet has a 70% difference in the proporton of mutant eyes. This is exhibited graphically in Figure 12.

The end of the TSP is defined by the first shift-up cultures in which a wild type phenotype occurs. This occurs in culture numbers 12 and 13 (Table 9). Most individuals in these cultures, which had developed at 22°C for 144 and 168 hours respectively before shifting to 29°C, were late third instar larvae and early

TABLE 9 Eye phenotypes of $\underline{ras^2\ v^{ts1}}$ adults in cultures shifted from 22°C to 29°C at different successive intervals.

	TIME OF SHIFT		NUMBER OF FLIES IN EACH PHENOTYPIC CLASS							
			FEMALES				MALES			
Culture number	Culture age (hr)	Developmental stage*	ras^2	I**	Dilute-raspberry	% +***	ras^2	I	Orange	% +
1	12	eggs			119	0			127	0
2	24	1(recently hatched)			127	0			141	0
3	36	1			75	0			122	0
4	48	1(some 11)			81	0			65	0
5	60	11		2	93	0		8	68	0
6	72	11(some 111)		4	87	0		6	81	0
7	84	111	3	12	51	4.3	1	10	66	1.3
8	96	111	1	9	64	1.4	5	12	79	5.3
9	108	111	8	14	70	8.7	8	3	48	13.2
10	120	111	5	34	49	5.7	2	28	73	2.1
11	132	111	15	49	27	16.5	11	60	41	9.8
12	144	111(some P)	29	37	18	34.4	33	41	26	33.0

TABLE 9 continued

Culture number	TIME OF SHIFT: Culture age (hr)	TIME OF SHIFT: Developmental stage *	NUMBER OF FLIES IN EACH PHENOTYPIC CLASS: FEMALES: ras^2	FEMALES: I**	FEMALES: Dilute-raspberry	FEMALES: % +***	MALES: ras^2	MALES: I	MALES: Orange	MALES: % +
13	168	P(some 111)	56	18	5	71.0	41	29	18	46.6
14	180	P	70	11	2	85.0	55	14	3	76.5
15	192	P	76	15	6	78.4	61	12	0	83.6
16	216	P	85	6	0	93.4	72	10	0	87.8
17	240	P	52	0	0	100	59	1	0	98
18	264	P	26	1	0	96.3	37	0	0	100
19	Not shifted		22	0	0	100	25	0	0	100

* Developmental stage: 1 = first instar larvae, 11 = second instar larvae, 111 = third instar larvae
P = pupae

** I = intermediate phenotype between raspberry and the specific $\underline{ras^2\ v^{ts1}}$ phenotype for either sex at 29°C. For the calculation of the percent wild type phenotype with respect to $\underline{v^{ts1}}$, these flies were included in the mutant class.

*** % + for each culture = percent of flies exhibiting wild type expression of $\underline{v^{ts1}}$.

TABLE 10 Eye phenotypes of $\underline{ras^2\ v^{ts1}}$ adults in cultures shifted from 29°C to 22°C at different successive intervals.

	TIME OF SHIFT		NUMBER OF FLIES IN EACH PHENOTYPIC CLASS							
			FEMALES				MALES			
Culture number	Culture age (hr)	Developmental stage	ras^2	I	Dilute-raspberry	% +	ras^2	I	Orange	% +
1	0	eggs	93			100	117			100
2	24	1(some 11)	76			100	89			100
3	48	11	151	3		99	164			100
4	64	111	65	15	2	79.3	71	12		85.5
5	72	111	49	16	84	32.9	45	18	96	28.3
6	75	111	12	49	83	8.3	23	49	137	11.8
7	96	111(some P)	3	41	126	1.8	6	24	93	4.9
8	108	P	0	9	112	0	0	10	98	0
9	120	P	0	3	56	0	0	2	75	0
10	144	P	0	0	89	0	0	0	80	0
11	Not shifted		0	0	37	0	0	0	34	0

pupae. However, the end of the TSP is not as well defined as the start (Figure 12). This probably reflects the greater asynchrony in developmental stage reached by individuals in these cultures at the time of shift-ups compared to the individuals in the earlier shift-downs which delineate the start of the TSP.

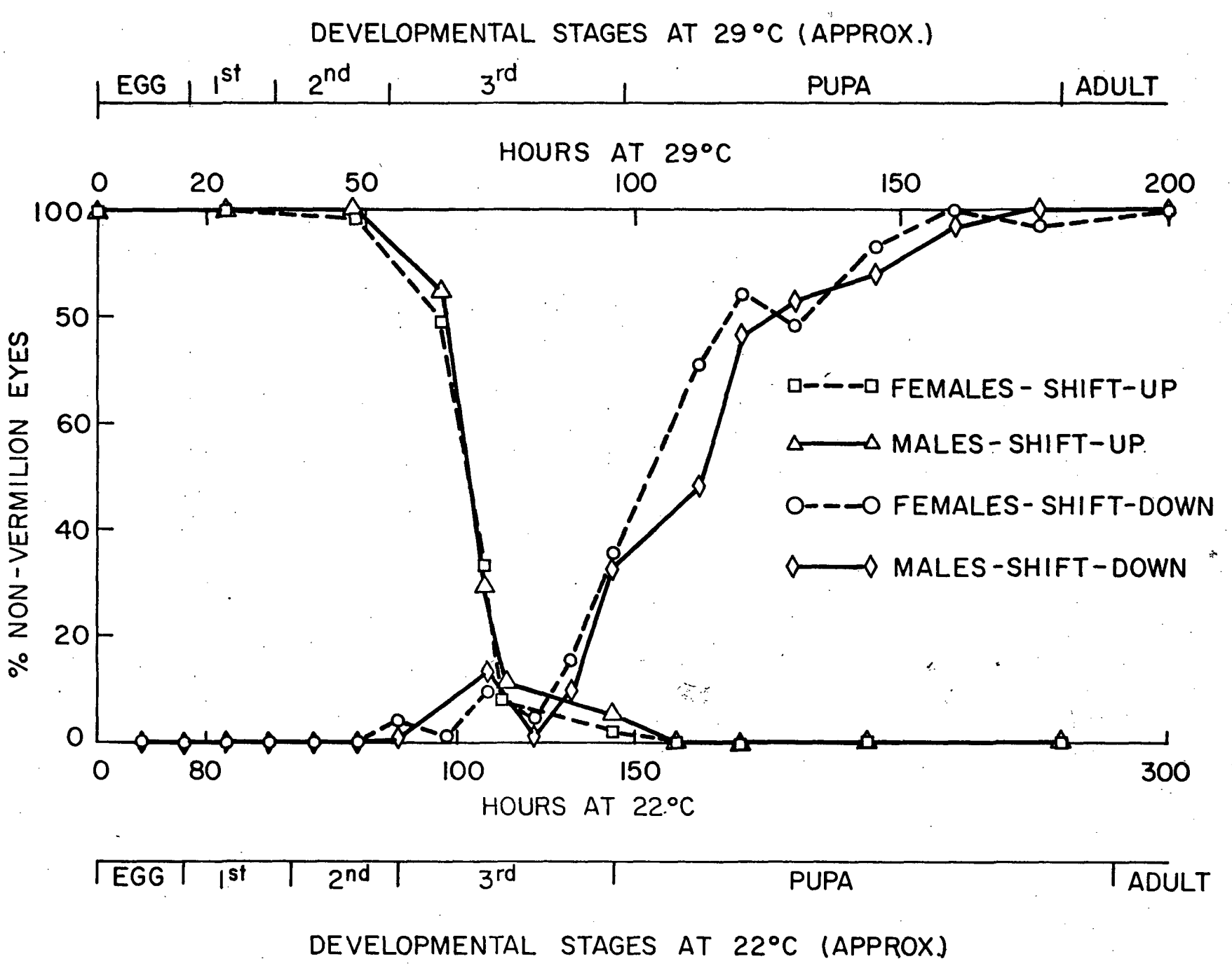
Thus, the phenocritical period for $\underline{v^{ts1}}$ expression occurs from the mid-third instar period and extends into the early pupal stage. Interestingly, this period appears to be the same for males and females, despite the significant difference in expression of the $\underline{v^{ts1}}$ phenotype in adult makes and females raised at 29°C. This is the earliest known temperature effect on pigment production. GRIGLIATTI AND SUZUKI (1970) found a TSP for eye pigments in a ts allele of ras in the last half of the pupal period as did SCHWINK (1961, 1962) for rosy. SURRARRER (1935) found a TSP in 20 - 25 hour pupae of mot - 28 and EPHRUSSI AND HEROLD (1945) showed the TSP of $\underline{w^{bl}}$ to be in 40 - 48 hour pupae.

C. ASSAYS OF TRYPTOPHAN PYRROLASE

I. Spectrophotometrically determined standards of kynurenine and protein.

(a) Kynurenine. The optical densities (OD) of successive dilutions of 0.14 mM standard kynurenine, subjected to the BRATTON AND MARSHALL (1939) determination, were measured at a wavelength of 560 mμ in a GILFORD spectrophotometer. Tubes of each dilution were

FIGURE 12 Determination of the temperature-sensitive period of $\underline{v^{ts1}}$ in shift studies.


DEVELOPMENTAL STAGES AT 29°C (APPROX.)
EGG
1st
2nd
3rd
PUPA
ADULT
HOURS AT 29°C
0
20
50
100
150
200
100
50
60
40
20
0
% NON-VERMILION EYES
FEMALES - SHIFT-UP
MALES - SHIFT-UP
FEMALES - SHIFT-DOWN
MALES - SHIFT-DOWN
0
80
100
150
300
HOURS AT 22°C
EGG
1st
2nd
3rd
PUPA
ADULT
DEVELOPMENTAL STAGES AT 22°C (APPROX.)

set up in duplicate, read against a reagent blank and a mean OD recorded.

The relationship between OD and kynurenine concentration is shown in Figure 13 and is linear to an OD of about 1. An OD change of 0.220 units is equivalent to 10 μM kynurenine under these conditions.

(b) Protein. Successive dilutions of a standard solution of crystalline bovine serum albumin were spectrophotometrically determined by the LOWRY method and are plotted in Figure 14. Tubes of each dilution were set up in duplicate and read at 600 mμ against a reagent blank. The relationship between protein concentration and OD is linear (Figure 14) with very good correlation within the protein limits employed.

II. Reaction kinetics of tryptophan pyrrolase (TP).

The progress of the reaction catalyzed by TP extracted from v^+ flies raised at 22°C was followed over a period of 5 hours under optimal assay conditions. Results of these determinations are shown in Table 11.

The data of Table 11 were used to plot enzyme activity as a function of incubation time (Figure 15). At the start of the reaction, activity is not proportional to time and there is a slight lag. Thereafter, a typical linear relationship can be seen, thereby indicating a first-order enzyme reaction to approximately 3 hours. The reaction rate slows down after about 3 hours probably because of gradual inactivation of the enzyme since the substrate concentra-

FIGURE 13 Relationship between concentration of kynurenine and optical density (OD) at 560 mμ.

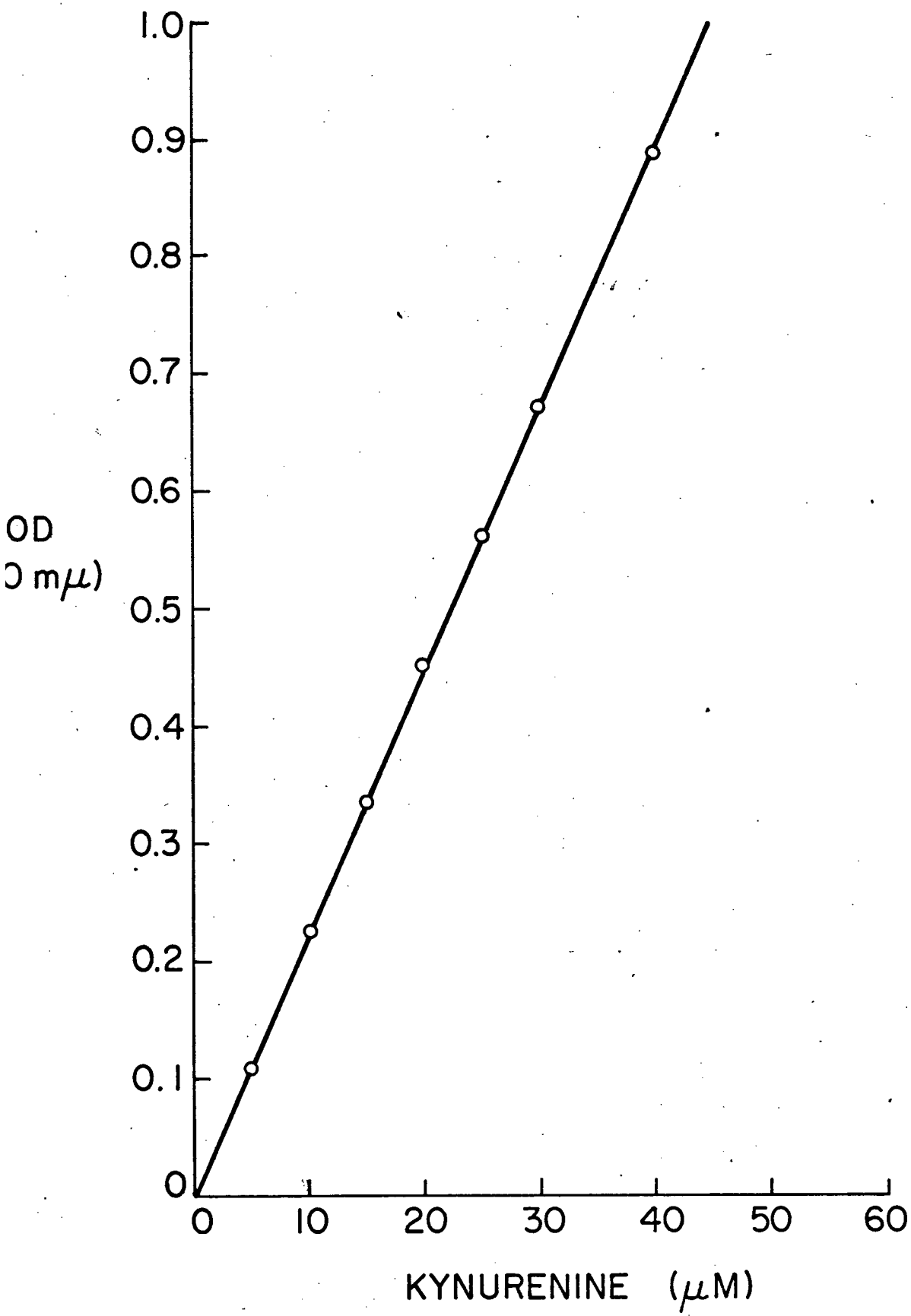
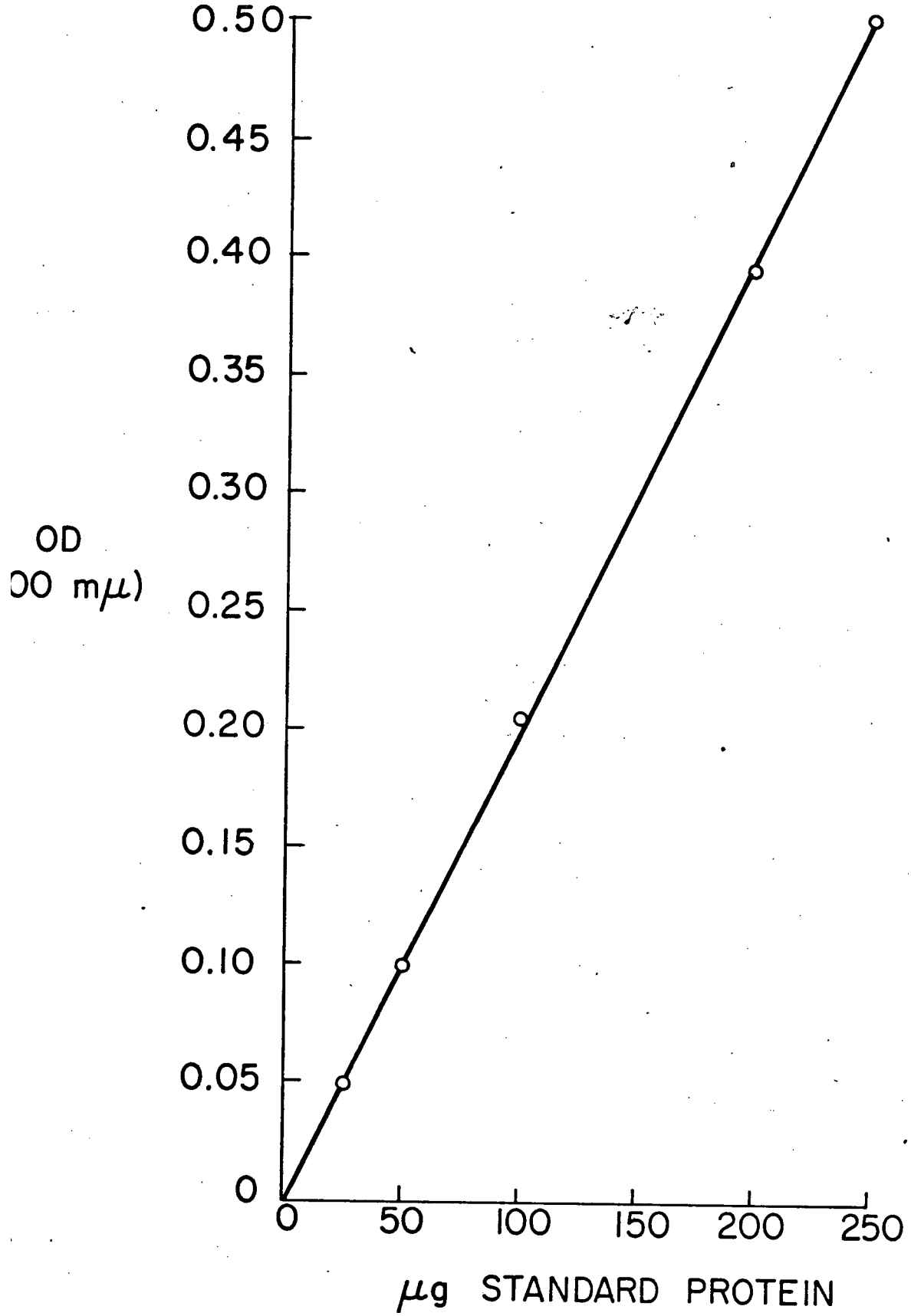

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD
D mμ)
0
10
20
30
40
50
60
KYNURENINE (μM)

FIGURE 14 Relationship between concentration of protein and optical density (OD) at 600 mμ.


0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
OD
00 mμ)
0
50
100
150
200
250
μg STANDARD PROTEIN

tion remaining is still not a rate-limiting factor. The linearity of the reaction rate until at least 3 hours suggests that $\underline{v}^+$ TP is quite stable under these conditions of assay.

Enzyme samples prepared in the same manner as wild type TP were obtained from $\underline{v^{ts1}}$ flies grown at 22° and 29°C. The time-activity relationship of TP from $\underline{v^{ts1}}$ strains was compared with that of $\underline{v}^+$ TP. Conditions were similar as for extracts of $\underline{v}^+$ TP except that 0.8 instead of 0.4 mls of the 29°C - reared $\underline{v^{ts1}}$ TP extract was used. The doubling was necessary since the OD of the kynurenine produced in the latter case is near the limit of sensitivity of the assay for incubation times of less than 2 hours. Even then, accumulation of product does not vary significantly from controls until at least 60 minutes of incubation.

The data obtained for these determinations are shown in Table 12 and plotted in Figure 15. Note that although the $\underline{v^{ts1}}$ flies were reared continuously at 22° or 29°C, all enzyme incubations were carried out at 41°C.

The data on TP activity as a function of incubation time for both 22° - and 29°C - reared $\underline{v^{ts1}}$ flies show greater deviation from linearity compared to $\underline{v}^+$ TP. However, accumulation of product increases approximately linearly as a function of incubation time between 90 and 180 minutes in extracts of both 22° - and 29°C - raised flies.

In the 22°C - grown $\underline{v^{ts1}}$ extracts, a greater lag period occurs before linear kinetics are attained and the reaction rate is at all

TABLE 11 Variation in v† TP activity as a function of time of incubation.

Incubation time (minutes at 41°C)	30	45	60	90	120	180	240	280
OD of experimental tube	0.152	0.254	0.333	0.495	0.631	0.941	1.238	1.270
OD of control tube	0.016	0.019	0.022	0.015	0.019	0.011	0.028	0.020
Net OD	0.136	0.235	0.311	0.480	0.612	0.930	0.210	0.250
Activity (µM kynurenine per ml of solution per ml of enzyme)	*0.062	0.105	0.139	0.216	0.274	0.419	0.545	0.562

* Calculations of TP activity were made as follows : for example for 60 minutes incubation time, net OD of the experimental tube was 0.311. From the kynurenine standard curve (Figure 13), this is equivalent to 0.0139 µM kynurenine/ml. This is the amount of kynurenine present in 0.8 mls of a 3.2 ml TCA filtrate. Therefore in 3.2 ml of the TCA filtrate there are : 0.0139 x 3.2/0.8 µM kynurenine/ml. The 3.2 mls of TCA filtrate contains the total kynurenine released from 5 µM l-tryptophan by 0.4 ml of enzyme extract. Hence, µM kynurenine/ml/ml enzyme extract/60 minutes incubation time = 0.0139 x 3.2/0.8 x 1.0/0.4 = 0.139

The TP activity in the other tubes was similarly calculated.

TABLE 12 Variation of TP activity from v^{ts1} flies with time of incubation.

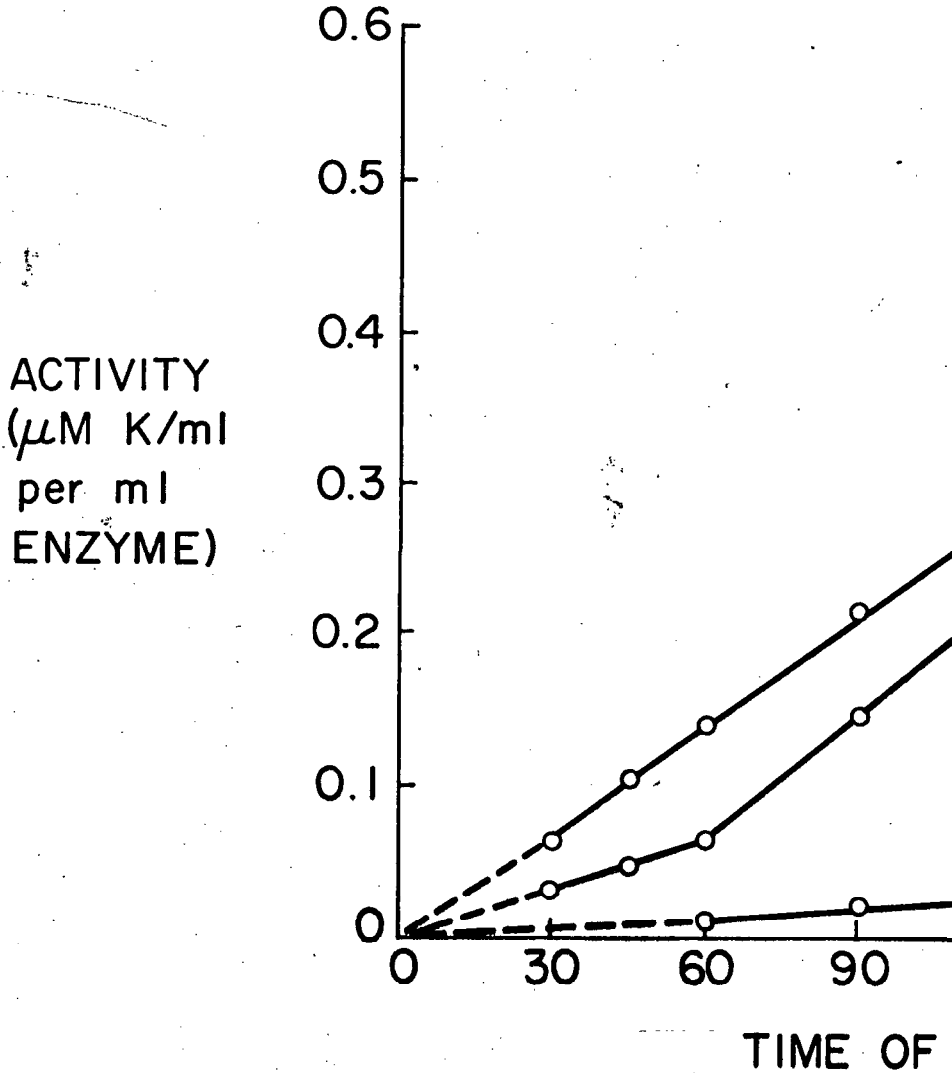
a) 22°C - RAISED FLIES

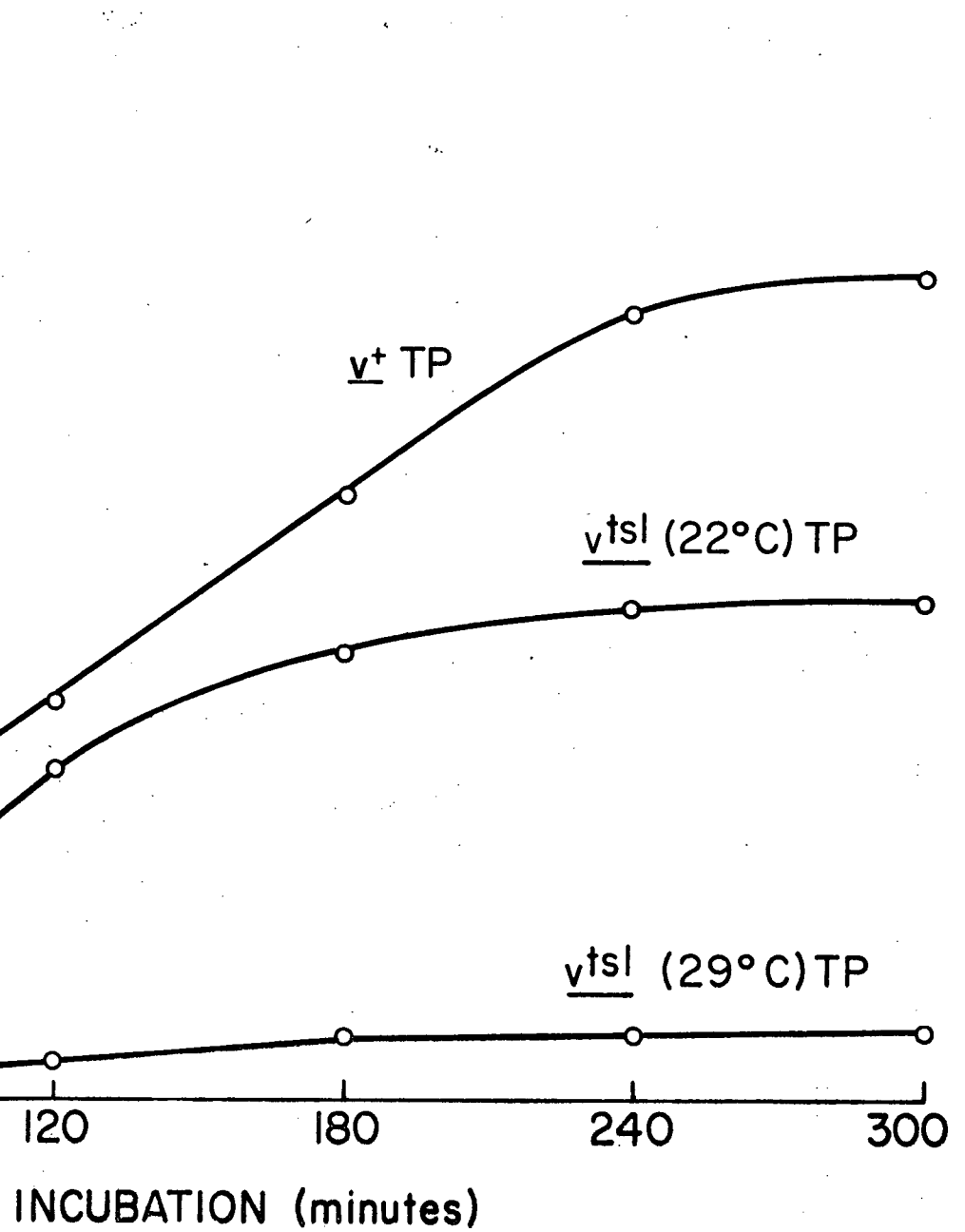
Time of incubation (minutes)	30	45	60	90	120	180	240	300
Activity (μM kynurenine/ml/ml enzyme) mean of 2 determinations	0.033	0.048	0.066	0.147	0.230	0.310	0.338	0.345

b) 29°C - RAISED FLIES

Time of incubation (minutes)	60	90	120	180	240	300
Activity (μM kynurenine/ml/ml enzyme) mean of 2 determinations	0.011	0.022	0.027	0.043	0.044	0.043

FIGURE 15 Variation of enzyme activity with time of incubation.


ACTIVITY
(μM K/ml
per ml
ENZYME)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
30
60
90
TIME OF


v+ TP
vtsl (22°C) TP
vtsl (29°C) TP
120
180
240
300
INCUBATION (minutes)

times reduced in comparison with $\underline{v^+}$ TP. The reaction rate of 22°C - grown $\underline{v^{ts1}}$ enzyme also declines somewhat earlier than wild type TP (Figure 15). This could indicate that $\underline{v^{ts1}}$ TP has a reduced stability when incubation is prolonged at 41°C.

TP of 29°C - grown $\underline{v^{ts1}}$ flies has a reaction rate which is not strictly linear with time of incubation (Figure 15). As expected from the eye phenotype, the reaction rate at all times of incubation is much reduced compared with either $\underline{v^+}$ or 22°C - grown $\underline{v^{ts1}}$ TP. The catalytic activity of the TP produced by the $\underline{v^{ts1}}$ mutant therefore is considerably reduced when the mutant is grown at 29°C. By contrast, TP activity of 22°C - reared $\underline{v^{ts1}}$ flies is near normal even when assayed at 41°C. Clearly, the elevated temperature of incubation is not necessarily deleterious to enzyme activity. The accumulation of low levels of product over a two hour incubation period in extracts of 29°C - reared $\underline{v^{ts1}}$ flies shows that some TP catalytic activity does remain under restrictive conditions.

Enzyme activity as a function of enzyme concentration.

Crude enzyme extracts from $\underline{v^+}$(22°C), $\underline{v^{ts1}}$(22°C) and $\underline{v^{ts1}}$ (29°C) were assayed for μM kynurenine/ml/2 hours of incubation in relation to increasing enzyme concentration. The data from these assays are shown in Table 13 and are plotted in Figure 16. Proportionality between TP activity and concentration essentially holds over the range 0.05 - 0.8 mls of $\underline{v^+}$ extract. The activity-concentration relationship for TP from $\underline{v^{ts1}}$(22°C) appears linear

at all but the low enzyme concentrations (Table 13 and Figure 16). Consequently, in all further assays of TP from $\underline{v^{ts1}}$ (22°C) flies, at least 0.4 ml of enzyme extract was used in a total assay volume of 2.0 mls to ensure proportionality in enzyme activity determinations.

TP activity of $\underline{v^{ts1}}$ (29°C) flies can also be seen in Table 13 and Figure 16. Between 0.4 and 0.8 ml of crude extract, TP activity was significantly lower than in extracts of $\underline{v^{ts1}}$ (22°C) at the same concentrations. Below 0.4 ml, corresponding ODs are too close to the control values to allow detection of significant differences in enzyme activity. While the extract of $\underline{v^{ts1}}$ (29°C) does not exhibit a strictly linear increase with increasing concentration of enzyme, TP activity does increase.

Since linear kinetics with enzyme concentration and time of incubation are important criteria of an enzyme catalyzed reaction, these two results are consistent with the suggestion that at 29°C, the $\underline{v^{ts1}}$ mutant synthesizes an enzyme which has very low but residual activity. An alternative interpretation is that the $\underline{v^{ts1}}$ mutant produces much less enzyme at 29°C than at 22°C but that what enzyme is made is kinetically, relatively normal. (These interpretations are elaborated on in the Discussion).

On the other hand the kinetics of TP activity from $\underline{v^{ts1}}$ (22°C) are almost normal when compared to $\underline{v^{+}}$ enzyme indicating that both the amount and the structure are not greatly different from wild type TP.

TABLE 13 Tryptophan pyrrolase activity at different concentrations of enzyme extract.

a) v^+ TP

mls of enzyme extract	0.05	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80
Activity (mean of 2 determinations)	0.015	0.031	0.056	0.083	0.123	0.145	0.171	0.196	0.228

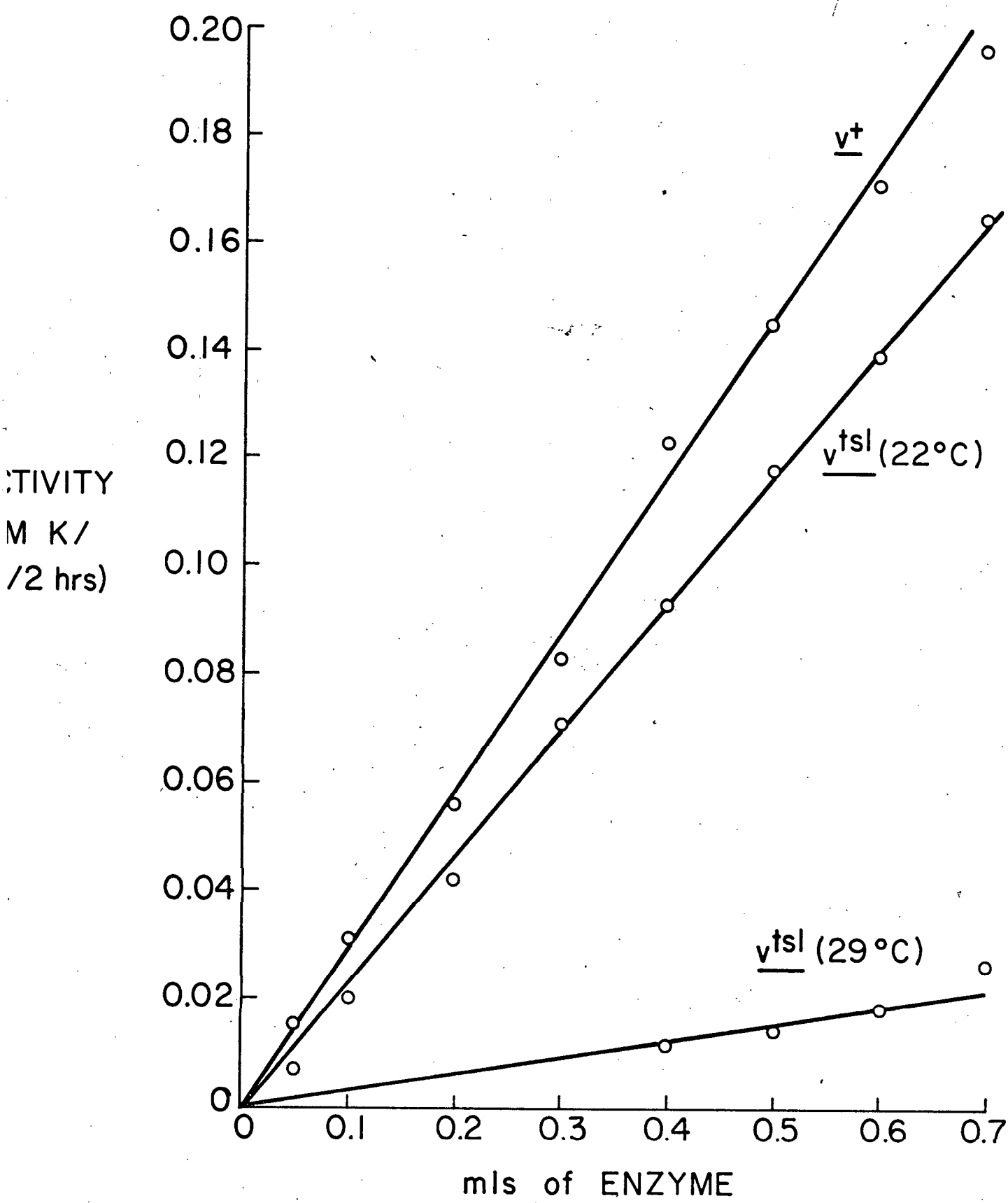
b) v^{ts1} (22°C)TP

mls of enzyme extract	0.05	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80
Activity (mean of 2 determinations)	0.007	0.020	0.042	0.071	0.093	0.118	0.139	0.165	0.182

c) v^{ts1} (29°C)TP

mls of enzyme extract	0.40	0.50	0.60	0.70	0.80
Activity (mean of 2 determinations)	0.011	0.014	0.018	0.026	0.032

Figure 16 Tryptophan pyrrolase activity at different concentrations of enzyme extract.


0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
mls of ENZYME
v+
vtsl (22°C)
vtsl (29°C)
TIVITY
M K/
/2 hrs)

Substrate effect and Michaelis-Menten Coustants (Kms)

Crude enzyme preparations from $\underline{v^+}$ and $\underline{v^{ts1}}$ flies raised at 22°C were used to determine the effect of varying substrate concentration on enzyme activity thereby providing optimal substrate concentrations and Kms for the enzymes from the two genotypes. The standard reaction system was used in each case except that the final reaction mixture concentration of l-tryptophan varied between 0.05 - 0.8 mM as indicated in Table 14. For $\underline{v^+}$ enzyme, 0.4 ml of enzyme extract was added to the standard reaction system and duplicates at each substrate concentration were established. Each tube was incubated for 2 hours at 41°C. Kynurenine formation was estimated in aliquots of the TCA supernatant as usual.

Two determinations were made at each substrate concentration with separately prepared and assayed enzyme. The mean of these two determinations at each substrate concentration is shown in Table 14. Figure 17 records TP activity in μM kynurenine/ml/g flies/2 hours as a function of substrate concentration in mM and shows that TP activity increases rapidly as substrate concentration is increased and peaks at about 7 mM l-tryptophan. An approximate rectangular hyperbola, typical of simple enzyme-substrate reactions, is obtained. The optimal substrate concentration is 5-7 mM l-tryptophan which is similar to previous determinations of this parameter (MARZLUF 1965; TARTOF 1969; BAILLIE AND CHOVNICK 1971).

When the reciprocal of the substrate concentration in moles

TABLE 14 Effect of varying l-tryptophan concentration on tryptophan pyrrolase activity in v^+ extracts.

mls of 20 mM l-tryptophan	0.05	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80
l-tryptophan final concentration (mM)	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0
OD_{560} (mean of 2 determinations)	0.180	0.320	0.451	0.530	0.601	0.621	0.638	0.641	0.640
Activity (µM kynurenine/ml /2 hours)	0.032	0.057	0.082	0.095	0.108	0.111	0.114	0.115	0.115
Specific activity (µM kynurenine/ml /g flies/2 hours)	0.240	0.429	0.612	0.714	0.807	0.834	0.858	0.862	0.862

is plotted against the reciprocal of the velocity of the reaction measured as µM kynurenine/ml/2 hours of incubation (Table 15) (LINEWEAVER - BURK 1934), a straight line provides the best fit (Figure 18). The line cuts the x axis at -1/Km of -640 M thereby providing an estimated Km for $\underline{v^+}$ TP of 1.56×10^{-3} M. This value corresponds closely to the value of 1.53×10^{-3} M reported by TARTOF (1969) but is somewhat higher than the 1×10^{-3} M reported by MARZLUF (1965). The intercept on the y axis gives a theoretical maximum velocity (Vmax) of 1.10 µM kynurenine/ml/g flies/2 hours.

A similar analysis was carried out for TP activity of $\underline{v^{ts1}}$ (22^oC) flies (Table 16, Figure 17). As can be seen from the Figure, the relationship between substrate concentration and TP activity is similar to that of the $\underline{v^+}$ enzyme except that the activity of the $\underline{v^{ts1}}$(22^oC) enzyme is lower than $\underline{v^+}$ enzyme at all substrate concentrations. The optimal substrate concentration is 5-7 mM l-tryptophan, again similar to $\underline{v^+}$ TP. From the LINEWEAVER-BURK regression plot (Figure 18), the Km for TP extracted from 22^oC - grown $\underline{v^{ts1}}$ flies is approximately 1.74×10^{-3} M which indicates only slightly less affinity for substrate than $\underline{v^+}$ TP. The Vmax of the $\underline{v^{ts1}}$(22^oC) TP reaction is obtained from the y axis intercept of Figure 18 and is 0.750 µM kynurenine/ml/g/2 hours. This is about 78% of $\underline{v^+}$ Vmax indicating that the rate of breakdown of the $\underline{v^{ts1}}$(22^oC) TP - substrate complex is only slightly retarded compared with $\underline{v^+}$ TP.

The effect of varying substrate concentration on TP activity of 29^oC - reared $\underline{v^{ts1}}$ flies was determined using the standard assay

TABLE 15 Lineweaver-Burk regression analysis of substrate effect on $\underline{v}^+$ tryptophan pyrrolase activity.

Substrate concentration (mM)	0.05	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80
Reciprocal of substrate concentration (1/s in M)	2000	1000	500	333	250	200	167	143	125
Velocity kynurenine/ml /2 hours)	0.032	0.057	0.082	0.095	0.108	0.111	0.114	0.115	0.115
Reciprocal of velocity (1/v in µM kynurenine/ ml/2 hours)	31.3	17.5	12.2	10.5	9.3	9.0	8.8	8.7	8.7

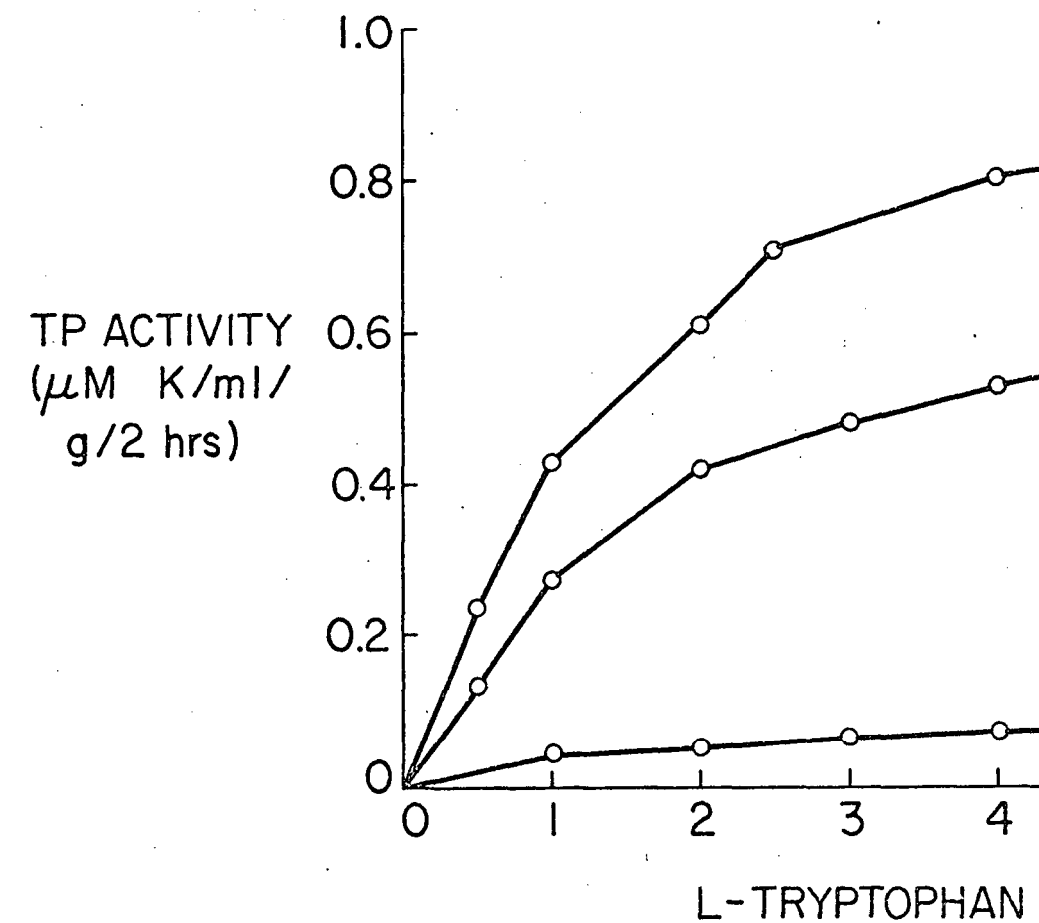
TABLE 16 Effect of varying l-tryptophan concentration on tryptophan pyrrolase activity in 22°C - grown v^{ts1} enzyme extracts and Lineweaver-Burk regression analysis.

l-tryptophan (mM)	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0
OD_{560} (mean of 2 determinations)	0.101	0.206	0.312	0.357	0.402	0.422	0.433	0.427	0.422
Activity (µM kynurenine/ ml/2 hours)	0.018	0.037	0.056	0.064	0.071	0.076	0.078	0.077	0.076
Specific activity (µM kynurenine/ ml/g flies/ 2 hours)	0.138	0.279	0.420	0.480	0.532	0.570	0.585	0.577	0.570
1/S (M)	2000	1000	500	333	250	200	167	143	125
1/v(µM kynurenine/ ml/2 hours)	54.3	26.9	17.9	15.6	14.1	13.2	12.6	12.9	13.2

TABLE 17 Effect of varying l-tryptophan concentration on tryptophan pyrrolase activity in 29°C - grown v_{ts1} enzyme extracts and Lineweaver-Burk regression analysis.

l-tryptophan (mM)	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0
OD_{560} (mean of 2 determinations)	0.106	0.162	0.207	0.214	0.246	0.233	0.228	0.215
Activity (µM kynurenine/ ml/ 2 hours)	0.0120	0.0180	0.0234	0.0240	0.0275	0.0260	0.0250	0.0240
Specific activity (µM kynurenine/ ml/g flies 2 hours	0.048	0.054	0.069	0.072	0.083	0.078	0.075	0.072
1/S (M)	1000	500	333	250	200	167	143	125
1/v (µM kynurenine/ ml/2 hours)	85.5	53	43.4	41.7	36.4	38.5	40	41.7

FIGURE 17 Tryptophan pyrrolase activity as a function of substrate concentration.


TP ACTIVITY
(μM K/ml/
g/2 hrs)
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
L-TRYPTOPHAN

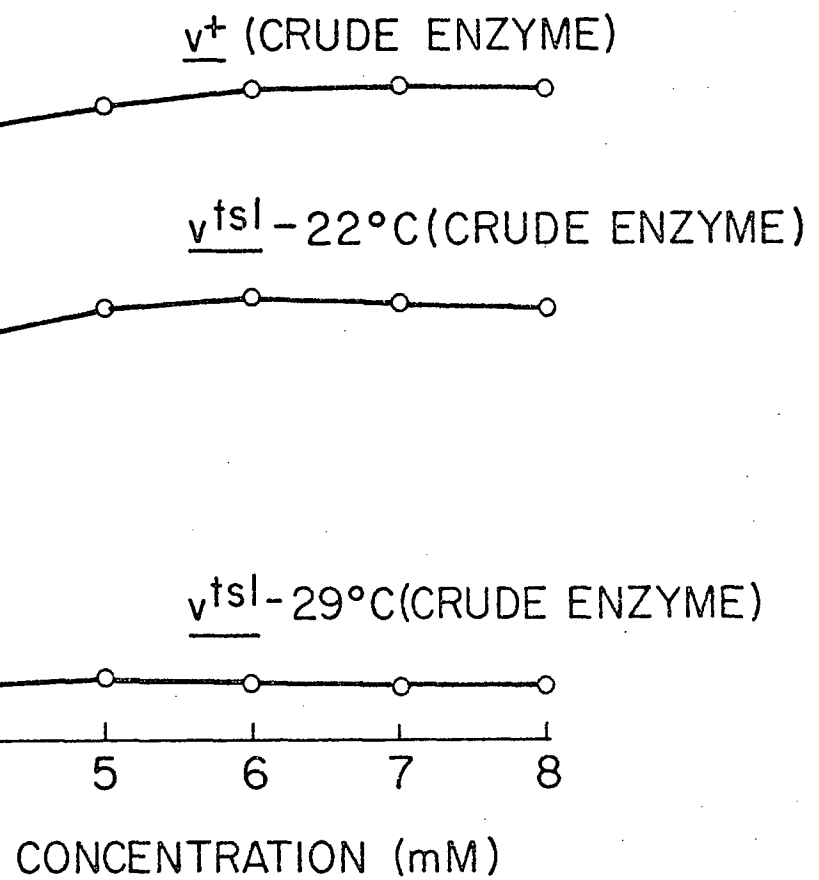
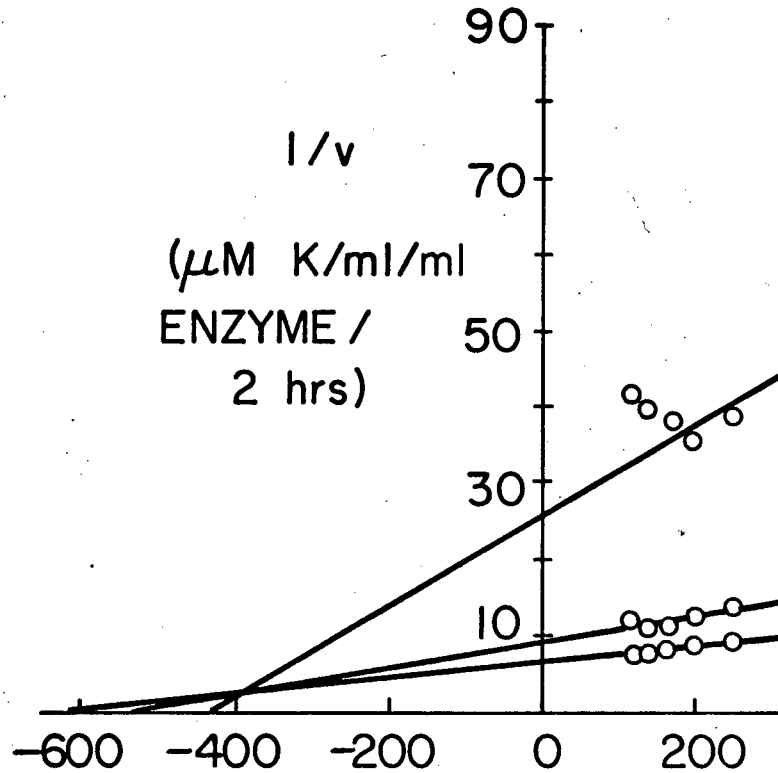
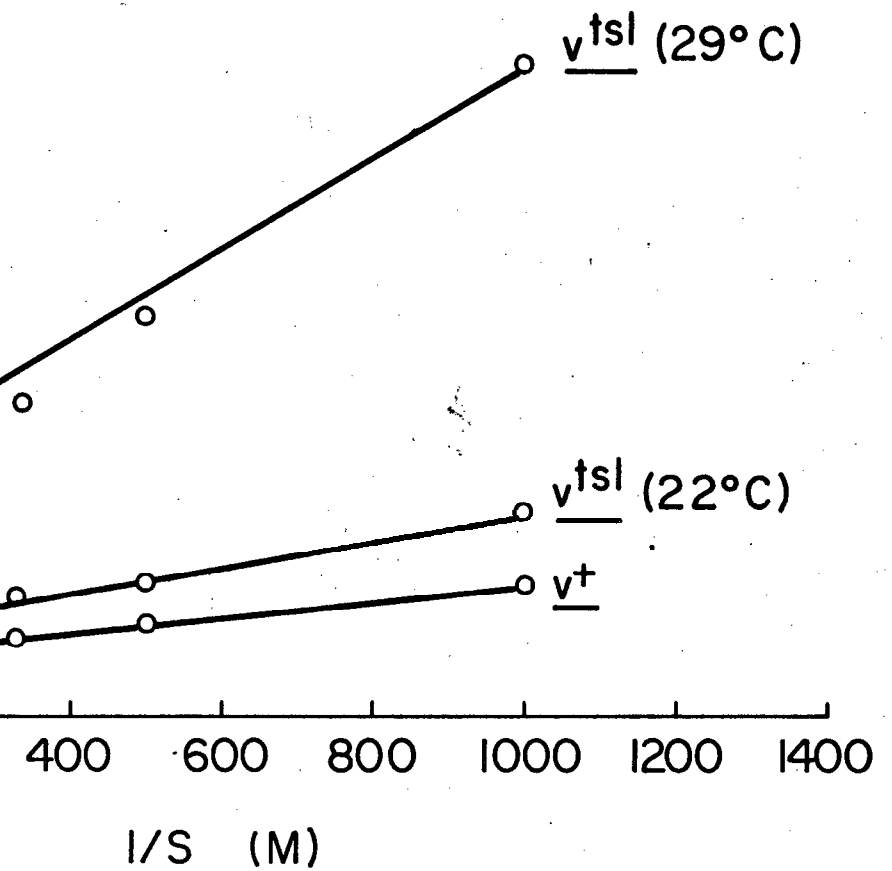

v+ (CRUDE ENZYME)
vtsl - 22°C (CRUDE ENZYME)
vtsl - 29°C (CRUDE ENZYME)
5
6
7
8
CONCENTRATION (mM)

FIGURE 18 Lineweaver-Burk regression plots.


90
70
50
30
10
1/v
(μM K/ml/ml
ENZYME /
2 hrs)
-600
-400
-200
0
200


v tsl (29° C)
v tsl (22° C)
v+
400
600
800
1000
1200
1400
I/S (M)

procedure except that 0.8 ml of enzyme was used in each eaction tube and 1.6 mls of TCA supernatant were analyzed for kynurenine content. Both of these modifications increase the OD range obtained for this enzyme preparation. The results of these determinations are given in Table 17 and plotted in Figure 17. It can be seen that TP activity increases gradually with increasing substrate concentration and peaks at an optimal substrate concentration of about 5 mM l-tryptophan. The plot is not a rectangular hyperbola characteristic of the relationship between enzyme activity and substrate concentration of both $\underline{v^+}$ and $\underline{v^{ts1}}$(22°C) TP. This reflects the very low activities detectable and is suggestive of an alteration in the enzyme-substrate relationship such that TP from 29°C - reared flies has less affinity for substrate than the enzyme from 22°C - raised $\underline{v^{ts1}}$ flies.

The LINEWEAVER-BURK linear regression treatment of these data (Table 17) is plotted in Figure 18 and indicates a difference in Km of the 29°C - grown $\underline{v^{ts1}}$ enzyme compared with either $\underline{v^+}$ TP or 22°C - grown $\underline{v^{ts1}}$ TP. From the x axis intercept of Figure 18 the Km value for $\underline{v^{ts1}}$(29°C) TP is approximately 2.3×10^{-3}M, a value significantly greater than that determined for $\underline{v^+}$ TP (1.56×10^{-3}M) and $\underline{v^{ts1}}$(22°C) TP (1.78×10^{-3}M). This suggests that the enzyme from the $\underline{v^{ts1}}$ mutant grown at 29°C has considerably less affinity for substrate than do the enzymes from the other two sources. This indicates that $\underline{v^{ts1}}$(29°C) TP possibly has tertiary or quaternary structural differences resulting in a less efficient catalytic re-

action compared to the wild type or $\underline{v^{ts1}}$(22°C) enzymes.

The Vmax for $\underline{v^{ts1}}$(29°C) TP, calculated from the y axis intercept of Figure 18, is equivalent to an activity of 0.12 µM kynurenine/ml/g flies/2 hours of incubation, which is about 10% of wild type TP. This shows that the rate of breakdown of the enzyme-substrate complex is severely retarded compared to both $\underline{v^{+}}$ and $\underline{v^{ts1}}$ (22°C) TP. This is usually thought to be due to an alteration in the structure of the catalytic site of the enzyme (DIXON AND WEBB 1964).

Partial purification procedure and specific activities of TP derived from v^{+} and v^{ts1} flies raised at 22° and 29°C.

TP was partially purified from $\underline{v^{+}}$ and $\underline{v^{ts1}}$ stocks in order: (i) to carry out a more precise comparison of enzyme activities between them; (ii) to establish whether the enzyme derived from 29°C - raised $\underline{v^{ts1}}$ flies could be concentrated by a procedure which does purify for the activity of $\underline{v^{+}}$ enzyme; and (iii) to analyze temperature effects on the enzymes derived from the three sources.

The crude enzyme preparations from the three sources were subjected to ammonium sulfate fractionation as outlined in the Materials and Methods. Protein measurements of crude and partially purified preparations were made so that specific activities could be obtained.

The steps in the purification procedure for $\underline{v^{+}}$ enzyme and the results obtained with a given initial weight of flies are

given to illustrate the methods involved in obtaining the purification data shown in Table 19. Different experiments varied only in that the initial weight of flies differed in each case.

(i) 5.3 g of $\underline{v^+}$ females and males were collected. Therefore, the volume of homogenizing buffer for a 1:5 weight to volume ratio was 26.5 mls.

(ii) After homogenization, centrifugation and filtration, the volume of crude supernatant was 15.7 mls (5.3 g dry weight of flies is equivalent to 15.7 mls of crude enzyme extract).

(iii) The supernatant was recentrifuged at 48,000 x g for an additional 30 minutes. After filtration, the supernatant volume was 12.6 mls.

(iv) Cold, saturated ammonium sulfate was added dropwise to bring the crude enzyme extract to 42% saturation, 9.1 mls of saturated ammonium sulfate solution were required. The total volume of the preparation was then 21.7 mls.

(v) The preparation was centrifuged at 30,000 x g for 10 minutes and the supernatant was collected by filtering through a Whatman No. 4 filter paper. The filtrate volume was then 13.6 mls.

(vi) Addition of 21.6 mls of saturated ammonium sulfate to the supernatant increased the concentration of ammonium sulfate to 59%. The preparation was centrifuged at 30,000 x g for 10 minutes, the pellet retained and dissolved in 1/6 of the initial homogenizing buffer volume (= 4.4 mls).

The protein content of the crude homogenate was assayed in duplicates of 0.1 and 0.2 ml of a 1:10 dilution of the fraction. Similarly, protein contents of the ammonium sulfate fractions were assayed in duplicates of 0.1 and 0.2 ml of a 1:20 dilution of the material precipitating between 40 and 60% ammonium sulfate saturation. Results of both of these determinations are presented in Table 18. As can be seen in the Table, ammonium sulfate fractionation results in a net increase in the amount of protein/ml of the fraction (18 mg protein/ml compared to 12 mg protein/ml in the crude extract).

Similar preparations of $\underline{v^{ts1}}$(22°C) TP and $\underline{v^{ts1}}$(29°C) TP were made and their protein contents determined. In each case the protein content was approximately the same as the equivalent $\underline{v^{+}}$ fraction.

As shown by the purification data in Table 19, ammonium sulfate fractionation increases the specific activity of $\underline{v^{+}}$ TP 2 - 3 fold (0.053 units compared to 0.024 units).

Similar preparations were made of TP extracted from $\underline{v^{ts1}}$ flies grown at both 22° and 29°C, and they were assayed under the same conditions as for $\underline{v^{+}}$ enzyme. The results of these determinations are indicated in Table 20.

The TP enzymes from both 22° - and 29°C - raised $\underline{v^{ts1}}$ flies also were concentrated by ammonium sulfate fractionation, slightly more so in the case of $\underline{v^{ts1}}$(29°C) TP. Since similar preparations of enzymes from all three sources yielded similar amounts of protein

TABLE 18 Protein contents of v^+ TP fractions.

a)				
mls of crude extract	0.1	0.1	0.2	0.2
OD_{600}	0.242	0.240	0.478	0.481
µg protein	123	120	240	242
mean mg protein/ml of crude extract		12*		
b)				
mls of 40-60% ammonium sulfate fraction	0.1	0.1	0.2	0.2
OD_{600}	0.180	0.178	0.362	0.359
µg protein	90	88	180	178
mean mg protein/ml of fraction		18		

* This was calculated as follows: Average ug protein/0.1 ml crude extract = 121.5. Therefore, in 1.0 ml of a 1:10 dilution there are 1215 µg/ml, and in the original crude homogenate there are 12,150 µg/protein/ml or approximately 12 mg/ml. Mean mg protein/ml of ammonium sulfate fraction was similarly calculated.

TABLE 19 Purification of v^+ tryptophan pyrrolase.

	TP ACTIVITY (µM KYNURENINE/ml/ml ENZYME/2 HOURS	VOLUME (ml)	mg PROTEIN PER ml	TOTAL ACTIVITY	SPECIFIC ACTIVITY (PER mg PROT.)
CRUDE EXTRACT	0.288*	15	12	4.32	0.024
40-60% AMMONIUM SULFATE FRACTION	0.957	4	18	3.83	0.053

* Activity was calculated as in the following example: 0.2 and 0.4 mls of enzyme extract were assayed in the standard reaction mixture with normal controls. For 0.2 ml of enzyme extract, the OD of 0.8 ml of a 3.2 ml TCA supernatant was 0.348 and the OD of the TCA control was 0.028. Therefore, net OD was 0.320, which is equivalent to 0.0143 µM kynurenine/ml/2 hours. Hence, in 3.2 ml supernatant there are 0.0143 x 3.2/0.8 µM kynurenine/ml/2 hours. This is equivalent to the kynurenine released by 0.2 ml of enzyme extract. Therefore, the kynurenine released by 1.0 ml of enzyme is 0.0143 x 3.2/0.8 x 1/0.2 = 0.286 µM kynurenine/ml/ml enzyme/2 hours. Similarly, 0.4 ml of enzyme released kynurenine calculated as 0.290 µM kynurenine/ml/ml enzyme/2 hours. Therefore, mean activity is 0.288 µM kynurenine/ml/ml enzyme/2 hours. Now, 1.0 ml of enzyme is equivalent to 1/3 g of flies and contains 12 mg protein/ml therefore, specific activity is 0.864 µM kynurenine/ml/g flies/2 hours or 0.024 units/mg protein. The activity and specific activity of the ammonium sulfate fraction were similarly calculated.

TABLE 20 Purification of v^{ts1}(22° and 29°C) tryptophan pyrrolase.

	TP ACTIVITY (μM/ML/ML ENZYME/2 HRS.)	VOLUME (MLS)	MG PROTEIN /ML	TOTAL ACTIVITY	SPECIFIC ACTIVITY (PER MG PROT.)
i) CRUDE EXTRACT					
a) v^{ts1}(22°C)	0.230	20	12.5	4.6	0.0184
b) v^{ts1}(29°C)	0.031	16	12.0	0.5	0.0026
ii) 40-60% AMMONIUM SULFATE FRACTION					
a) v^{ts1}(22°C)	0.778	5	19	3.9	0.0410
b) v^{ts1}(29°C)	0.069	4	18	0.3	0.004

in all fractions assayed (Tables 18 and 20), differences in enzyme activities between them are not due to differences in protein contents per se. Thus, measurements of enzyme activities on a dry weight of flies basis, permit valid comparisons of TP activities between these different strains.

Since ammonium sulfate fractionation concentrates the activities of both v^{ts1}(22°C) TP and v^{ts1}(29°C) TP (Table 20), this supports the contention that the mutant makes an enzyme which is approximately normal if grown at 22°C but which is catalytically deficient if grown at 29°C. Therefore, the catalytic deficiency could be due to a structural alteration in tryptophan pyrrolase synthesized by v^{ts1} at 29°C or it could result from the synthesis of a reduced amount of normal TP at 29°C.

Temperature effects on TP activity in v^{+}, v^{ts1}(22°C) and v^{ts1}(29°C) flies.

(a) v^{+} TP: The effect of varying incubation temperature on TP activity was studied with crude preparations of the enzyme. Results of these studies are shown in Table 21. Means of determinations made in 2 tests are plotted in Figure 19. The data show that the reaction rate increases to 41°C after which it declines. This decline may indicate that the enzyme loses stability at these higher temperatures over a 2 hour incubation period.

The data of Table 21 were used to construct an Arrhenius plot to establish the energy of activation for the v^{+} enzyme.

TABLE 21 Effect of temperature of incubation on v^+ TP activity and Arrhenius plot values.

TEMPERATURE (^oC)	22°	25°	30°	35°	37°	41°	45°	50°	55°
ACTIVITY (μM KYNURENINE/G /2 HOURS)	0.221	0.271	0.394	0.572	0.706	0.865	0.784	0.623	0.410

ARRHENIUS PLOT VALUES

T (ABSOLUTE TEMPERATURE ^oK)	295	298	303	308	310	314	318	323	328
$1/T \times 10^5$	339	336	330	325	323	318	314	310	305
v(μM KYNURENINE/ML/G /2 HOURS)	0.221	0.271	0.394	0.572	0.706	0.865	0.784	0.623	0.410
$LOG_{10}v$	$\bar{1}.3444$	$\bar{1}.4330$	$\bar{1}.5955$	$\bar{1}.7574$	$\bar{1}.8488$	$\bar{1}.9370$	$\bar{1}.8943$	$\bar{1}.7938$	$\bar{1}.6128$

The Arrhenius equation relating a velocity constant K to the absolute temperature of incubation (T) is given by:

$$2.3 \log K = B - Ea/RT$$

where: $\log K = \log_{10}$ of velocity constant

B = constant

R = gas constant (1.98 cal/mole/degree)

Ea = energy of activation in cal/mole

The values obtained for the Arrhenius plot are given in Table 21 and the plot of the absolute temperature against the log of the activity is seen in Figure 20. The plot for $\underline{v^+}$ TP gives a straight line of best fit with an inflection point at a temperature corresponding to 41°C. The inflection point separates a zone where increasing temperature results in increasing enzyme activity from a zone where increasing temperature results in gradual inactivation of the enzyme. The slope of the line describing the increasing activity with increasing temperature is given by Ea/2.3R so that,

$$Ea = \text{slope} \times 2.3 \times 1.98 \text{ cal/mole}$$

$$= 0.3000/10 \times 10^{-5} \times 2.3 \times 1.98 \text{ cal/mole}$$

$$= 13{,}660 \text{ cal/mole}$$

This value corresponds reasonably well with the value of 12,800 cal/mole determined by MARZLUF (1965).

(b) $\underline{v^{ts1}}$(22°C) TP: Thermal effects on the catalytic activity of TP in $\underline{v^{ts1}}$ flies reared at 22°C were measured in a similar fashion. These results and the Arrhenius plot values of three parallel experiments using independently prepared and assayed

$\underline{v^{ts1}}$(22°C) TP are seen in Table 22. The Arrhenius values are plotted in Figure 20. It is clear that more variability exists in this case compared to $\underline{v^{+}}$ enzyme. However, the data permit the following general conclusions to be drawn:

(i) Optimal TP activity in $\underline{v^{ts1}}$(22°C) flies is more dependent on a limited temperature range (35°-41°C) in comparison with $\underline{v^{+}}$ TP (in which good activity is recovered in the range 30°-50°C). TP activity of 22°C - reared $\underline{v^{ts1}}$ flies falls off at an increased rate at high temperatures compared with $\underline{v^{+}}$ TP. However, the optimal temperature for $\underline{v^{ts1}}$(22°C) TP activity is 41°C which is the same as $\underline{v^{+}}$ TP showing that the former enzyme is not significantly different in thermal properties to the wild type enzyme.

(ii) The variability in assays of $\underline{v^{ts1}}$(22°C) TP activity with increasing incubation temperature results in an Arrhenius plot which is not a particularly satisfactory straight line. However, assessing the slope from the line of best fit as drawn, provides the following data for an energy of activation determination:

$$\begin{aligned} Ea &= \text{slope} \times 2.3 \times 1.98 \text{ cal/mole} \\ &= 0.3000/7.5 \times 10^{5} \times 2.3 \times 1.98 \text{ cal/mole} \\ &= 18{,}220 \text{ cal/mole} \end{aligned}$$

Thus, an apparent increase in the energy of activation is obtained compared with v^{+} TP.

(c) $\underline{v^{ts1}}$(29°C)TP: Owing to the low level of TP activity in 29°C - reared $\underline{v^{ts1}}$ flies, thermal effects on this enzyme were determined using 0.4 ml of ammonium sulfate fractionated enzyme

TABLE 22 Effect of temperature of incubation on v^{ts1} ($22^{o}C$) TP activity.

TEMPERATURE (^{o}C)	22^{o}	25^{o}	27^{o}	30^{o}	35^{o}	37^{o}	41^{o}	45^{o}	50^{o}
ACTIVITY (μM KYNURENINE/ML /G/2 HOURS)									
(i)	0.024	0.083	0.126	0.168	0.398	0.467	0.676	0.525	0.086
(ii)	0.061	0.142	0.178	0.226	0.464	0.575	0.731	0.641	0.130
(iii)	0.041	0.123	0.175	0.200	0.455	0.548	0.750	0.544	0.102
MEAN	0.042	0.116	0.166	0.198	0.439	0.530	0.719	0.570	0.106

ARRHENIUS PLOT VALUES

T (ABSOLUTE TEMPERATURE ^{o}K)	295	298	300	303	308	310	314	318	323
$1/T \times 10^{5}$	339	336	333	330	325	323	318	314	310
LOG_{10} v	$\bar{2}.6232$	$\bar{1}.0645$	$\bar{1}.2068$	$\bar{1}.2967$	$\bar{1}.6427$	$\bar{1}.7243$	$\bar{1}.8567$	$\bar{1}.7559$	$\bar{1}.0253$

TABLE 23 Effect of temperature of incubation on v^{ts1} (29°C) TP activity.

TEMPERATURE (°C)	27°	30°	35°	37°	41°	45°	50°
ACTIVITY (μM KYNURENINE/ ML/G/ 2 HOURS							
(i)	0.030	0.106	0.153	0.188	0.202	0.107	0.111
(ii)	0.056	0.132	0.169	0.198	0.214	0.133	0.023
MEAN	0.042	0.119	0.161	0.193	0.208	0.120	0.017

ARRHENIUS PLOT VALUES

T(ABSOLUTE TEMPERATURE °K)	300	303	308	310	314	318	323
$1/T \times 10^5$	333	330	325	323	318	314	310
LOG_{10} v	$\bar{2}.6233$	$\bar{1}.0755$	$\bar{1}.2068$	$\bar{1}.2856$	$\bar{1}.3181$	$\bar{1}.0792$	$\bar{2}.2305$

FIGURE 19 Effect of temperature of incubation on tryptophan pyrrolase activity of $\underline{v^{+}}$, $\underline{v^{ts1}}$(22°C - raised) and $\underline{v^{ts1}}$(29°C - raised) flies.

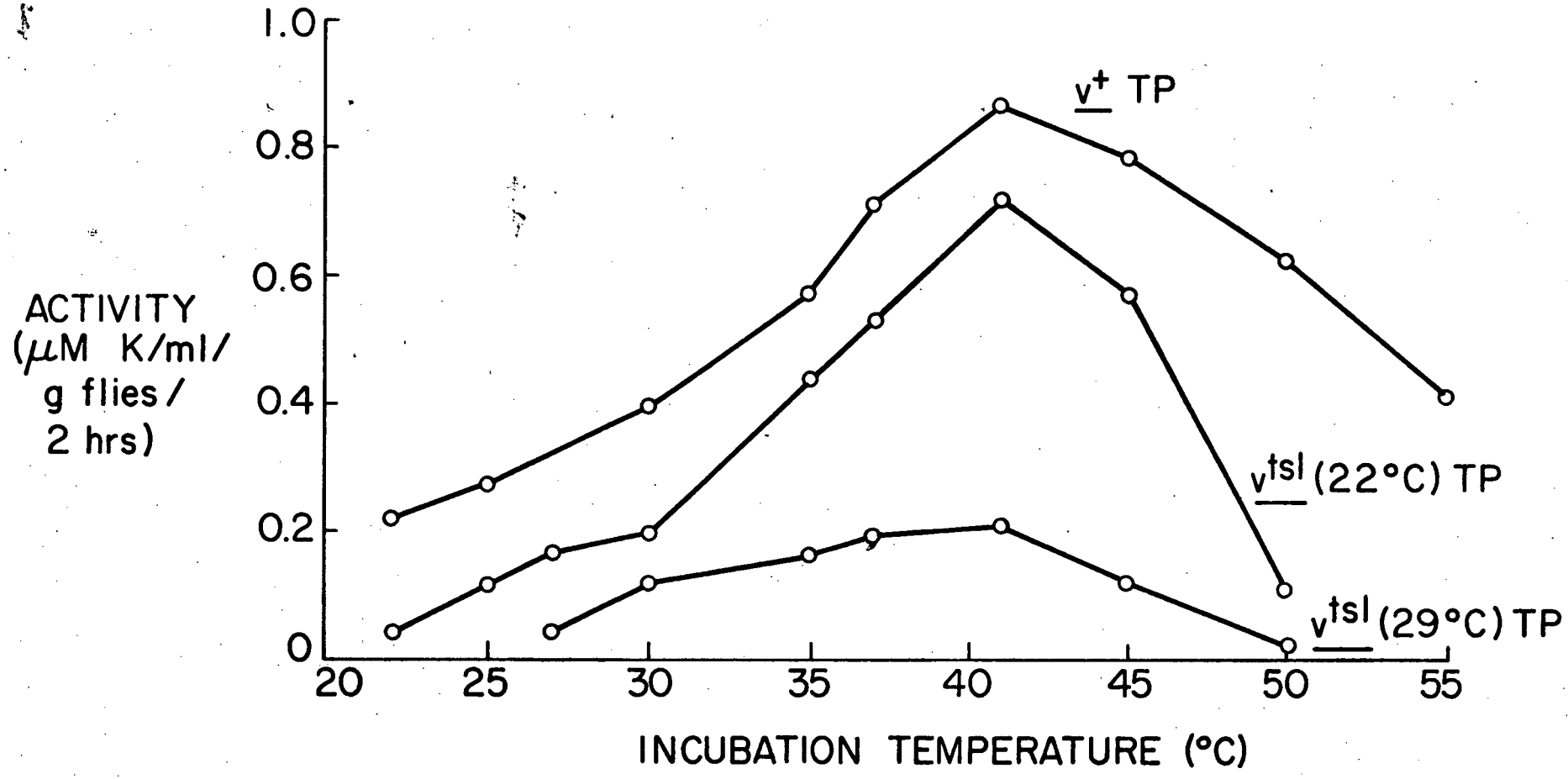
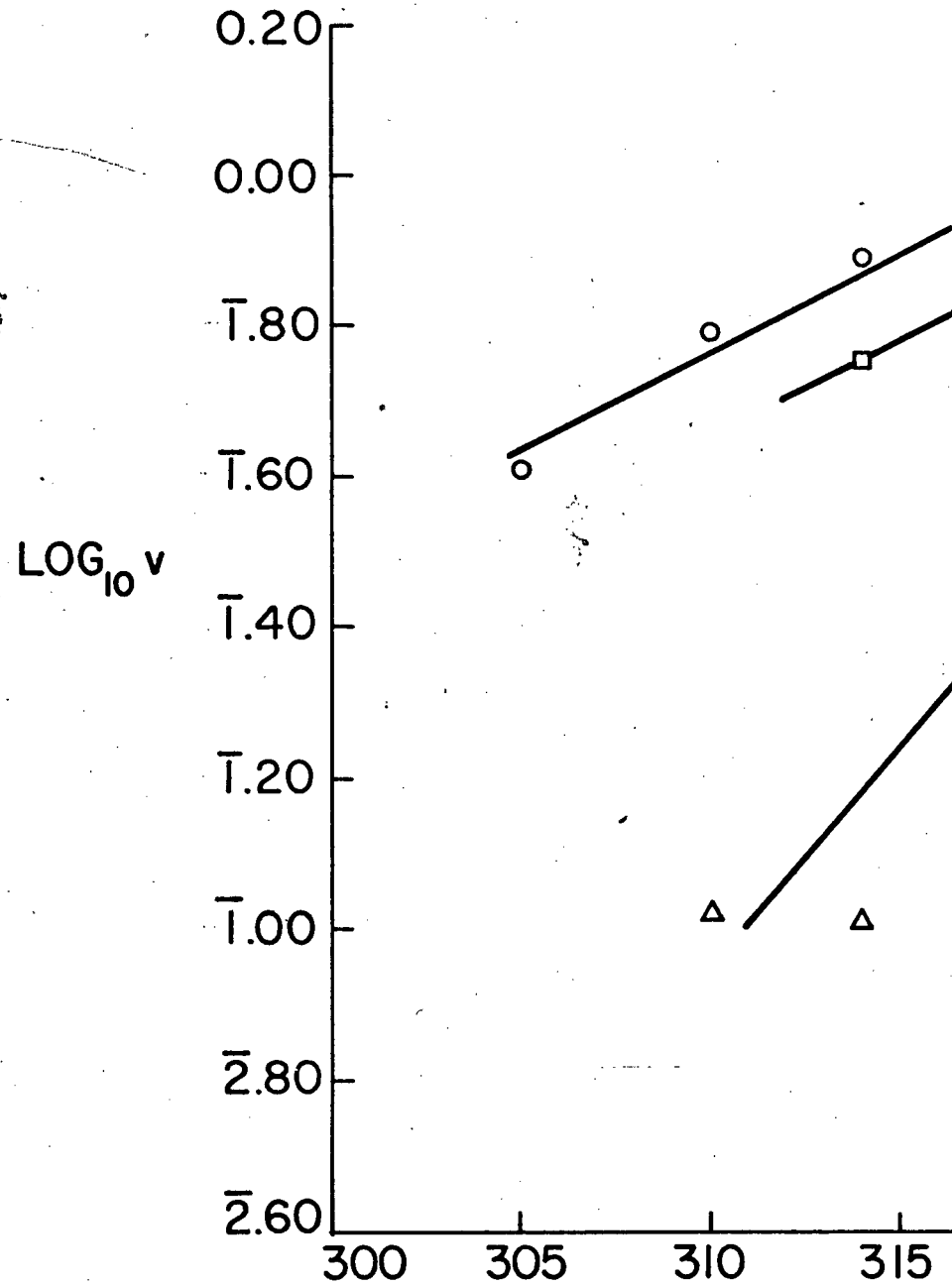
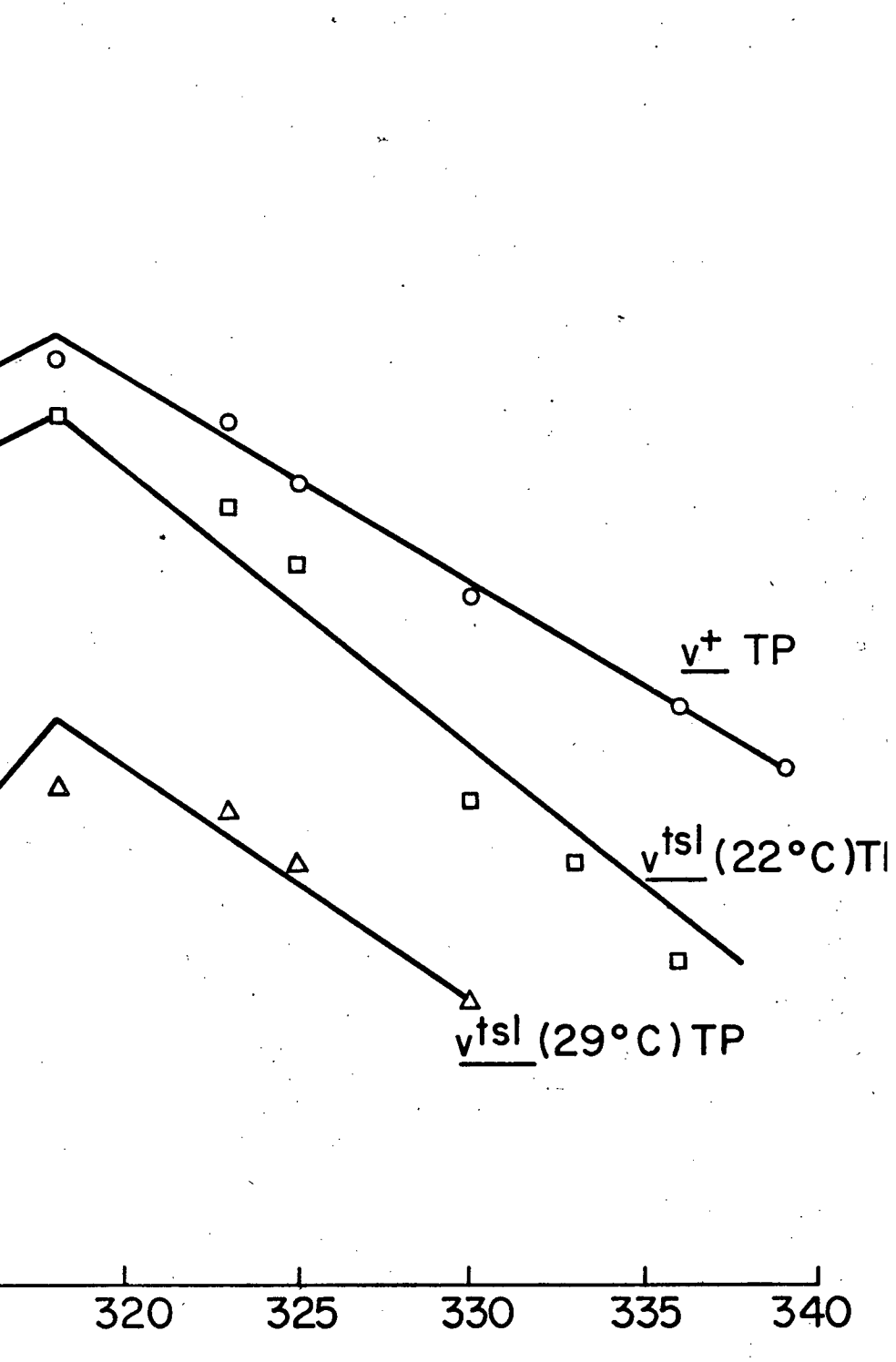

1.0
0.8
0.6
0.4
0.2
0
ACTIVITY
(μM K/ml/
g flies/
2 hrs)
v+ TP
vtsl (22°C) TP
vtsl (29°C) TP
20
25
30
35
40
45
50
55
INCUBATION TEMPERATURE (°C)

FIGURE 20 Arrhenius plots for $\underline{v^+}$, $\underline{v^{ts1}}$(22°C - raised) and $\underline{v^{ts1}}$(29°C - raised) TPs.


0.20
0.00
$\bar{1}$.80
$\bar{1}$.60
$\bar{1}$.40
$\bar{1}$.20
$\bar{1}$.00
$\bar{2}$.80
$\bar{2}$.60
LOG_{10} v
300
305
310
315


v+ TP
vtsl (22°C)TP
vtsl (29°C) TP
320
325
330
335
340

under standard reaction conditions except that the incubation temperature ranged from 27° to 50°C. Results of two separate determinations at each temperature with independently prepared and assayed ammonium sulfate fractions are indicated in Table 23.

The relationship between enzyme activity and temperature of incubation is not readily analysable (Figure 19). Activity increases with increasing incubation temperature and maximum TP activity again occurs at 41°C. However, the peak is not as defined as with $\underline{v^+}$ and $\underline{v^{ts1}}$(22°C) TPs, probably reflecting the very low enzyme activities recovered. Although activity falls off more rapdily above 41°C compared with $\underline{v^+}$ and $\underline{v^{ts1}}$(22°C)TP, the difference is not pronounced, suggesting only slightly increased thermolability at higher temperatures.

The Arrhenius plot values, given in Table 23, are graphed in Figure 20 and do not yield a satisfactory straight line of best fit. Calculations of the energy of activation of $\underline{v^{ts1}}$(29°C) TP based on these data therefore are provisional at best. Using the tabulated values rather than the graphical version, the energy of activation is about 17,000 cal/mole, elevated from that obtained for $\underline{v^+}$ enzyme (13,660 cal/mole) but in the range of that calculated for $\underline{v^{ts1}}$(22°C) TP (18,200 cal/mole).

The enzymes from all three sources appear to have an optimal <u>in vitro</u> incubation temperature range of 37°- 41°C. Above that, inactivation of the enzymes occur during a 2 hour incubation. This inactivation appears to be more severe with $\underline{v^{ts1}}$(29°C)TP, but is

only slightly greater with $\underline{v^{ts1}}$(22°C)TP compared with $\underline{v^{+}}$ TP. Thus, the temperature-sensitivity of the $\underline{v^{ts1}}$ allele cannot be attributed to a significant increase in thermolability of TP at least under these *in vitro* conditions.

III. Comparison of TP activities in various strains of *Drosophila melanogaster*.

Dosage compensation at the enzyme level (males with one dose of $\underline{v^{+}}$ have at least as much TP activity as females with two $\underline{v^{+}}$ doses) has already been reported by TARTOF (1969); TOBLER, BOWMAN AND SIMMONS 1971; and BAILLIE AND CHOVNICK (1971). These results have been repeated for $\underline{v^{+}}$ and extended to include $\underline{v^{ts1}}$ and $\underline{v}$ deficiency heterozygotes with a view to clarifying the nature of the phenomenon.

In addition, the relationship between the visible eye colour phenotype and TP activity could be determined. Various genotypes involving $\underline{v^{+}}$, $\underline{v^{1}}$, $\underline{v^{ts1}}$, $\underline{v^{0f}}$ and $\underline{Df(1)v^{L3}}$ were constructed and males and females, where appropriate, were assayed for TP activity.

Wild type dosage compensation.

Males and females of the Oregon-R wild type strain were grown at 22° and 29°C, collected separately and crude enzyme preparations made of each in identical manner. Assays of TP activity were

performed under the standard reaction conditions and activities were determined in µM kynurenine/ml/g flies/2 hours of incubation at 41°C. The results are shown in Table 24 with the number of separate determinations shown in parenthesis after each activity value. The activities obtained from homogenates made from equal weights of wild type males and females grown at 22° and 29°C respectively, were designated as 100% TP activity for comparison of TP activities from wild type males and females alone.

These data indicate that little difference in TP activity results from growing wild type flies (males or females) at 22° or 29°C. There is only a slight reduction in enzyme activities in enzyme preparations from all wild type sources if the flies are grown at 29° rather than 22°C.

Dosage compensation at the enzyme level in which males with one dose of the $\underline{v^+}$ gene have at least the TP activity of their homozygous sibs, irrespective of whether the flies are grown at 22° or 29°C, clearly exists. In fact, in agreement with the data of TARTOF (1969); TOBLER, BOWMAN AND SIMMONS (1971); and BAILLIE AND CHOVNICK (1971), the males are consistently slightly higher in TP activity than the females, indicating some overcompensation of enzyme activity.

Similar dosage compensation has been observed with other enzymes controlled by sex linked loci in Drosophila melanogaster such as glucose - 6 - phosphate dehydrogenase and 6 - phosphogluconate dehydrogenase (STEELE, YOUNG AND CHILDS 1969; SEECOF, KAPLAN AND

FUTCH 1969; for a review of this subject see LUCCHESI 1973).

Comparative studies were conducted on the enzyme produced by the $\underline{v^{ts1}}$ mutant and the results obtained paralleled the reversal of dosage compensation seen at the phenotypic level in 29°C - reared $\underline{v^{ts1}}$ flies.

Crude enzyme extracts of 22° and 29°C - reared $\underline{v^{ts1}}$ males and females were obtained. Assays of TP activity of these extracts, as well as enzyme homogenates made from equal weights of $\underline{v^{ts1}}$ males and females, were performed under the same conditions as for the equivalent wild type studies. The results are shown in Table 25, where the percent of wild type activity is based on the results of Table 24, from which the activities of $\underline{v^{+}}$ males and females grown at 22° and 29°C respectively, are taken as 100% TP activity.

The data of Table 25 reveal some interesting aspects of the effect of the $\underline{v^{ts1}}$ mutation on TP activity in males and females: (i) When grown at 22°C, $\underline{v^{ts1}}$ males have a slight but reproducible increase in TP activity compared to $\underline{v^{ts1}}$ females grown and assayed under the same conditions. This overcompensation parallels the situation found in the wild type strain; (ii) remarkably, this dosage compensation in enzyme activity is reversed if the $\underline{v^{ts1}}$ strain is raised at 29°C; the hemizygous males now have considerably less TP activity than their homozygous sibs (5% of 29°C wild type activity compared with 17% in the females). Thus, the males with one dose of the $\underline{v^{ts1}}$ mutation have significantly less TP

TABLE 24 TP activities in males and females of Oregon-R wild type strain.

	TP ACTIVITIES AND DEVIATIONS FROM THE MEAN OF FLIES GROWN AT A GIVEN TEMPERATURE		PERCENT OF WILD TYPE ACTIVITY AT A GIVEN TEMPERATURE	
	22°C	29°C	22°C	29°C
WILD TYPE MALES AND FEMALES	0.861 ± 0.03(3)*	0.852 ± 0.03(2)	100	100
WILD TYPE MALES	0.920 ± 0.04(2)	0.895 ± 0.03(2)	107	105
WILD TYPE FEMALES	0.810 ± 0.02(2)	0.808 ± 0.03(2)	94	94

* Numbers in parentheses indicate number of independently prepared and assayed determinations.

activity than the females with two doses of $\underline{v^{ts1}}$, an effect which is correlated with the marked visible difference seen in the eye phenotypes; when grown at 29°C, the males are much more vermilion-like than the females.

To further investigate these effects, heterozygotes for a vermilion deficiency and either $\underline{v^{+}}$ or $\underline{v^{ts1}}$ were generated and raised at both 22° and 29°C. The results of enzyme assays on these heterozygotes are shown in Table 26. Only one determination at each temperature was made with these stocks.

As seen in Table 26, the activity of females heterozygous for a deficiency of the v locus is approximately half that of normal wild type females, irrespective of the temperature at which the flies are grown. This result might be expected if the TP activity of females is a simple addition of the activities contributed by each $\underline{v^{+}}$ allele. Such an interpretation has been advanced by TOBLER, BOWMAN AND SIMMONS (1971) and BAILLIE AND CHOVNICK (1971), to account for similar results obtained for v/+ and Df(1)v/+ females.

The $\underline{Dfv^{L3}}/\underline{v^{ts1}}$ females, raised at 22°C, are decidedly vermilion in phenotype (although distinguishable from $\underline{v^{1}}$ homozygotes) and have a TP activity of 0.134 μM kynurenine/ml/g flies/2 hours of incubation. This is much lower than the value in $\underline{v^{ts1}}$/Y(22°C) males of 0.667 units, even though both genotypes have the same single dose of the $\underline{v^{ts1}}$ allele. Thus, there would seem to be factors, other than a simple dosage of the $\underline{v^{ts1}}$ allele, involved in the control of TP activity in $\underline{v^{ts1}}$/Y(22°C) males and/or $\underline{Dfv^{L3}}/\underline{v^{ts1}}$ females.

TABLE 25 TP activites in v^{ts1} males and females raised at 22° and 29°C.

	TP ACTIVITIES AND DEVIATIONS FROM THE MEAN OF FLIES GROWN AT A GIVEN TEMPERATURE		PERCENT OF WILD TYPE ACTIVITY AT A GIVEN TEMPERATURE	
	22°C	29°C	22°C	29°C
v^{ts1} MALES AND FEMALES (EQUAL WEIGHTS)	0.645 ± 0.04(3)	0.091 ± 0.001(3)	75	10.5
v^{ts1} MALES	0.667 ± 0.03(2)	0.042 ± 0.01(2)	78	5
v^{ts1} FEMALES	0.593 ± 0.03(2)	0.145 ± 0.02(2)	70	17

TABLE 26 TP activities in *vermilion* deficiency heterozygotes.

GENOTYPE	TP ACTIVITIES IN FEMALES RAISED AT A GIVEN TEMPERATURE		PER CENT WILD TYPE ACTIVITY AT A GIVEN TEMPERATURE	
	22°C	29°C	22°C	29°C
$\underline{Df(1)v^{L3}}/+$	0.439	0.458	51	54
$\frac{Df(1)v^{L3}}{v^{ts1}}/+$	0.134	0.047	15.5	5.6

These results are compatible with a dosage compensation mechanism in which the amount of activity of a locus is dependent on dosage of several or entire X chromosome regions rather than the locus itself. However, as will be presented in the Discussion other considerations make the latter possibility quite plausible as well.

When grown at 29°C, the TP activity of $\underline{Dfv^{L3}}/\underline{v^{ts1}}$ females drops to a value closely approximating that obtained with $\underline{v^{ts1}}$/Y males of about 5 - 6% of the equivalent wild type TP activity obtained at this temperature (Table 24.) This result suggests a greater thermolability of TP in $\underline{v^{ts1}}$ males compared to $\underline{v^{ts1}}/\underline{v^{ts1}}$ females. Phenotypes of $\underline{v^{ts1}}$/Y males and $\underline{Dfv^{L3}}/\underline{v^{ts1}}$ females are quite indistinguishable if both are raised at 29°C.

Measurements of TP activities in v strains.

The TP activities in males and females of the $\underline{v^{1}}$ strain and females of the attached-X strain, $\underline{C(1)RM,v^{0f}}$ f/Y, were determined under the same conditions of enzyme preparation and assay as for wild type except that in the assay, 0.8 ml of enzyme extract was used in each incubation mixture and kynurenine was determined in 1.6 ml of TCA filtrate instead of 0.8 ml. Both of these changes were made to bring the OD readings into recordable range.

As shown in Table 27, all v strains tested, whether raised at 22° or 29°C, had negligible TP activities in accordance with

results obtained for these and other v strains by BAGLIONI (1959, 1960); KAUFMAN (1962); MARZLUF (1965); and TARTOF (1969). All TP activities shown in Table 27 are less than 1% of the equivalent v^+ activities grown at either 22° or 29°C.

TABLE 27 TP activities in v strains raised at 22° and 29°C.

GENOTYPE	TP ACTIVITY (μM KYNURENINE/ML/G FLIES/2 HOURS OF INCUBATION)	
	22°C (MEAN ± DEVIATION FROM MEAN)	29°C (MEAN ± DEVIATION FROM MEAN)
v^1 MALES AND FEMALES EQUAL WEIGHTS)	0.007 ± 0.004(2)	0.006 ± 0.003(2)
v^1 MALES	0.005 ± 0.003(2)	0.005 ± 0.003(2)
v^1 FEMALES	0.006 ± 0.003(2)	0.007 ± 0.002(2)
C(1)RM, v^{0f} f/Y FEMALES	0.005 ± 0.003(2)	0.006 (1)

DISCUSSION

The initial object of this investigation was to recover a ts mutation in a gene which controls the activity of a known and assayable enzyme. In addition to providing a method for determining the molecular nature of temperature-sensitivity, the mutant enzyme can be used as a biochemical marker to follow development.

The studies performed in this project fell into three main categories:

(i) The induction and recovery of ts v mutations by mutagenesis and genetic screening;

(ii) The phenogenetics of a ts v mutation for comparison with the known genetic and developmental properties of the v locus;

(iii) A biochemical analysis of the properties of tryptophan pyrrolase (TP), the enzyme known to be controlled by the v locus.

These three aspects will be discussed separately, and then integrated into a model explaining the phenotypic and molecular expression of the v locus.

I. Induction and recovery of v mutations

The potent DNA alkylating agent, EMS, which was used to induce v mutations in the present study, is known to induce missense ts mutations in prokaryotes (KREIG 1963). It has been assumed that EMS also induces a preponderance of missense ts mutations in

Drosophila (SUZUKI 1970). FRISTROM (1970) has argued, however, that ts mutations may be mainly of a deletion type change. Although direct evidence on this point (such as amino acid changes in a protein specified by an EMS-induced mutation, or reversion studies) is lacking, the available indirect evidence argues against FRISTROM's position.

Thus, the majority of EMS-induced mutations in Drosophila, whether lethal or visible, conditional or nonconditional, dominant or recessive, behave as single site point mutations in mapping and complementation studies (SUZUKI 1970). Furthermore, very few EMS-induced lethal mutations are associated with chromosomal rearrangements or deletions (LIM AND SNYDER 1968), although in a recent study, an EMS-induced, non-ts behavioural mutant, wob has been shown to result from a translocation involving the X, second and third chromosomes (GRIGLIATTI, KAUFMAN AND SUZUKI 1973). However, none of several hundred EMS-induced ts lethals analyzed has been found to carry a rearrangement (SUZUKI 1970; TASAKA AND SUZUKI 1973). This contrasts with the finding that of 10 γ-ray-induced ts lethal mutations, 3 were found to be associated with X - autosome translocations (KAUFMAN AND SUZUKI 1974).

The recovery of a ts allele of v with its known effect on tryptophan pyrrolase provides the potential for clarifying the nature of temperature-sensitivity. Recently, MÜLLER-HILL (unpublished observations) has shown that temperature-sensitivity of a mutant for alcohol dehydrogenase results from thermolability of the

enzyme *in vitro*.

In the screening for *v* mutations, the precise frequency of induction of *v* mutations could not be calculated because the total number of chromosomes tested was not counted, the primary object being the recovery of a ts mutation. Nevertheless, it can be pointed out that cytogenetic analysis shows the *v* locus to be located in salivary chromosome band 10A1-2 (GREEN 1952; LEFEVRE 1969) which is one of the largest of all X chromosome bands (LEFEVRE 1969) and is heavily compacted, darkly staining and rich in DNA (RUDKIN 1965). By these criteria, the *v* locus should present a good target for mutation by EMS if, as LEFEVRE (1967) points out, the amount of DNA available for breakage and induced mutation in a given region of the gametic X chromosome is in direct proportion to the amount of DNA in the corresponding portion of the salivary gland X chromosome (based on data of KAUFMAN 1946, and LEFEVRE 1967). Indeed, LINDSLEY AND GRELL (1968) list 5 spontaneous and 7 induced *v* point mutations and GREEN (1952) tested 6 spontaneous and 16 induced *v* mutations, several of which are additional alleles. More recently, LEFEVRE (1967) recovered 26 *v* mutations from the progeny of irradiated males. In the present study, 33 *v* mutations were recovered from the progeny of 13,000 EMS - treated males, thereby demonstrating that the *v* locus is indeed highly mutable.

Significantly, only two of the *v* mutations obtained in this study exhibited temperature-sensitivity. Additionally, none of the representative sample of previously derived *v* alleles was

temperature-sensitive (Table 4). Thus the paucity of ts v mutations recovered in these screens is probably a real reflection of the very low frequency of their occurrence. This may not be surprising in view of the fact that only three mutable sites, separable by crossing over, have been found in the v locus (BAILLIE AND CHOVNICK 1971; SCHALET 1971). These results contrast strikingly with WRIGHT's (1968) report of 3 ts alleles of lethal myospheroid among 1500 mutagenized chromosomes and the high propertion of ts alleles of Y chromosome loci (KEISS AND KAUFMAN - unpublished).

Newly-induced v mutations were detected in females heterozygous for v or a deletion of the locus. One ts allele was recovered in each screen and neither complemented with v point mutants nor was lethal in hemi- or homozygotes. As only deletions for the v locus appear to be lethal (LEFEVRE 1969), we assume that the two v^{ts} mutations are indeed point mutants.

Both v^{ts1} and v^{ts2} are phenotypically "leaky" at the restrictive temperature, that is the mutations do not result in a total loss of v^{+} function. This property is typical of many ts mutations recovered in Drosophila (SUZUKI 1970) and probably reflects a partial rather than a complete loss of function of a thermolabile gene product. Since v^{ts1} was far less leaky at 29°C than v^{ts2}, it was selected for further investigation.

II. Phenogenetics of v^{ts} and v mutations.

The phenogenetic and biochemical studies of v^{ts} and v mutations were predicated on the assumption that the v locus is, or contains, the structural gene for TP. The most compelling evidence for a locus being the structural gene for a particular enzyme is that electrophoretic variants map at the locus. In the absence of technique for distinguishing v mutations by variation in electrophoretic mobility of TP, indirect evidence has been obtained which strongly suggests that v is indeed the structural gene for TP.

Repeatedly it has been shown that mutation at the v locus causes a specific loss in TP activity in constrast with other mutations, such as cn and st which similarly produce a bright-red colour but which result in elevated TP activities (BAGLIONI 1959, 1960; GLASSMAN 1965; TOBLER, SIMMONS AND BOWMAN 1968). Reciprocal transplantation studies indicate that cn and st control distally sequential steps to that controlled by v in the metabolic pathway leading to brown eye pigment formation (BEADLE AND EPHRUSSI 1936, 1937; WAGNER AND MITCHELL 1955) and therefore their elevated TP activities could result from the accumulation of kynurenine due to the metabolic blocks distal to v. This demonstrates that the v locus controls TP activity.

TARTOF (1969) has shown that in suppressed v^{k} flies, a TP is synthesized which is kinetically different to wild type and other

suppressed $\underline{v}^s$ TPs. This is probably due to an alteration in the structure of the TP controlled by $\underline{v}^k$, since su(s) mutations, by themselves, do not cause any change in the activity or kinetics of wild type TP.

BAILLIE AND CHOVNICK (1971) clearly demonstrated that a linear increase in TP activity is a direct function of increase in the dosage of $\underline{v}^+$ alleles, supporting the contention that each $\underline{v}^+$ allele codes the information required for a unit of TP activity.

Finally, the interactions of the enzymes controlled by the various $\underline{v}^s$ alleles with mutations at the su(s) locus, strongly suggest that the extent of restoration of TP activity is dependent upon specific changes in TP structure directed by the particular $\underline{v}^s$ allele (TARTOF 1969; JACOBSON 1971; TWARDZICK, GRELL AND JACOBSON 1971).

As was shown in the REVIEW, the v cistron is probably simple in organization, consisting of just the linear array of nucleotides necessary to encode a TP subunit. Therefore, all v mutations probably represent changes in the nucleotide sequence of the structural gene which give rise to equivalent amino acid changes in the TP subunit. This hypothesis is compatible with the mapping and complementation properties and, as will be discussed later, correlates with the structure of active TP.

Since the two $\underline{v}^{ts}$ mutations recovered in this investigation differ phenotypically, and their complementation properties and the mapping of $\underline{v}^{ts1}$ show that they are point mutations within the v cistron, they probably represent different missense mutations in

the v structural gene which effect different thermo-sensitive changes in the conformational properties of TP resulting in a reduction in enzyme activity at the restrictive temperature.

In both v^{ts1} and v^{ts2}, growth at the permissive temperature results in an approximately normal v^{+} phenotype suggesting that the amount and the structure of TP synthesized at this temperature are normal. It would seem unlikely that v mutations would affect the rate of synthesis of TP at one temperature and not at another so it is unlikely that v^{ts1} or v^{ts2} are mutations in regulatory elements. Biochemical evidence, to be presented subsequently, concerning the effect of the v^{ts1} mutation on the properties of TP, supports this hypothesis.

Suppressibility of v^{ts1}

The extensive analyses of $su(s)^2$ - v^s interactions have established that only spontaneous v alleles are suppressible, whereas some spontaneous and all induced v mutations are unsuppressible (GREEN 1952; MARZLUF 1965; TARTOF 1969). The known molecular basis of the mechanism of suppression of v^s alleles by su(s) mutations implicates variation in the changes in structure of TP directed by v^s and v^u alleles as the most probable cause of this distinction. Thus, v^u alleles invariably have negligible TP activity, whereas some enzyme activity can be recovered from certain v^s mutants under partial starvation or substrate-adapted conditions

(RIZKI 1966; BAILLIE AND CHOVNICK 1971). Therefore, v^s mutations might result in lesions in the TP enzyme which are not essential to catalytic activity under suppressed conditions, whereas v^u mutations could cause either an irreversible change in a part of the enzyme necessary for activity or, conceivably, could control the synthesis of a greatly reduced amount of enzyme which would then provide insignificant activity under any metabolic conditions.

In the present study, v^{ts1}, a chemically induced mutation, was shown to be unsuppressed by $su(s)^2$ (Figure 7). Since v^{ts1} is a leaky v mutation, both phenotypically and enzymatically, this suggests that suppressibility does not depend on the amount of residual TP activity available, but rather on the structural basis of the inactivity.

Mapping of v^{ts1}

The vermilion alleles have been the subject of fine-structure recombination studies (GREEN 1952, 1954; BARISH AND FOX 1956; LEFEVRE 1971; SCHALET 1971), and a summary of the map of the locus is presented in Figure 1. At least three sites have been separated by crossing over, with the majority of the mutants so far localized falling into two distinct regions. The spontaneous, suppressible mutations v^1, v^2 and v^k are located in the lefthand site of the cistron and have not been separated from the induced, unsuppressible mutation v^{48a}. The induced mutation v^{65c} and the spontane-

ous, unsuppressible allele $\underline{v^{36f}}$, occupy the righthand site of the cistron. The middle site is represented by only one allele $\underline{v^{E1}}$, which is EMS-induced and unsuppressible.

In this investigation, the EMS-induced, unsuppressible, ts allele, $\underline{v^{ts1}}$, was shown to map to the right of $\underline{v^{1}}$ (2 recombinants in 20,610 chromosomes) but did not recombine with $\underline{v^{36f}}$ in a sample of 15,553 progeny of $\underline{v^{ts1}}/\underline{v^{36f}}$ heterozygotes. Thus, map position within the v cistron is not strictly correlated either with suppressibility or mode of origin of the allele.

An unexpected result arising from the intracistronic mapping of $\underline{v^{ts1}}$ was the recovery of confirmed exceptional chromosomes from $\underline{ras^{2}}$ $\underline{v^{ts1}}$ m /+ $\underline{v^{1}}$ + heterozygotes which were absent from the progeny of $\underline{ras^{2}}$ $\underline{v^{ts1}}$ m /+ $\underline{v^{36f}}$ + heterozygotes (Tables 7 and 8). Out of 20,610 chromosomes sampled from the $\underline{ras^{2}}$ $\underline{v^{ts1}}$ m /+ $\underline{v^{1}}$ + females, 48 confirmed + + + and 4 confirmed $\underline{ras^{2}}$ + m chromosomes were recovered. In both cases, generation of these chromosomes by conventional crossing over requires a double crossover within the very short genetic interval between ras (32.8) and m (36.1). Disregarding interference, the expected frequency of such double crossovers is approximately 6×10^{-6}, far lower than the frequency (2×10^{-4}) with which the exceptions were recovered. Thus, it would seem unlikely that within such a short genetic interval, double crossovers could generate the required frequency of recombinant chromosomes without postulating abnormally high negative interference.

A more likely explanation for the production of these exception-

al chromosomes is a conversion-type event, although this too has the difficulty of reconciling the known low frequencies of such events in Drosophila with the high frequencies found here. In an intensive study of possible conversion events at the rosy (ry) locus, CHOVNICK, BALLANTYNE AND HOLM (1971) found the frequencies of these events to be of the order of 4 - 21 x 10^{-6}, depending on the particular ry alleles tested.

In known conversion events in Neurospora, MURRAY (1965) has found that of two mutants within a locus, one is converted to wild type more frequently than the other. In the present study this non-reciprocality was demonstrated. Thus, v^{ts1} converted v^{1} to wild type (resulting in the 48 + + + exceptional chromosomes) at a much higher frequency than v^{ts1} was converted to wild type by v^{1} (resulting in the 4 ras^{2} + + m chromosomes). Although this is suggestive of conversion, a definitive demonstration that it occurs at the v locus will depend on repeating the experiments described here, using an attached-X chromosome in which both chromosomes are marked with diagnostic flanking markers, so that it can be shown that the reciprocal double mutant chromosomes do not occur, a necessary condition for true conversion events.

Another possibility for the relatively frequent production of exceptional chromosomes from ras^{2} v^{ts1} m /+ v^{1}+ heterozygotes is that some unknown property of the v^{ts1} mutant may be involved. The present studies provide no evidence for this possibility unless the high frequency itself is indicative of something novel.

Since it is normally accepted that the closer together two mutants are in a cistron, the more likely negative interference

and/or conversion-type events occur (CHOVNICK, BALLANTYNE AND HOLM 1971), it was surprising that no exceptional chromosomes were recovered from the $\underline{ras^2}$ $\underline{v^{ts1}}$ $\underline{m}$ / + $\underline{v^{36f}}$ + females. The lack of recombination between $\underline{v^{ts1}}$ and $\underline{v^{36f}}$ found in this experiment probably indicates that $\underline{v^{36f}}$ is more closely linked to $\underline{v^{ts1}}$ than is $\underline{v^1}$, and therefore exceptions generated, either by high negative interference or conversion, might be expected to occur with at least the frequency found in the $\underline{v^{ts1}}$ - $\underline{v^1}$ recombination experiment. As this did not occur, the reason for the high frequency of wild type and $\underline{ras}$ - $\underline{m}$ exceptions found from $\underline{ras^2}$ - $\underline{v^{ts1}}$ $\underline{m}$ / + $\underline{v^1}$ + females remains obscure.

Developmental nonautonomy of $\underline{v^{ts1}}$

The developmental nonautonomy of $\underline{v}$ mutations in which genotypically $\underline{v}$ eye tissue is modified to express a $\underline{v^+}$ phenotype by the presence of $\underline{v^+}$ tissue, has been extensively documented (STURTEVANT 1932; BEADLE AND EPHRUSSI 1936, 1937; SHAPARD 1960), and has been attributed to the production of a diffusible substance by $\underline{v^+}$ tissue which is transferred to developing $\underline{v}$ eyes and converts their phenotype to wild type (BEADLE AND EPHRUSSI 1936).

In the present investigation, $\underline{v^{ts1}}$ was shown to be nonautonomous in $\underline{y}$ $\underline{v^{ts1}}/\underline{v^+}$: $\underline{y}$ $\underline{v^{ts1}}/0$ gynanders at both the permissive and restrictive temperatures for the $\underline{v^{ts1}}$ mutation. Thus, both eyes of all 7 gynanders recovered at 22°C and of the 2

gynanders recovered at 29°C were wild type even though one eye and a varying proportion of the bodies of all 9 gynanders were genotypically y v^{ts1}/0. This clearly demonstrates that v^{ts1} is completely nonautonomous and recessive to v^{+}.

In all 6 y v^{ts1}/v^{1} : y v^{ts1}/0 gynanders recovered at 22°C, both eyes were wild type indicating that v^{1} is nonautonomous and recessive to v^{ts1} at the permissive temperature for the latter. That is to say, at 22°C v^{ts1} behaves like v^{+} in gynanders, producing a diffusible substance which can convert a genotypically v^{1} eye to v^{+}. At 22°C, y v^{ts1}/v^{1} flies have a vermilion index of 1.5 (Table 4), that is, are nearly vermilion. However, in combination with contralateral y v^{ts1}/0 tissue this is altered to an index of 4.5 (or virtually wild type). This shows that the activity of v^{ts1} in a single dose greatly exceeds v^{ts1} activity when it is carried in an X/X zygote. This is a striking demonstration that the compensatory mechanism for sex-linked gene dosage is either increased activity in single X-bearing flies or reduced activity in X/X flies.

Both eyes of all 3 y v^{ts1} : y v^{ts1}/0 gynanders recovered at 29°C were clearly vermilion, demonstrating that the temperature-sensitive expression of v^{ts1} is unaltered in a gynander.

Temperature-sensitive period (TSP) of v^{ts1}

The developmental interval during which a change in culture

temperature elicits an alteration in the eye colour phenotype of $\underline{v^{ts1}}$ was shown to commence in the early to middle third-instar larva and to end in the early pupa (Figure 12).

The most common interpretation of the molecular basis of a TSP in Drosophila is that this period represents the time in development during which the gene product controlled by a ts gene must be biologically active to allow normal development of a wild type adult fly (SUZUKI 1970). In the absence of a directly analyzable gene product of a ts locus whose developmental fluxes in activity have been followed, this conclusion has been based on a variety of phenotypic and developmental studies of many ts mutations whose indirect affects on tissue, tissue products or organs are amenable to analysis (GRIGLIATTI AND SUZUKI 1970; TARASOFF AND SUZUKI 1971; FOSTER AND SUZUKI 1971; GRIGLIATTI, SUZUKI AND WILLIAMSON 1972; POODRY, HALL AND SUZUKI 1973).

The gene product of $\underline{v^{ts1}}$, that is TP, is known. This provides a potential probe for determining the relationships between the actual period in development when this product is synthesized, the period when it is catalytically active, and the TSP based on the eye colour phenotype. Although this investigation was not directly concerned with these relationships, some temporal correlations between the TSP found for $\underline{v^{ts1}}$ by phenotypic studies, the ontogenetic variation in TP activity and the time of brown pigment deposition in the developing eye, warrant attention.

KAUFMAN (1962) has shown that TP activity, assayed in the whole

organism, is detectable from the second instar larva through to the adult. The level of TP activity increases as development proceeds from the second instar larva and reaches a pre-adult maximum in early pupae of 6 - day culture age at 25°C.

RIZKI (1968) was able to detect the autofluorescence characteristic of kynurenine, for a defined period only, in the fat body of the third instar larva. This period of kynurenine accumulation begins at about 6 - 8 hours after the commencement of the third instar and appears to decline towards the end of this instar. According to the data of KAUFMAN (1962), TP activity rises approximately four-fold between the end of the second instar and the end of the third instar. Thus, this rapid increase in TP activity approximately coincides with the period during which accumulation of kynurenine, the product of TP activity, is occurring in the fat body.

The TSP determined for the $\underline{v^{ts1}}$ mutation starts during the early to middle third instar and appears to end during the early pupal period. Therefore, the TSP approximately corresponds to both the time in development during which there is a rapid increase in the activity of the enzyme controlled by the $\underline{v}$ locus and to the accumulation of the product of the enzyme reaction in the fat body cells in which TP appears to be synthesized and is active (RIZKI 1963, 1966, 1968). Moreover, this same period in development is critical for the induction of TP activity and kynurenine accumulation in $\underline{v^{s}}$ larvae either by partial starvation (GREEN 1952) or by

addition of substrate (tryptophan) to the medium (RIZKI 1966).

This is at least circumstantial evidence that the TSP for $\underline{v^{ts1}}$ corresponds to the developmental period during which the enzyme controlled by the locus is biologically active. Alternatively, the evidence that the TP formed at 22°C is no longer thermolabile could suggest that the TSP defines translational changes.

The suggestion that there is a temporal correlation between the TSP for $\underline{v^{ts1}}$ and increase in TP activity makes no prediction as to the time during which transcription occurs nor when the enzyme is synthesized. It could be synthesized in advance of the TSP and remain catalytically inactive until metabolic or genetic conditions trigger its activity, or it could be synthesized just prior to the TSP. One approach to determining the correlation between TP synthesis and activity would be to compare the time at which cross-reacting material specific for TP protein is first obtainable from the fat body with the time at which maximum increase in TP specific activity occurs in isolated fat bodies. Unfortunately, as yet the preparation of TP from Drosophila apparently does not result in a sufficiently pure enzyme to elicit specific antibodies against it in mammals (MARZLUF 1965; EZELL - personal communication).

Brown pigment first appears in the developing eye about 48 - 50 hours after puparium formation in cultures grown at 25°C (CLANCY 1940; ZIEGLER 1961; PHILLIPS, FORREST AND KULKARNI 1973).

This is long after the end of the TSP of $\underline{v^{ts1}}$ (Figure 12) and shows that the TSP is not correlated with brown pigment deposition in the eye.

III. Biochemical analysis of v^{ts1}

In this investigation it was shown that the v^{ts1} mutation markedly reduces the catalytic activity of the TP synthesized by v^{ts1} flies raised at 29°C. This conclusion is based on the great decrease in enzyme reaction rate when TP, extracted from 29°C - raised v^{ts1} flies, is assayed under reaction conditions which were shown to be optimal for TP extracted from v^+ flies [v^+(29°C) TP]. Thus, when both enzymes are assayed at an incubation temperature of 41°C for 5 hours, the rate of accumulation of the product (kynurenine) of the enzyme reaction is far slower at all times and declines at an earlier time with the TP of v^{ts1} flies raised at 29°C [v^{ts1}(29°C) TP] (Tables 11 and 12, Figure 15).

By contrast, the reaction rate of TP extracted from v^{ts1} flies raised at 22°C [v^{ts1}(22°C) TP] is only slightly slower than v^+ TP and does not decline after 3 hours of incubation at 41°C like v^{ts1}(29°C) TP (Table 12 and Figure 15).

The data indicate that v^{ts1}(29°C) TP has a reduced stability if incubated at a temperature which is optimal for both v^+ and v^{ts1}(22°C) TP. The accumulation of product over the period incubated indicates that v^{ts1} flies do, in fact, synthesize TP if raised at 29°C but this enzyme is catalytically defective. The similar kinetics exhibited by v^+ TP and v^{ts1}(22°C) TP with respect to the release of product over a 5 hour incubation period at an optimal *in vitro* temperature, demonstrates that the latter enzyme

is virtually normal catalytically and has stability similar to wild type *in vitro*.

The relationship between time of incubation and reaction rate for both $\underline{v^+}$ and $\underline{v^{ts1}}$(22°C)TP is quite linear up to 3 hours which shows that these enzymes have extraordinarily good *in vitro* stability in comparison with many other enzymes (DIXON AND WEBB 1964). For both $\underline{v^+}$ and $\underline{v^{ts1}}$(22°C) enzymes there was only a slight lag at the start of the incubation period (Figure 15) during which accumulation of product was not linear with time in comparison with the more extensive departure from linearity in the first 30 minutes of incubation observed for $\underline{v^+}$ enzyme by BAILLIE AND CHOVNICK (1971) with essentially the same assay conditions. BAILLIE AND CHOVNICK attributed this lag period to the presence of endogenous inhibitors such as pteridines and allopurinol both of which have been shown to act as *in vitro* inhibitors of TP activity and are present in crude Drosophila extracts (GHOSH AND FORREST 1967; BECKING AND JOHNSON 1967). These inhibitors can be removed from the enzyme extracts by Norit treatment prior to enzyme homogenization (BAILLIE AND CHOVNICK 1971; present study).

The conclusion that $\underline{v^{ts1}}$(29°C) is catalytically deficient is also supported by the results of the effects of increasing enzyme concentration on enzyme activity (Table 13 and Figure 16). The relationship is essentially linear for both $\underline{v^+}$ and $\underline{v^{ts1}}$(22°C) TP presumably because increasing the enzyme concentration increases the number of normal catalytic sites available to the excess of

substrate. By contrast, although the $\underline{v^{ts1}}$(29°C) TP activity does increase with increasing enyzme concentration, the relationship is not strictly linear (Figure 16) suggesting that the substrate binding properties of this enzyme are defective.

These data do not differentiate between possible molecular explanations. If synthesized at 29°C and subsequently assayed for activity at 41°C, $\underline{v^{ts1}}$(29°C) TP could undergo temperature-dependent conformational changes which interfere with enzyme-substrate binding. An alternative possibility is that the $\underline{v^{ts1}}$ mutation reduces the amount of normal enzyme synthesized by the $\underline{v}$ locus at 29°C. However, this latter suggestion is less likely since it would be expected that linear kinetics would then occur as structurally normal enzyme is made.

A more direct approach to the possible difference between $\underline{v^{+}}$ and $\underline{v^{ts1}}$(22°C) TP, and $\underline{v^{ts1}}$(29°C) TP in their enzyme-substrate interactions, is the effect of varying substrate concentration on the enzyme reaction rate. Increasing the substrate concentration first increases the reaction rate of $\underline{v^{+}}$ TP in an approximately linear manner and then the rate slows down so that the relationship is described by a rectangular hyperbola (Figure 17). This indicates that the active sites of the enzyme are increasingly saturated with substrate with first-order kinetics until, at a substrate concentration of between 5 and 7 mM l-tryptophan, complete saturation of the enzyme occurs and zero-order kinetics prevail.

The Km of 1.56×10^{-3} M found for $\underline{v^+}$ TP is consistent with the values of 1.53×10^{-3} M reported by TARTOF (1969) and 1.48×10^{-3} M recorded by BAILLIE AND CHOVNICK (1971) and is about three times the value found for rat liver (KNOX AND MEHLER 1950) and Pseudomonas (POILLON, MAENO, KOIKE AND FEIGELSON 1969) TPs. The Drosophila $\underline{v^+}$ TP activity is therefore quite low in comparison with rat liver and tryptophan-adapted Pseudomonas TP. MARZLUF (1965) has calculated that if the turnover number of the enzyme is about the same for the different organisms, then the specific activity of the crude enzyme extracted form Pseudomonas is at least 500 times that of the Drosophila enzyme.

In Drosophila, TP activity has been measured in extracts from the whole organism, whereas specific tissue and cells have been assayed for enzyme activity in rat liver and Pseudomonas respectively. If fat bodies of third instar larvae of Drosophila were assayed, the TP specific activity undoubtedly would be considerably higher than in whole organism extracts.

The $\underline{v^{ts1}}(22^{\circ}C)$ TP shows similar kinetics to $\underline{v^+}$ TP with increasing substrate concentration and the activity peaks at about 5mM l-tryptophan showing that the enzyme is saturated with substrate at about the same substrate concentration as is $\underline{v^+}$ TP (Figure 17). The Km of $\underline{v^{ts1}}(22^{\circ}C)$ TP is slightly elevated, (1.74×10^{-3} M), compared to $\underline{v^+}$ TP but this relatively small increase probably reflects only slightly less affinity for substrate.

However, the $\underline{v^{ts1}}$(29°C) TP demonstrates a marked change in the kinetics of the enzyme-substrate relationship. The reaction rate increases only very gradually with increasing substrate concentration and exhibits non-linear kinetics (Figure 17). The reaction rate peaks at about the same substrate concentration as for the $\underline{v^{+}}$ and $\underline{v^{ts1}}$(22°C) enzymes but because zero-order kinetics, in which reaction rate is independent of substrate concentration, are not attained it is uncertain whether the enzyme has been saturated with substrate. The Km for $\underline{v^{ts1}}$(29°C) TP based on these data is higher (2.4×10^{-3} M) than for either $\underline{v^{+}}$ or $\underline{v^{ts1}}$(22°C) TP.

Km is a direct measure of the rate of formation of the enzyme-substrate complex and significant increases in its value are usually interpreted as resulting from a structural alteration in the enzyme which reduces the efficiency of the binding of substrate to enzyme (DIXON AND WEBB 1964). Therefore, the increase in Km of $\underline{v^{ts1}}$(29°C) TP suggests that the mutation results either directly in an altered substrate binding site or it could cause conformational changes in the enzyme synthesized at 29°C which indirectly lowers the efficiency of enzyme-substrate binding.

The maximum initial velocity (Vmax) attained by $\underline{v^{ts1}}$(22°C) TP is 78% of $\underline{v^{+}}$ TP, whereas for v^{ts1}(29 C) TP, it is only 10%, indicating that the rate of breakdown of the E-S complex is significantly slower with the $\underline{v^{ts1}}$(29°C) enzyme. This very low Vmax value could be due to a decreased E-S complex concentration or to its slower breakdown into free enzyme and product.

The very low activities recovered from $\underline{v^{ts1}}$(29°C) TP therefore would appear to be based on altered kinetic properties of the enzyme which are contingent on changes in structure of the enzyme, rather than on a reduction in the amount of functionally normal enzyme synthesized at 29°C.

It is possible that $\underline{v^{ts1}}$(29°C) TP might be more susceptible than $\underline{v^{ts1}}$(22°C) or $\underline{v^{+}}$ TP to the effect of a small molecular weight *in vivo* inhibitor such as Cu^{++}, or the -SH group inhibitors, hydroxylamine and sodium azide all of which have been shown to inhibit $\underline{v^{+}}$ TP *in vitro* (MARZLUF 1965). However, if this were the case, ammonium sulfate fractionation would be expected to remove the inhibitors and therefore result in a relatively greater increase in $\underline{v^{ts1}}$(29°C) TP specific activity compared with $\underline{v^{+}}$ or $\underline{v^{ts1}}$(22°C) TP. This, in fact, did not occur. Ammonium sulfate fractionation resulted in a similar two to three fold increase in the specific activities of the enzymes from the three sources over their respective specific activities in crude preparations (Tables 19 and 20).

The defect in TP caused by the $\underline{v^{ts1}}$ mutation therefore appears not to be in a site vital for catalytic activity such as the active centre or cofactor binding, otherwise the activity and kinetic properties of the enzyme synthesized at 22°C might be expected to be more affected. Since the amount of enzyme synthesized by the $\underline{v^{ts1}}$ mutant appears to be approximately normal at 22°C and it is unlikely that a regulatory mutation would reduce the amount of

enzyme at 29°C but not at 22°C, the most likely primary lesion caused by the $\underline{v^{ts1}}$ mutation is missense substitution of an amino acid which affects the conformational properties of the enzyme if it is synthesized at 29°C. The proposed conformational change in the enzyme formed at 29°C would then be indirectly responsible for the catalytic deficiency and altered kinetic properties of $\underline{v^{ts1}}$(29°C) TP assayed *in vitro* at 41°C. The critical question is whether this proposed conformational change in $\underline{v^{ts1}}$(29°C) TP reduces enzyme activity by making the enzyme more thermolabile or by making formation of an active enzyme, perhaps by cofactor-mediated aggregation of identical subunits, more difficult.

Temperature-sensitive phenotypes in procaryotes have been shown to result from increased heat lability of enzyme directed by missense mutations in their structural genes which impose conformational changes in the tertiary or quaternary structures of the enzymes (JOCKUSCH 1964, 1966; WITTMAN, WITTMAN-LIEBOLD AND JAUREGUI-ADELL 1965).

In the present study, an increase in incubation temperature from 22°C to 41°C for the *in vitro* assay of $\underline{v^{+}}$ TP led to a four-fold increase in its activity (Table 21 and Figure 19). The *in vitro* $\underline{v^{+}}$ TP activity peaks at 41°C, a temperature which is higher than the normal biological temperatures encountered by wild type Drosophila and certainly much higher than the laboratory temperature of 22°C at which this inbred line is maintained. Indeed, at culture temperatures above 29°C, viability of Drosophila strains

is severely affected (PARSONS 1973; present study). Nevertheless, in all previous *in vitro* assays of v^+ TP an optimal incubation temperature of at least 37°C has been established (BAGLIONI 1960, KAUFMAN 1962, MARZLUF 1965, TOBLER, SIMMONS AND BOWMAN 1967, TARTOF 1969, BAILLIE AND CHOVNICK 1971, TOBLER, BOWMAN AND SIMMONDS 1971) and temperatures in this range are commonly employed to obtain maximum *in vitro* activity from many different Drosophila enzymes (GRELL 1962, GLASSMAN 1965, CHOVNICK *et al*. 1967, 1969, MacINTYRE AND O'BRIEN 1969).

While it could be argued that optimal *in vitro* assay temperatures have dubious significance for the *in vivo* situation since they represent a balance between the accelerating effect of increasing temperatures on the rate of the enzyme reaction and their effect on the rate of destruction of the enzyme protein, nonetheless at least for the *v* locus there are consistent correlations between the *in vivo* effects of various *v* mutations, rearrangements and heterozygotes as determined by variations in the *v* phenotype at normal culture temperatures, and their corresponding TP activities measured at higher *in vitro* temperatures. The $su(s)^2\ v^{36f}$ genotype, for example, is vermilion in phenotype at a culture temperature of 25°C and its TP activity at an assay temperature of 37°C is just 4.4% of wild type, whereas the phenotype of $su(s)^2\ v^k$ is clearly wild type at 25°C and its TP activity is 21% of wild type at an assay temperature of 37°C (TARTOF 1969).

In assays of v^+ TP, a progressive decline in activity

occurred as the incubation temperature was increased above 41°C (Figure 19), probably because the enzyme becomes unstable and gradually denatures. In comparable assays of $\underline{v^{ts1}}$(22°C) TP, a similar rise in enzyme activity occurred between 22°C and 41°C, thereafter declining more rapidly than $\underline{v^{+}}$ TP with increasing incubation temperature (Table 22 and Figure 19). These results demonstrate that the enzyme extracted from $\underline{v^{ts1}}$ flies raised at 22°C is not thermolabile at any *in vitro* assay temperature below 41°C and suggest that only slightly increased thermolability occurs above 41°C.

Interestingly, the ammonium sulfate fractionated $\underline{v^{ts1}}$ (29 C) TP showed an approximate two-fold increase in activity between 22°C and 41°C. While the rise in activity did not parallel that found for the other two enzymes, the maximum enzyme activity was again close to 41°C (Table 23 and Figure 19), although there was no distinctive peak at this temperature as was determined for the $\underline{v^{+}}$ and $\underline{v^{ts1}}$(22°C) enzymes. The activity of $\underline{v^{ts1}}$(29°C) TP declines rapidly at incubation temperatures higher than 41°C and is almost zero at 50°C, a temperature at which both $\underline{v^{+}}$ and $\underline{v^{ts1}}$(22°C) TP still show appreciable activity (Figure 19). However, since the $\underline{v^{ts1}}$(29°C) TP has only a fraction of the activity of either $\underline{v^{+}}$ or $\underline{v^{ts1}}$(22°C) TP at any temperature of incubation, the rapid decline in activity of $\underline{v^{ts1}}$(29°C) TP above 41°C is probably insignificant since it does not represent a greater proportional decrease than that of the other two enzymes.

The key finding in these results is that $\underline{v^{ts1}}$(29°C) TP is no more thermolabile in an *in vitro* assay system than either $\underline{v^{+}}$ or $\underline{v^{ts1}}$(22°C) TP since it demonstrates very low activity at an incubation temperature of 22°C which is increased, rather than decreased, when the incubation temperature is raised through 29°C to 41°C. At an *in vivo* temperature of 29°C the enzyme is obviously defective because the $\underline{v^{ts1}}$ flies raised at this temperature exhibit a v phenotype, hence the TP of $\underline{v^{ts1}}$ flies raised at 29°C is probably already structurally and catalytically deficient before extraction and assay at any temperature is begun. Increasing the temperature of incubation would then simply increase the rate of the enzyme reaction by providing more energy for enzyme-substrate formation, however deficient this might be with $\underline{v^{ts1}}$(29°C) TP, and increasing its rate of breakdown into free enzyme and product, thereby apparently increasing enzyme activity until a temperature (41°C) is reached at which destruction of the enzyme begins to outweigh the thermodynamic effect of increasing temperature. Since the effect of increasing incubation temperature on $\underline{v^{ts1}}$(22°C) TP *in vitro* activity is approximately similar, in proportion, then it could be surmised that the primary effect of the $\underline{v^{ts1}}$ mutation, which must be the same in both $\underline{v^{ts1}}$ (22°C) and $\underline{v^{ts1}}$(29°C) TP, causes a permanent loss of TP function if the enzyme is synthesized at 29°C, whereas at 22°C it is only partial.

The primary lesion produced by $\underline{v^{ts1}}$ therefore most likely effects conformational changes either in the aggregated enzyme

itself, or in the inactive monomers which are then prevented from forming an active multimeric aggregate at 29°C, but which result in only slight malfunction in the enzyme if it is synthesized at 22°C. Thus, the TP synthesized at 22°C is not temperature-sensitive at an *in vitro* temperature of 29°C although synthesis and subsequent activity of the enzyme at an *in vivo* temperature of 29°C are impaired.

Measurement of the activation energies (Ea) of the reactions catalyzed by the $\underline{v^+}$, $\underline{v^{ts1}}$(22°C) and $\underline{v^{ts1}}$(29°C) enzymes reveal that different amounts of energy are required to form an activated enzyme-substrate complex in the three cases. The Ea for $\underline{v^+}$ TP is 13,660 cal/mole determined for $\underline{v^{ts1}}$(22°C) and $\underline{v^{ts1}}$(29°C) TPs. (Tables 21, 22 and 23 and Figure 20). The Arrhenius plots for all 3 enzymes demonstrate discontinuity of slopes and in each case approximate to two straight lines meeting at inflection points separating a zone of enzyme activation by increasing termperature form a zone of inactivation of the proteins by heat (Figure 20). The increase in Ea for the $\underline{v^{ts1}}$(22°C) and $\underline{v^{ts1}}$(29°C) TPs implies that these activated E-S complexes require more energy to form than the $\underline{v^+}$ E-S complex. This is usually interpreted as resulting from a structural change in the enzyme (DIXON AND WEBB 1964). The data do not reveal any distinctive difference in Ea between $\underline{v^{ts1}}$(22°C) and $\underline{v^{ts1}}$(29°C) TP, although this should be regarded as provisional since the low levels of activity of $\underline{v^{ts1}}$(29°C) TP give disproportionately large fluctuations in the slope of the Arrhenius plot, hence the measurement of the Ea of this enzyme

is very approximate using this method.

Apart from this measurement, the $\underline{v^{ts1}}$(29°C) TP is consistently different in kinetic parameters to both $\underline{v^{+}}$ and $\underline{v^{ts1}}$(22°C) TPs, whereas only slight variations generally occur between the latter two enzymes. Together with the consistently low TP activities obtained from 29°C - raised $\underline{v^{ts1}}$ flies under any *in vitro* conditions, this means that the mutational effects of $\underline{v^{ts1}}$ possibly are associated with an irreversible alteration in the structure of the enzyme formed during development at 29°C. This interpretation makes no commitment as to the precise nature of the structural alteration in the TP enzyme controlled by the $\underline{v^{ts1}}$ mutation at 29°C; indeed both catalytic and regulatory (allosteric) sites in the enzyme could be affected by a conformational change produced by a single amino acid substitution in the TP protein.

In microorganisms, direct correlations have been found between phenotypic temperature-sensitivity at a restrictive temperature and a loss in activity of the protein product at the same *in vitro* temperature. The best analyzed ts mutations in microorganisms have been the Tobacco mosaic virus (TMV) coat protein mutations, in which single amino acid replacements in the protein render it directly heat labile by causing conformational changes which denature the protein at a given temperature (JOCKUSCH 1966).

The advanced techniques for selecting ts mutations and

purifying coat proteins in TMV, enabled JOCKUSCH to directly measure the rates of denaturation of purified TMV coat proteins at given temperatures. The increased heat lability of ts mutant coat proteins was measured by loss of solubility at pH 5 after placing in a water bath at the same temperature at which the morphology of the virus coat protein is temperature-sensitive. At pH 5, normal coat protein heated to the restrictive temperature, and ts coat protein heated to the morphological permissive temperature, are both maximally soluble, whereas the ts coat protein heated to the restrictive temperature becomes completely insoluble, indicating irreversible denaturation. The ts coat protein crystals appear in the electron microscope as a disordered aggregation in contrast to the ordered rod-like crystals of normal and ts coat protein under permissive conditions. JOCKUSCH noted that aggregation of protein subunits before heat treatment was begun had a stabilizing effect on ts coat proteins. In the present study this aggregation of TP protein subunits probably did not take place during synthesis of $\underline{v^{ts1}}$ TP at 29°C, thereby rendering the protein disordered before extraction of the enzyme.

Two further properties of the ts TMV coat proteins are relevant to the present studies: the ts mutations are commonly leaky, that is the change in protein structure caused by the ts mutation usually produces slight malfunction at the permissive temperature and incomplete loss of function at the restrictive temperature. This type of "leakiness" is demonstrated by $\underline{v^{ts1}}$

TP. Furthermore, the ts coat proteins are all quaternary proteins, that is they consist of monomeric subunits aggregated together usually by prosthetic groups, and the ts mutations cause a configurational change in the subunits which prevent aggregation at the restrictive temperature. There is good evidence that TP consists of identical subunits, possibly held together by the heme prosthetic group. Therefore, the $\underline{v^{ts1}}$ mutation possibly could cause a change in normal subunit interaction leading to the formation of the active multimeric enzyme.

LANGRIDGE (1968 a, b) has studied the thermal characteristics and intracellular behaviour of an extensive array of a specific class of ß galactosidase ts mutants in E. coli. All of the ts mutants resulted from suppression of different ß galactosidase amber mutations by $su^{+}I$ which inserts serine at the position corresponding to the UAG triplet, thus permitting synthesis of the complete protein. By this means, 52 ts variants of ß galactosidase were obtained, differing from each other only in the position of serine substitution for the original amino acid.

LANGRIDGE found that the temperature responses of these suppressed enzymes depend mainly on the position of the amino acid substitution rather than on the type of amino acid inserted. The altered enzymes produced by suppression are thus representative of the missense type; they possess relatively high catalytic activity and they do not limit the growth of bacteria containing them at ordinary temperatures. In these respects, $\underline{v^{ts1}}$ TP is similar,

it too possesses relatively high catalytic activity at permissive temperatures and apparently does not limit the growth of $\underline{v^{ts1}}$ flies at 22°C.

LANGRIDGE (1968 a, b) has shown that the difference in amino acid side chains following serine substitution in amber mutants generally causes a moderate change in the hydrophilic nature of the outside of the enzyme molecule. Despite the mildness of the change, 60% of the altered enzymes had less than half the *in vitro* stability of the normal enzyme at the restrictive temperature (57°C). Thus, small changes in conformation can lead to great changes in thermal properties.

The serine-substituted enzymes were examined for changes in substrate affinity by measuring the ability of normal substrate (lactose) to competitively inhibit the binding of a substrate analogue (ONPG). All enzymes except one had normal binding properties as shown by the inhibition constants. Three of the enzymes with reduced temperature stability but normal substrate binding were tested for changes in activation energy. The changes were not significantly different from the Ea of normal enzyme (12,400 ± 650 cals/mole). LANGRIDGE concluded from these data that increased sensitivity of an enzyme to heat as a result of mutational change is seldom accompanied by temperature-dependent changes in substrate affinity or catalysis. Alterations in kinetic responses to temperature, as distinct from changes in stability, have been found only for mutants with reduced substrate

affinity.

The properties of the $\underline{v^{ts1}}$ mutant are consistent with these conclusions; the $\underline{v^{ts1}}$(29°C) TP has altered kinetic responses to temperature, but does not appear to have radical changes in temperature-dependent stability <u>after</u> it has been synthesized at 29°C.

Another point of comparison between the ts galactosidase mutants and the $\underline{v^{ts1}}$ mutation is that LANGRIDGE found many of the former mutant enzymes did not exhibit strictly linear kinetics with time, substrate concentration or temperature, results which have already been discussed for $\underline{v^{ts1}}$(29°C) TP. This may indicate that ts mutant enzymes, as a class, do not follow completely predictable changes in their properties under restrictive conditions. For example, LANGRIDGE found an extremely large negative apparent heat of activation for one ts mutant enzyme which probably reflects the marked increase in activity of the enzyme as the temperature is lowered. Evidence shows that this particular enzyme is partially dissociated into inactive monomers at high temperatures and that lowering the temperature facilitates reassociation into the active tetrameric structure. Other ts β galactosidase mutants had apparent increased heats of activation despite reduced Kms (therefore, seemingly greater affinity for substrate), whereas ts enzymes with expected increased heats of activation and increased Kms were also encountered. Therefore, even in the relatively well understood ts β galactosidase mutants of <u>E. coli</u>. it is

difficult to ascribe cause and effect relationships for ts enzymes from kinetic considerations alone.

DUNSMUIR AND HYNES (1973) have recovered 4 ts mutations affecting the activity of acetamidase in the simple eukaryote, Aspergillus nidulans. Three of the ts mutants were in the structural gene for the enzyme and the other one was in an apparent positive regulator gene.

The ts mutants had interesting effects on the activity of acetamidase. One structural gene mutant and the one regulator gene mutant both had very low acetamidase activity, in comparison with wild type, if raised at the permissive (25°C) or restrictive (40°C) temperatures and assayed at 37°C (the optimal in vitro assay temperature for wild type acetamidase activity). The other two ts structural gene mutants when raised at 40°C had very low enzyme activities at an assay temperature of 37°C compared with either wild type raised at 40°C or the mutants raised at 25°C. Significantly, all acetamidase assays performed at 25°C on the various strains gave essentially the same results as at 37°C. Therefore, assaying at the in vivo permissive temperature did not permit restoration of activity to the mutant enzymes. This result is similar to that obtained for assaying $\underline{v^{ts1}}$(29°C) TP at the in vivo permissive temperature (22°C); a decrease rather than an increase in enzyme activity occurred. Conversely, assaying $\underline{v^{ts1}}$(22°C) TP at the in vivo restrictive temperature (29°C) resulted in an increase in activity, whereas if the enzyme is

thermolabile at 29°C a decrease in activity would have been expected.

In only one of the ts acetamidase structural gene mutants studied by DUNSMUIR AND HYNES could the *in vivo* temperature-sensitivity exhibited by all ts strains at 40°C be accounted for by increased thermolability of the enzyme. In the other two ts structural gene mutants and the one ts regulator gene mutant, no difference in heat-induced enzyme inactivation compared to wild type was seen. The loss of enzyme activity in the ts regulator gene raised at the restrictive temperature was due to a greatly reduced rate of enzyme synthesis.

In one of the two ts structural gene mutants which did not produce a thermolabile enzyme, acetamidase activity could be greatly increased by shifting growing cultures from the restrictive to the permissive temperature for 8 hours. This increase in enzyme activity was mostly independent of new protein synthesis since neither the protein synthesis inhibitor, cycloheximide, or the transcription inhibitor, actinomycin D, prevented the rapid increase in activity after shifting to 25°C. DUNSMUIR AND HYNES suggest that this ts mutation therefore affects the assembly of normally synthesized enzyme subunits into active enzyme at 40°C but that the permissive temperature allows immediate inhibitor-insensitive subunit assembly. The MW of acetamidase is about 150,000 which is compatible with the proposed subunit structure. Thus, the mechanism of action proposed for the $\underline{v^{ts1}}$ mutation is

by no means unique and finds support in similar interpretations advanced to explain the effects of some ts mutations in organisms far simpler than Drosophila.

A molecular model of the functioning of the v locus, which reconciles diverse genetic and biochemical observations, should also explain the effects of different doses of v^+ and v alleles on TP activity in males and females.

Wild type TP is dosage compensated. Thus, v^+/Y males and v^+/v^+ females have essentially equivalent TP activities (KAUFMAN 1962; TOBLER, SIMMONS AND BOWMAN 1967; TARTOF 1969; BAILLIE AND CHOVNICK 1971; TOBLER, BOWMAN AND SIMMONS 1971; present study). This dosage compensation does not change if v^+/Y males and v^+/v^+ females are raised at 29°C and TP activities also are virtually unaltered by changes in culture temperature (Table 24). Therefore, a culture temperature of 29°C apparently has little effect on the regulation of the functional activity of the v^+ locus.

The TP activities of v^{ts1}/Y males and v^{ts1}/v^{ts1} females raised at 22°C are also essentially equivalent even though their net enzyme activity is only about 75% of wild type males and females (Table 25). However, if raised at 29°C, v^{ts1}/Y males have appreciably less TP activity than v^{ts1}/v^{ts1} females (5% of wild type TP activity compared with 17% for the females (Table 25). This difference is correlated with the more vermilion-like eye colour of v^{ts1}/Y males and, incidentally, shows that there is no clear-cut threshold of TP activity which distinguishes a v pheno-

type from a v^+ phenotype. Flies with up to 5% of wild type TP activity are clearly vermilion, (v^{ts1}(29°C) males and $su(s)^2\ v^{36f}$ for example), whereas a TP activity of 15-20% of wild type, such as in v^{ts1} females at 29°C and $su(s)^2\ v^1$ for example, is sufficient to provide an intermediate eye colour. Activities above 20% of wild type allow a wild type phenotype (KAUFMAN 1962; TARTOF 1969).

A single v^+ dose in a female specifies about 50% of the TP activity obtained from v^+/v^+ females and v^+/Y males as shown by the TP activities obtained from $v^+/Df(1)v^{L3}$ females in this study (Table 26) and from In(1)FM6/Df(1)v females (BAILLIE AND CHOVNICK 1971). Hence the enzyme activity obtained from a female is the result of a simple addition of the activities contributed by each v^+ allele. BAILLIE AND CHOVNICK (1971) found that females with 3 v^+ doses, [C(1)Dx, y f / y^+ Y v^+], had, as expected, approximately one and one-half times the activity of normal v^+/v^+ females. In males with 2 v^+ doses, (v^+/y^+ Y v^+), there is a similar simple addition of the TP activities contributed by each v^+ allele, except that each contributes twice the activity of each v allele in a female. Thus, v^+/y^+ Y v^+ males have about twice the TP activity of v^+/Y males, but they have approximately four times the activity of v^+/Df(1)v females (BAILLIE AND CHOVNICK 1971; TOBLER, BOWMAN AND SIMMONS 1971).

Regulation of v^+ activity therefore is different in males and females and normally acts to bring the TP activities of males to

the same level as females. It is therefore difficult to understand why females with 2 doses of the leaky $\underline{v^{ts1}}$ allele should have over twice the TP activity of males with one dose when both are raised at the restrictive temperature. Furthermore, $\underline{v^{ts1}}$ males and females exhibit normal dosage compensation at the permissive temperature and a culture temperature of 29°C does not affect the mechanisms responsible for wild type dosage compensation.

In $\underline{v^{ts1}}/\underline{Df(1)v^{L3}}$ females raised at 22°C, a TP activity of 15.5% of wild type was recorded (Table 26). This activity is less than expected on the basis of the TP activity of $\underline{v^{ts1}}/\underline{v^{ts1}}$ females raised at 22°C and the 50% reduction in TP activity of $\underline{v^{+}}/\underline{Df(1)v}$ females compared with $\underline{v^{+}}/\underline{v^{+}}$ females. Thus, $\underline{v^{ts1}}/\underline{v^{ts1}}$ females raised at 22°C have about 70% of wild type TP activity, hence a TP activity of about 35% of wild type would be predicted for $\underline{v^{ts1}}/\underline{Df(1)v^{L3}}$ females raised at 22°C. Moreover, the TP activity of $\underline{v^{ts1}}/\underline{Df(1)v^{L3}}$ females raised at 22°C is much less than the predicted 50% of the TP activity of 22°C - raised $\underline{v^{ts1}}$/Y males and is the clearest indication that the allelic control of TP activity is radically different in $\underline{v^{ts1}}$ males and females.

The TP activity of $\underline{v^{ts1}}/\underline{Df(1)v^{L3}}$ females, raised at 29°C, decreases to a value of about 5% of wild type which is approximately the same TP activity derived from $\underline{v^{ts1}}$/Y males raised at 29°C (Table 25). This value probably represents the upper limit of TP activity obtainable from males and females with a single

dose of the $\underline{v^{ts1}}$ mutation and raised at 29°C. Furthermore, the significant difference between the TP activities obtained from females with single and double doses of $\underline{v^{ts1}}$ at both 22°C and 29°C, points to the possibility of additional interactions among enzyme subunits when 2 doses of $\underline{v^{ts1}}$ are present in a female which are not available with just one dose. This extra interaction among subunits could lead to better correction of mutant enzyme than in single-dose females at the permissive or restrictive temperatures, and single-dose males at the restrictive temperature, thereby allowing more TP activity in these $\underline{v^{ts1}}/\underline{v^{ts1}}$ females.

Recently, the biochemical mechanisms involved in the dosage compensation of a number of X-linked structural genes in Drosophila have been at least partially resolved but the genetic regulation of these processes remains obscure. Three sex-linked structural genes for the enzymes 6-phosphogluconate dehydrogenase (6PGD), glucose-6-phosphate dehydrogenase (G6PD) and tryptophan pyrrolase (v) respectively have the following properties in common:

(i) wild type enzyme activities in males and females of all 3 genes are equivalent. (KAZAZIAN et al. 1965; YOUNG, 1966; SEECOF et al. 1969; TOBLER, SIMMONS AND BOWMAN, 1971; BOWMAN AND SIMMONS, 1973).

(ii) the enzyme activities of deficiency heterozygotes and null allele heterozygotes in females are, in all cases, approximately

50% of +/+ enzyme activities.

(iii) both X-linked wild type loci of all 3 genes are expressed in a female, each contributing approximately 50% of the respective total enzyme activities (KAZAZIAN AND YOUNG, 1966; SEECOF, KAPLAN AND FUTCH, 1969; TOBLER, SIMMONS AND BOWMAN, 1971; BOWMAN AND SIMMONS, 1973).

(iv) the regulation of dosage compensation appears to be a property of the gene or its immediate genetic environment rather than a property of the X-chromosome as a whole, or a combination of X-chromosome and autosomes. This conclusion is based on the observations that in rearrangements involving any of the 3 structural genes studied, exact compensation of dosage of wild type alleles is retained irrespective of the size of the rearrangement involving the wild type allele (BOWMAN AND SIMMONS, 1973). For example, if the $\underline{v^+}$ allele is duplicated on the Y chromosome, or is carried as a hyperploid segregant on an autosome due to an insertional translocation, the proportional increase in TP activity is dosage compensated very precisely. That is, for each additional $\underline{v^+}$ allele in a male, twice the TP activity of the same supernumerary gene located on the same chromosome in a female is obtained. This is true even though the extra $\underline{v^+}$ allele may contribute less TP activity, because of position effect depression, than when it is located on the X chromosome. The dosage compensation remains the same irrespective of the length of the duplicated segment containing the wild type allele, hence it is probable that the

regulatory functions accomplishing this compensation are closely linked to the structural genes involved. No mechanism is effectively operating to adjust overall enzyme levels to wild type. Therefore, it is hard to imagine a genic balance or modifier mechanism, as proposed by STERN (1960) and MULLER AND KAPLAN (1966), acting to compensate enzyme activities to wild type. LUCCHESI (1973), however, has adduced other evidence to show that dosage compensation of X-linked genes may depend on the activity of an autosomal gene which is itself dosage-dependent and whose product is necessary for the transcription of all X-linked genes.

HOLMQUIST (1972) has established that the probable mechanism involved in doubling the amount (KAZAZIAN, 1966) and activity of enzymes controlled by sex-linked loci in males compared to females, is an increased transcription rate of these loci. Thus, each X salivary gland chromosome band in the 16A-17E region in a male was shown to transcribe approximately 0.7 units of ^{3}H-uridine pulse-labelled RNA for each 1.0 unit transcribed by two similar bands in the two X chromosomes of the female. Hence a 40% increase in RNA synthesis by male salivary gland X chromosome bands presumably could effect a 100% increase in enzyme levels by increasing the rate of mRNA transcription while not increasing synthesis of non-mRNA chromosomal RNA.

This possible mechanism of dosage compensation does not clarify the results obtained for TP activities of $\underline{v^{ts1}}/Y$ males and $\underline{v^{ts1}}/\underline{v^{ts1}}$ females raised at 29°C. The $\underline{v^{ts1}}/Y$ males raised

at 29°C would be expected to transcribe sufficient TP-mRNA to permit equivalent enzyme activity to the $\underline{v^{ts1}}/\underline{v^{ts1}}$ females. However, $\underline{v^{ts1}}/\underline{v^{ts1}}$ females have nearly three times the TP activity of $\underline{v^{ts1}}/Y$ males which suggests that the $\underline{v^{ts1}}$ mutation, at the restrictive temperature, disturbs the normal mechanism involved in regulation of compensation.

Many of the properties of v mutations and their effects on TP can be explained by assuming that the v locus codes the identical subunits which may comprise active TP. The homomultimeric nature of the active enzyme would account for the lack of complementation between any v alleles irrespective of their individual properties. Further support for the homomultimeric structure of active TP is adduced from a comparison of its properties with those of rat liver and Pseudomonas TP and from a consideration of its probable molecular weight.

Pseudomonas TP has been shown, by direct physical techniques, to be composed of 4 polypeptide chains of equivalent mass (POILLON, MAENO, KOIKE AND FEIGELSON, 1969). The subunits are devoid of enzyme activity and only the tetrameric form, stabilized exclusively by non-covalent interactions, is the enzymatically active form. Drosophila TP has many properties in common with the Pseudomonas enzyme. Thus, both enzymes have a heme prosthetic group which is necessary for enzyme activity. Both wild type enzymes are substrate-inducible and possibly also substrate-stabilized (MARZLUF 1965; RIZKI 1968; POILLON et al. 1969). Activation by

2-mercaptoethanol is necessary for full activity of the crude enzymes from both organisms and is an absolute requirement for the partially purified TPs (TARTOF 1969; BAILLIE AND CHOVNICK 1971; POILLON et al. 1969). The pH optima and inhibition profiles are also very similar (MARZLUF, 1965). Considering that the structures of many basic enzymes are conserved during evolution it is possible that Pseudomonas and Drosophila TPs have a similar structural organization and that minor amino acid changes only have occurred phylogenetically. Furthermore, the molecular weight of Drosophila TP is approximately the same as the known molecular weight of Pseudomonas TP (BAILLIE AND CHOVNICK 1971).

More recently, preliminary results obtained with electrophoretic techniques have shown that Drosophila v^+ TP migrates as a single band and appears to consist of a tetramer of molecular weight 160,000 daltons, consisting of 4 identical subunits of about 40,000 daltons each. (FUCHS - unpublished observations).

TARTOF (1969) reported that certain v^s mutations when heterozygous with v^+, behave in a superadditive fashion rather than providing strictly additive TP activities as expected of heterozygous null alleles. That is, some v^s/v^+ heterozygotes yield more activity than the predicted 50% of v^+/v^+ TP activity, based on the virtually zero TP activity of the v^s/v^s homozygotes. The amount of excess enzyme activity contributed by the v^s allele is strictly related to its suppressibility. Thus, v^k is more suppressible than v^1 which, in turn, is more suppressible than v^{36f},

based on the amount of restored TP activity when all mutants are homozygous for $\underline{su(s)^2}$. The heterozygotes, $\underline{v^k}/\underline{v^+}$, $\underline{v^1}/\underline{v^+}$ and $\underline{v^{36f}}/\underline{v^+}$ likewise can be ranked in that order in their degree of superadditivity; $\underline{v^k}/\underline{v^+}$ has a TP activity 77.5% of wild type, $\underline{v^1}/\underline{v^+}$, 67.2% and $\underline{v^{36f}}/\underline{v^+}$, 63%. By contrast, the unsuppressible alleles, $\underline{v^{51c}}$ and $\underline{v^{48a}}$ when heterozygous with $\underline{v^+}$, yield almost exactly the expected 50% of wild type TP activity (TARTOF 1969).

The superadditivity obtained for TP activities from assays of $\underline{v^s}/\underline{v^+}$ heterozygotes in each case was closely paralleled by mixing equal aliquots of enzyme extracts obtained from equal weights of $\underline{v^s}/\underline{v^s}$ and $\underline{v^+}/\underline{v^+}$ flies and assaying for resultant TP activities. The material obtained from $\underline{v^s}$ flies, which was shown to be responsible for the superadditive effect, is almost certainly a protein since it is thermolabile, ammonium sulfate precipitable and non-dialyzable. Hence, it is clear that some $\underline{v^s}$ mutants, at least, form a potentially functional enzyme protein. Since $\underline{v^k}/\underline{v^k}$ extracts have virtually zero TP activity the reason advanced by TARTOF to explain the superadditive effect is interaction among enzyme subunits specified by $\underline{v^+}$ and $\underline{v^k}$ respectively such that additional enzyme activity is achieved compared with $\underline{v^+}/\underline{Df(1)v}$ or $\underline{v^+}/\underline{v^u}$.

The $\underline{v^s}$ alleles therefore could direct the synthesis of normal quantities of TP subunits but these subunits are variably altered structurally. MARZLUF (1965), for instance, has shown that $\underline{su(s)^2\ v^1}$ homozygotes produce a partially active TP which

is indistinguishable from wild type in its kinetic properties. Thus, the $\underline{v^1}$ allele allows the synthesis of an apparently normal enzyme under suppressed conditions. On the other hand, TARTOF (1969) has shown that TP produced by $\underline{su(s)^2\ v^k}$ homozygotes has altered optimal pH and Km and therefore differs structurally from wild type TP. Moreover, BAILLIE AND CHOVNICK (1971) demonstrated that unsuppressed $\underline{v^k}$ TP has about the same molecular weight as $\underline{v^+}$ TP even though it has no enzyme activity.

Therefore, $\underline{v^s}$ mutants probably do not affect the rate of synthesis of TP since sufficient subunits must be present to interact with the $\underline{v^+}$ product to produce the superadditive effect. The $\underline{v^s}$ alleles are not nonsense mutations otherwise the molecular weight of unsuppressed $\underline{v^k}$ TP should be less than $\underline{v^+}$ TP. Moreover, the polarity in their degree of suppressibility does not correlate with their map positions within the $\underline{v}$ locus. The degree of suppressibility is $\underline{v^k} > \underline{v^1} > \underline{v^{36f}}$ but, as previously shown in the REVIEW, $\underline{v^k}$ and $\underline{v^1}$ map to the same site, whereas $\underline{v^{36f}}$ maps to the right. Thus, the $\underline{v^s}$ mutations most probably represent different missense mutations at positions other than the active centre (which probably would not be post-translationally suppressible). These missense mutations probably change the conformational properties of the subunits such that in $\underline{v^s}$ hemi- or homozygotes correct folding of the inactive monomers into an active enzyme multimer is disallowed.

The proposed interactions among identical TP subunits, which

can account for most of the effects of mutation at the v locus on TP activity, are probably analogous to those which control 6-phosphogluconate dehydrogenase (6-PGD) activity. The 6-PGD structural locus is located on the X chromosome at 0.9 map units since electrophoretic variants of the enzyme map to this position (YOUNG 1966). Certain strains have a single 6-PGD electrophoretic band whose mobility is greater than the single 6-PGD band of other strains. Crosses between strains containing the fast migrating 6-PGD band (6-PGDA) and strains containing the slow migrating 6-PGD band (6-PGDB), yield females which have three 6-PGD electrophoretic bands: a 6-PGDA band and a 6-PGDB band, and a wider, more densely staining intermediate band representing the 6-PGDA - 6-PGDB aggregate.

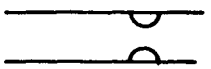
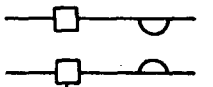
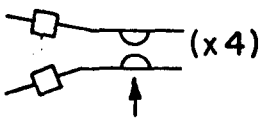
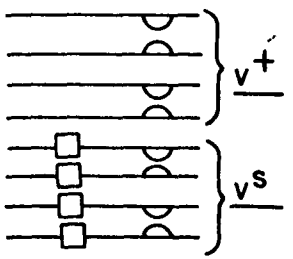
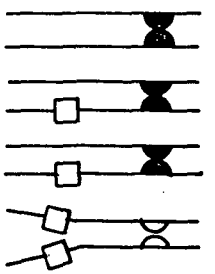
Thus, a female heterozygous for the 6-PGDA and 6-PGDB alleles produces both subunits in equal quantity which randomly assemble to form dimers in the proportion 1 6-PGDA dimer : 2 6-PGDA - 6-PGDB dimers : 1 6-PGDB dimer. The monomers are enzymatically inactive and wild type, active 6-PGD therefore consists of a dimer of identical subunits.

Similarly, it is proposed that v^S and v^+ alleles in heterozygous females, produce the subunit products Pv^S and Pv^+ respectively, in equal proportion. Each monomer contains an active centre but this is not enzymatically active unless a tetramer is formed. The monomers also contain conformational and regulatory sites which interact with a variety of cofactors and inhibitors *in vivo*.

The Pv^+ and Pv^S subunits are free to tetramerize randomly with the restriction that the Pv^S subunits are unable to aggregate into active enzyme because of local misfolding due to conformational changes brought about by the $\underline{v}^S$ missense mutations. In $\underline{v}^S/\underline{v}^+$ heterozygotes, tetramer formation is strongly favoured and therefore occurs soon after synthesis of the monomers. At equilibrium, the tetramers formed from $\underline{v}^S/\underline{v}^+$ heterozygotes and their unit TP activity are shown in Figure 21 and compared with the tetramers and their TP activities formed by $\underline{v}^+/\underline{v}^+$ and $\underline{v}^S/\underline{v}^S$ females and $\underline{v}^+/Y$ and $\underline{v}^S/Y$ males. For simplicity of representation, the tetramers are shown as dimers since this does not affect the proposed mechanism.

As shown in the Figure, $\underline{v}^+/Y$ males synthesize twice as many TP monomers per $\underline{v}^+$ allele as $\underline{v}^+/\underline{v}^+$ females, hence their activities are equivalent. The $\underline{v}^S$ hemi- and homzygotes fail to form any active tetramers because conformational changes prevent the configuration of the active centres necessary for TP activity. Tetramers must still form in $\underline{v}^S$ hemi- or homozygotes since the molecular weight of unsuppressed $\underline{v}^S$ TP is approximately the same as $\underline{v}^+$ TP, (TARTOF 1969; BAILLIE AND CHOVNICK 1971). In $\underline{v}^S/\underline{v}^+$ females, random interaction among the equal numbers of Pv^S and Pv^+ subunits produce tetramers in the ratio, 1 $(Pv^+)_4$: 2 $(Pv^+)_2$ $(Pv^S)_2$: 1 $(Pv^S)_4$. Each $(Pv^+)_2(Pv^S)_2$ tetramer potentially has a full unit of TP activity, therefore the maximum TP activity recoverable from $\underline{v}^S/\underline{v}^+$ females is 75% of wild type.

FIGURE 21 Model of interactions among tryptophan pyrrolase subunits from various genotypes and consequent enzyme activities.

GENOTYPE	SEX	SUBUNITS FORMED*	DIMERS** (RATIOS)	NO. ACTIVE DIMERS***	POTENTIAL TP ACTIVITY/DIMER	PHENOTYPE
$\underline{v^+}/\underline{v^+}$	F	 (x4)	 (x4) ACTIVE CENTRE OF DIMER	4	25 % 25 % 25 % 25 % 100 % TOTAL	+
$\underline{v^+}/Y$	M	AS ABOVE	AS ABOVE	4	AS ABOVE	+
$\underline{v^s}/\underline{v^s}$	F	 (x4) CONFORMATIONAL ERROR IN THE POLYPEPTIDE CHAIN	 (x4) NO ACTIVE CENTRE FORMED	0	0 %	v
$\underline{v^s}/Y$	M	AS ABOVE	AS ABOVE	0	0 %	v
$\underline{v^s}/\underline{v^+}$	F	 $\underline{v^+}$ $\underline{v^s}$		3	25 % 25 % 25 % 0 % 75 % TOTAL	+

*An aribitrary number of subunits is represented; this number remains the same for each genotype.

**Dimers are represented as an association of subunits which changes the configuration of the active centres, permitting enzyme activity.

***Represents the proportional number of dimers from which a unit of TP activity is obtained.

In practice, the realized TP activities of these heterozygotes would depend upon such factors as the extent of restored catalytic activity of each $(Pv^+)_2(Pv^s)_2$ tetramer and their relative susceptibilities to inhibition. That is, the various conformational "mistakes" present in the Pv^s subunits may variably prevent a full unit of TP activity in $(Pv^+)_2(Pv^s)_2$ tetramers. According to this model, the TP activities found for $\underline{v^s}/\underline{v^+}$ females should vary between 50% and 75%. This, in fact, has been found (TARTOF 1969).

The precise nature of the molecular interactions required to form active enzyme from inactive monomeric subunits is not predicted, although the heme prosthetic group is probably involved since loss of this from the $\underline{v^+}$ enzyme renders it inactive and *in vitro* preincubation of TP from one $\underline{v^s}$ allele ($\underline{v^k}$) with methemoglobin under unsuppressed conditions, restores a small amount of enzyme activity (5% of wild type) (BAILLIE AND CHOVNICK 1971). The substrate, l-tryptophan, may also have a role in stabilizing tetramer formation as $\underline{v^+}$ TP is substrate-inducible *in vivo* (RIZKI 1968) and *in vitro* preincubation of $\underline{v^+}$ extracts with the substrate analogue, α-methyl tryptophan, stimulates enzyme activity (BAILLIE AND CHOVNICK 1971).

Partial larval starvation of $\underline{v^s}$ alleles also permits a varying restoration of $\underline{v^+}$ eye colour, (GREEN 1952; SHAPARD 1960), and TP activity (MARZLUF 1965; TOBLER, SIMMONS AND BOWMAN 1967), possibly by reducing the amount of an endogenous inhibitor such as a pteridine which may interact with differentially sensitive sites

on $\underline{v}^s$ TPs, thereby disturbing tetramer formation.

As previously discussed, the unsuppressible $\underline{v}$ mutations, whether spontaneous or induced, are probably missense mutations which directly affect the active centre or another essential catalytic site in the enzyme. Therefore, in $\underline{v}^u/\underline{v}^+$ heterozygotes never more than 50% of wild type activity is recovered because only 50% of correct catalytic sites are available.

No complementation is observed between any combination of $\underline{v}$ alleles; $\underline{v}^s$ with $\underline{v}^s$, $\underline{v}^s$ with $\underline{v}^u$ or $\underline{v}^u$ with $\underline{v}^u$. For combinations of different $\underline{v}^s$ alleles this may be due to the extent of the conformational changes in the respective TP subunits which do not permit any significant aggregation into active enzyme. While the interpretation that $\underline{v}^u$ represent mutations in the active site of the enzyme is preferred, the data discussed do not rule out the possibility that they are mutations in regulatory elements located within the $\underline{v}$ cistron, although this is unlikely based on the size of the $\underline{v}$ cistron and the location of $\underline{v}^u$ alleles at each of the 3 identifiable sites within the cistron. In any case either interpretation is compatible with the lack of complementation between $\underline{v}^s$ and $\underline{v}^u$ or between different $\underline{v}^u$ alleles. Thus, if $\underline{v}^u$ are missense mutations in the active site of the enzyme no active, homomultimeric enzyme will be formed by $\underline{v}^s/\underline{v}^u$ heterozygotes since $\underline{v}^s$ TP subunits are conformationally disordered and $\underline{v}^u$ subunits are catalytically deficient. If the $\underline{v}^u$ mutants are regulatory in nature, then it might be expected that they contribute no, or

very few, TP subunits to the $\underline{v}^{s}/\underline{v}^{u}$ heterozygotes, thereby again failing to complement any $\underline{v}^{s}$ alleles. A definitive designation of the nature of $\underline{v}^{u}$ alleles awaits a determination of whether they form inactive TP protein of normal molecular weight.

In $\underline{Dfv}/\underline{v}^{+}$ heterozygotes, wild type phenotypes result in all cases (LEFEVRE 1969, and Table 6). According to the model illustrated in Figure 21, this is because the identical, correct subunits specified by the single $\underline{v}^{+}$ cistron can interact to produce an active enzyme. However, in $\underline{Dfv}/\underline{v}^{ts1}$ heterozygotes, a more vermilion-like phenotype results at the permissive temperature than in $\underline{v}^{ts1}/\underline{v}^{ts1}$ homozygotes or $\underline{v}^{ts1}/\underline{v}$ heterozygotes (Tables 4 and 6). This can be explained by correction between slightly altered $\underline{v}^{ts1}$ subunits and partial correction between $\underline{v}^{ts1}$ and $\underline{v}$ TP subunits at the permissive temperature. Since no product is formed by a deficiency, there will only be 50% of $\underline{v}^{ts1}$ TP subunits capable of interacting in $\underline{Dfv}/\underline{v}^{ts1}$ heterozygotes compared with 100% in $\underline{v}^{ts1}/\underline{v}^{ts1}$ homozygotes, and 50% $\underline{v}^{ts1}$ TP subunits and 50% $\underline{v}$ TP subunits in $\underline{v}^{ts1}/\underline{v}$ heterozygotes, thereby accounting for the more $\underline{v}$ - like phenotype of $\underline{Dfv}/\underline{v}^{ts1}$.

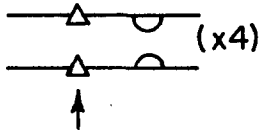
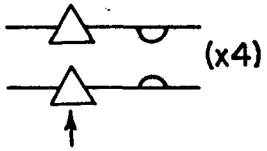
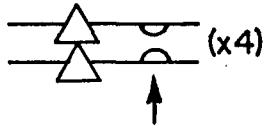
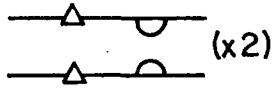
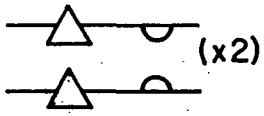
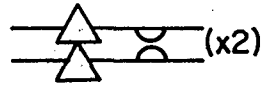
The $\underline{v}^{ts1}$ mutation is probably an unusual $\underline{v}$ allele in that it could be a missense mutation which primarily affects the conformational properties of the enzyme subunits differentially at different temperatures. This effect could be exaggerated as the temperature at which the enzyme is assembled is increased so that at the restrictive temperature the catalytic activity of the enzyme

is secondarily affected because of "warping" of the active centres due to conformational distortion of the tetramer. These possibilities are illustrated in Figure 22.

The lack of complementation between $\underline{v}^{ts1}$ and $\underline{v}^{ts2}$ at the restrictive temperature, thus could be seen as a failure of TP subunits, conformationally deficient in different regions, to aggregate together to form an active, homomultimeric enzyme. At the permissive temperature, these conformational changes are not nearly as severe, therefore, the subunits specified by $\underline{v}^{ts1}$ and $\underline{v}^{ts2}$ respectively in a $\underline{v}^{ts1}/\underline{v}^{ts2}$ heterozygote, are capable of aggregating together into an almost normally active enzyme, hence a wild type phenotype is observed. In combination with nonconditional $\underline{v}$ alleles, $\underline{v}^{ts1}$ is phenotypically more vermilion-like at the permissive temperature than $\underline{v}^{ts1}$ hemi- or homozygotes because the more severe structural alterations in the TP subunit controlled by the non- ts $\underline{v}$ alleles, do not permit the degree of aggregation into active enzyme that $\underline{v}^{ts1}$ subunits allow. At the restrictive temperature for $\underline{v}^{ts1}$, heterozygotes of $\underline{v}^{ts1}$ and non-ts $\underline{v}$ alleles are more vermilion-like than at the permissive temperature (Table 4) because there is an increased alteration in the structure of the $\underline{v}^{ts1}$ TP subunit leading to a less enzymatically active aggregation with the non- ts $\underline{v}$ TP subunits.

As is shown in Figure 22, enzyme activities of $\underline{v}^{ts1}/\underline{v}^{ts1}$ females and $\underline{v}^{ts1}$/Y males raised at 22°C are not 100% of wild type presumably because the conformational change in the monomers

FIGURE 22 Model of interactions among enzyme subunits controlled by v^{ts1} at the permissive and restrictive temperatures and consequent tryptophan pyrrolase activity.

GENOTYPE	SEX	TEMP	SUBUNITS FORMED	DIMERS (RATIOS)	NO. ACTIVE DIMERS	ACTIVITY (% WT) EXPECTED	ACTIVITY (% WT) ACTUAL	PHENOTYP
v^{tsl}/v^{tsl}	F	22°C	 (x4) CONFORMATIONAL CHANGE SPECIFIED BY v^{tsl}	 (x4) ACTIVE CENTRE OF DIMER	4	100 %	70 %	+
		29°C	 (x4) EXAGGERATED CHANGE AT 29 °C	 (x4) NO ACTIVE CENTRE FORMED	4 MOSTLY INACTIVE DIMERS	?	17 %	v
v^{tsl}/Y	M	22°C	AS ABOVE	AS ABOVE	AS ABOVE	100 %	78 %	+
		29°C	AS ABOVE	AS ABOVE	AS ABOVE	?	5 %	v
$v^{tsl}/Df v^{L3}$	F	22°C	 (x2)	 (x2)	2	35 %	15.5 %	v
		29°C	 (x2)	 (x2)	2 MOSTLY INACTIVE DIMERS	<17 %	5 %	v

probably causes some slight distortion of the active centres of the assembled tetramer. If raised at 29°C, the conformational changes are exaggerated and thereby cause severe distortions of the active centres. Hence the TP activities of $\underline{v^{ts1}}/\underline{v^{ts1}}$ females and $\underline{v^{ts1}}$/Y males are much reduced.

This model does not explain why $\underline{v^{ts1}}/\underline{v^{ts1}}$ females should retain more enzyme activity than $\underline{v^{ts1}}$/Y males at 29°C. Another difficulty is that the enzyme activity of $\underline{v^{ts1}}/\underline{Dfv^{L3}}$ females is less than expected at 22°C. Since they should produce half the number of the same subunits as formed by $\underline{v^{ts1}}/\underline{v^{ts1}}$ females at 22°C, then it is expected that they should yield half the enzyme activity. However, instead of an enzyme activity which is about 35% of wild type (half of the 70% of wild type activity produced by $\underline{v^{ts1}}/\underline{v^{ts1}}$ females at 22°C), the activity of $\underline{v^{ts1}}/\underline{Dfv^{L3}}$ heterozygotes is only 15.5% of wild type. The activity of $\underline{v^{ts1}}/\underline{Dfv^{L3}}$ females raised at 29°C is about 5% of wild type. As expected, this is approximately half of the TP activity produced by $\underline{v^{ts1}}/\underline{v^{ts1}}$ females at 29°C (17%).

This is a very simplistic model and obviously has value only as a working hypothesis of the functioning of the v locus in general and the $\underline{v^{ts1}}$ mutation in particular. As more direct data is accumulated by the development of electrophoretic and immunological techniques for purifying and analyzing TP, some of the unresolved questions concerning this locus and the $\underline{v^{ts1}}$ mutation undoubtedly will be clarified.

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