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BIOCHEMICAL AND EVOLUTIONARY GENETICS OF TREHALASE IN DROSOPHILA

City University of New York PH.D. 1980

BIOCHEMICAL AND EVOLUTIONARY GENETICS OF TREHALASE IN *DROSOPHILA*

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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ABSTRACT

This thesis has examined the evolutionary implications of genetic variation in the physicochemical properties of the trehalase gene-enzyme system in Drosophila. The arguments presented have been developed in the framework of the hypothesis of "Conditional Neutrality". This concept defines those mechanisms which result in compensation for alterations in the physicochemical properties of allozymes and lead to their selective equivalence. It is suggested that such homeostatic compensation has evolved as a result of selective pressures operating at the level of the selective unit of which the allozyme is part. A selective unit is envisioned as the smallest set of genes responsible for the production of a given phenotype which may be modified by natural selection. In this context, it is suggested that the molecular evolution of proteins can be viewed in a manner analogous to Wright's "Shifting Balance Theory Of Evolution", in that allozymes could enter into and oscillate between both selective and neutral phases depending upon the existence and stability of compensatory mechanisms. It is demonstrated that differences in the physicochemical properties of allozymes are required for molecular evolution by selective processes. Mechanisms are suggested whereby natural selection could operate on quantifiable enzyme properties (K_m and V_{max}) in a semi-quantitative model based on the utilization of trehalose as a substrate for insect flight. In the case of an unregulated enzyme, it is apparent that an optimum K_m and V_{max} would exist depending upon the rate of substrate utilization. In regulated enzyme systems, the evolution of

K_m and V_{max} would depend upon the properties of the regulatory molecule in relation to the demands imposed by product utilization and the amenability of the regulatory molecule to co-evolve.

Specific activity of soluble trehalase from Drosophila melanogaster has been increased a minimum of 10 times by preparative electrophesis using selective unstacking procedures. However, substantial purity was suggested by the presence of a single major protein band on analytical polyacrylamide gels. A comparison of K_m of crude (desalted) and electrophoretically purified trehalase revealed no difference (0.650 and 0.666 mM, respectively). Similarly, estimated K_m for 0-3 day, 12-15 day D. melanogaster and 0-4 day and 12-15 day D. simulans were identical with a mean value of 0.726 mM. These data indicate that no exhaustive purification or ageing procedures are required for K_m comparisons for these species. However, there were differences in maximal catalytic activity per gram wet weight of souble trehalase; the catalytic activity per gram wet weight of both age samples of D. simulans enzyme exceeded that of both samples from D. melanogaster. In both species, the younger flies showed higher activity. These data indicate that differences in V_{max} between enzyme forms must be interpreted cautiously.

Qualitatively, trehalase activity increases sharply in pharate pupae relative to all other preadult stages, and is maintained at this level in young adults. Electrophoretic mobility of the enzyme remained constant in all life cycle stages.

Trehalase from D. melanogaster is inhibited by a variety of compounds including amino acids and sugars, but the presence of a large

proteinaceous, divalent metal ion dependent regulatory molecule could not be substantiated.

Trehalases in the Drosophila melanogaster subgroup are hypothesized to be encoded by at least two structural gene loci, although in the majority of species only one is expressed. Species differences in electrophoretic mobility, thermal stability and/or K_m and V_{max} may be explained by hypotheses which combine the differential expression of these loci and the presence of fixed electromorphs at an expressed locus.

Within species, there is an apparent lack of electrophoretic genetic variation, yet electrophoretic bands in D. melanogaster and D. simulans individuals can be shown to be heterogeneous by the application of thermal denaturation techniques. The difference in thermal stability of two strains of D. melanogaster has been shown to segregate according to expected Mendelian ratios. The similarity of Michaelis constants, K_m, of these enzyme forms in five strains of D. melanogaster and two of D. simulans suggest that they are physiologically equivalent, and hence selectively neutral. No statement can be made concerning the parameter V_{max} until the genetic interpretation of this parameter is established. Trehalases from D. teissieri and D. yakuba are characterized by identical kinetic properties although they differ from both D. simulans and D. melanogaster. The enzyme from D. mauritiana has similar electrophoretic mobility and thermal lability as D. simulans but kinetically resembles D. teissieri and D. yakuba.

On the basis of these data, it is suggested that the trehalase enzyme locus is selectable, but that the alleles present within species are selectively neutral. The generation of species specific trehalases within the melanogaster subgroup of Drosophila has most likely involved

the action of natural selection and genetic drift on both structural and regulatory elements of the trehalase gene.

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I. GENERAL INTRODUCTION

A major generalization of evolutionary theory equating the rate of evolution with the amount of genetic variation expressed in populations has prompted studies which attempt to characterize the genetic variability of structural and regulatory gene loci in natural populations. The technique of gel electrophoresis was first applied to this problem by Lewontin and Hubby and Harris in 1966. Since these reports, surveys of prokaryotic and eukaryotic species have established the ubiquitous nature of genetic variation in structural genes coding enzymes and proteins. Considerations of the evolutionary significance and mechanisms for the maintenance of such large amounts of variation led to the generation of the neutralist and selectionist explanations. The controversy generated by these hypotheses has been a central theme of population genetics for the last decade.

Two basic investigative methodologies have been employed in an attempt to resolve this controversy; the statistical and experimental approaches. The statistical approach attempts to test the goodness of fit of available electorphoretic data with the predictions of the neutralist and selectionist theories. The experimental approach investigates the structural and functional properties of proteins and their relationship to selective and non-selective forces. Although it may be argued that the statistical approach is a more valid test of the two theories because of their mathematical formulation, the experimental approach is useful in that it can assess the conclusions of statistical testing procedures and be used to produce realistic

alterations in each theory. Despite these investigations, the controversy still flourishes.

The recent application of systematic biochemical procedures has detected the presence of "hidden" genetic variation. Although the existence of such variability has long been predicted, the data available to date indicate a bimodality of polymorphism, that is, polymorphic loci became more polymorphic when examined by systematic procedures, whereas monomorphic loci tended to remain so. These data, in combination with the information from statistical and experimental studies indicated the necessity to modify both selectionist and neutralist hypotheses. Nevertheless, it appears that both neutral and selectively maintained genes may co-exist in natural populations.

Neutral alleles may exist in populations as a consequence of several factors. These include an effective population size sufficiently small to increase the importance of stochastic factors relative to selective ones, alternatively by the functional equivalence of gene products. This latter aspect may arise by two mechanisms; from the physicochemical identity of the allelic products, and/or by some form of compensation either inherent in protein structure or due to biochemical and physiological mechanisms. Such compensation would minimize the biological differences among allelic products and thereby shield such genes from selective constraints.

The scope of this thesis involves the formalization of such biological compensation in terms of the hypothesis of "conditional neutrality". The argument advanced stresses the homeostatic features of biological systems and their relationship to enzymes which are

investigated electrophoretically in natural populations. The approach necessitates that the role of an enzyme to the overall biochemical and physiological process be defined (i.e. the selective unit) before any attempt is made to ascertain the evolutionary significance of genetic variants at the specified enzyme locus.

The metabolic pathway leading to the synthesis and degredation of the dissacharide trehalose has been chosen to investigate the feasibility of this model. Trehalose has long been known to be the major reserve carbohydrate in the hemolymph of higher insects, and as such plays an important role in physiological processes dependent upon energy reserves. These include insect flight, chitin synthesis, and possibly resistance to cold. The relationship of insect flight to trehalase metabolism is of particular interest in that its duration involves the catabolism of trehalose by a single enzyme trehalase.

In this context, it is apparent that the trehalose pathway offers an excellent opportunity to examine the evolutionary significance of genetic variation. The relationship between the enzyme trehalase and a number of physiological processes, particularly duration of flight, should permit the quantification of the selective unit and subsequently an assessment of the role of genetic variation at the trehalase locus in the evolutionary dynamics of this physiological process.

The investigations to be reported in this thesis are concerned with the catabolism of trehalose by the enzyme trehalase. The work completed to data involves: the formalization of arguments involving conditional neutrality and the selective differentiation of allozymes, the purification physicochemical and developmental characterization of the enzyme from Drosophila melanogaster, and the genetic and

biochemical characterization of the enzyme from strains which comprise the D. melanogaster subgroup. These data are discussed in terms of the evolutionary arguments surrounding the selectionist-neutralist controversy.

The thesis is divided into four sections: literature review of the selectionist-neutralist hypothesis, the trehalose metabolic pathway and its associated physiological correlates. Chapter 1 is concerned with the formalization of the hypothesis of conditional neutrality and the operation of selection at an allozyme locus. Chapter 2 summarizes the purification, physicochemical properties and developmental investigations performed on species Drosophila. Chapter 3 investigates the biochemical and evolutionary genetics of trehalase in the melanogaster subgroup of Drosophila.

Literature Review

I. Population and Biochemical Genetics

1. Genetic variation

Knowledge of the amount and distribution of genetic variation contained within and among species is an essential aspect of the study of evolution. It has long been known that genetic variation is the substrate for evolutionary processes including both speciation and adaptation. Studies concerned with the amount and distribution of genetic variation in natural populations have by technological necessity concentrated on structural gene loci rather than regulatory genes. Structural genes are defined as the sequence of nucleotides which are transcribed and subsequently translated into the primary structure of the protein. Regulatory genes, in population studies, are rather vaguely defined as genetic elements which control the expression of structural genes. This may entail such factors as tissue distribution, regulation of activity, among others.

(i) Structural genes

The utilization of gel electrophoresis in population genetics was pioneered by Lewontin and Hubby (1966), Hubby and Lewontin (1966) and Harris (1966) as a means of indirectly assessing the amounts of structural genetic variation in populations of Drosophila pseudoobscura and humans. Subsequent electrophoretic surveys of proteins indicated the almost ubiquitous nature of the genetical variation detected

within this class of genetic loci. Extensive reviews and compilation of the available data are presented by Selander and Kaufman (1973), Lewontin (1974), Powell (1975a,b), Nei (1975), Selander (1976) and Fuerst, Chakraborty and Nei (1977). Li (1978) and Lewontin (1974) have described a number of striking features of such electrophoretic surveys. 1) There appears to be an upper limit to and relative constancy of average heterozygosity within species (Lewontin 1974, Powell 1975a). 2) The distribution of allele frequencies in populations is U shaped (c.f. Ayala et al. 1974, Ohta 1976, Li 1978) and 3) there seems to be a large excess of rare alleles observed in this distribution, 4) closely related species and geographic populations have for the most part not diverged to a significant extent (Ayala et al. 1974).

The electrophoretic techniques employed in the majority of such investigations involved the use of a single resolution criteria and were always known to underestimate the amount of genetical variation present although the extent was and still is a matter of considerable speculation. Several factors were postulated which would result in this underestimation. As a consequence of the redundancy of the genetic code only 32% of all codon substitutions would produce identical protein molecules. Of the remaining, 68% of genetic substitutions could be detected and only 32% (i.e. 22% of 68%) of these were believed to be able to be detected electrophoretically. The existence of such "hidden variation" prompted King and Ohta (1975) to propose the term electromorph to designate genetic heterogeneity of electrophoretic bands.

The existence of electromorphs has had several consequences on the mathematical quantification of the neutral gene theory. In their

original formulation Kimura and Crow envisioned the number of possible allelic states to be infinite. Clearly this assumption was not valid in terms of the techniques available. The step-wise or charge state model of electrophoretic mutation was proposed by Ohta and Kimura (1973) as an alternate to the infinite allele model. Under the step-wise model the effective number of alleles (ne) in a population can be defined as:

$$ne = \sqrt{1 + 8NU} \qquad (1)$$

where N is the effective population size, and U the mutation rate, as compared to

$$ne = \sqrt{1 + 4NU} \qquad (2)$$

by the infinite allele model.

Although several studies, Cobbs and Prakash (1977), Ramshaw and Eanes (1978a) have presented evidence which supports the step-wise model, it is apparent that so-called standard electrophoretic procedures (single electrophoretic criteria usually at high pH) can detect partial as well as unit charges. This contention is supported by the considerations of Johnson (1974 and 1977) and by the starch gel electrophoretic survery of sequenced hemoglobins by Fuerst and Ferel (in preparation) who have shown that 30-40% of these hemoglobins variants migrate in a non step-wise fashion. These data suggest the applicability of a model proposed by Li (1976) which is intermediate with respect to the infinite and step wise models and predicts the effective number of alleles as:

$$ne = \sqrt{(1 + 4NU)^2 - 4N\mu} \qquad (3)$$

where U = the sum of stepwise μ and nonstep-wise ν mutation rate.

(ii) Hidden alleles

The existence of amino acid substitutions in proteins which

produce undectable changes in electrophoretic mobility has been known for some time. Several biochemical procedures are available which can detect an, as yet unknown, proportion of such variants. Those commonly employed in population studies involve: systematic alteration of gel concentration (e.g. gel sieving as termed by Johnson), pH variation (exemplified by Singh et al. 1976) heat denaturation studies (Bernstein et al. 1973) and isoelectric focusing (Synder 1978a). General methods for establishing the homogeneity of electrophoretic bands have been described by Ressler (1973) and Chrambach et al. (1976). Other techniques are mentioned by Singh et al. (1976). It should be cautioned that several procedures, for example, the use of kinetic constants Km and Vmax do not necessarily indicate the presence of structural variants. For example, Km may be modified by naturally occurring factors, and Vmax is determined in part by total enzyme concentration.

A number of studies (Chakraborty and Nei 1976, Nei and Li 1975) have attempted to mathematically determine the number of such hidden alleles under the assumption of neutrality. Milkman (1979) claimed that thermostability analysis in bacterial enzymes are only sensitive in detecting amino acid substitutions which separate the free energy of molecules by over 400 cal/mole. However, it should be cautioned, that it is not certain if the methodology utilized in this calculation was based on equal catalytic activities of enzymes or equal concentrations of protein molecules. The former approach is erroneous (see Segal 1975).

The existence of "hidden" genetic variation has been established in a number of proteins in a variety of organisms with several techniques.

The detection of electrophoretically silent alleles in the α and β chains of hemoglobin in primates, rabbits and humans by direct amino acid analysis has been reported by Von Ehrenstein (1966), Gallizzi (1972), Boyer (1974) and Carrick, Bricker and Barrick (1974). Milkman (1979) reports the failure of heat denaturation techniques to significantly alter estimates of ne (effective number of alleles) in Drosophila and bacteria. However, this preliminary report may be misleading because rare alleles are not expected to contribute substantially to this parameter but as will be discussed may be important in the overall distribution of genetic variation.

Johnson (1976) detected five additional alleles in a survey of two α-glycerophasphate allozymes in Colias butterflies by gel-sieving. Siebenaller and Somero (1978) noted a difference in pressure sensitivity in substrate binding, co-enzyme binding and catalytic rate in electrophoretically identical LDH M_4 isozymes in a deep water and shallow water species of fish. However this result may not necessarily indicate a difference in enzyme structure. Kahler et al. (1979) varied pH and % gel concentration in a survey of Est-1 and Est-2 and Ta - 1 and Ta - 2 locus in Avena barbata Hordeum vulgaris and Zea mays but could not detect a significant increase over the number of alleles detected by starch gel electrophoresis. Johnson (1978) indicated that genetic variation in Adh in D. mojavensis may be masked by conformational changes induced by NAD binding. Cobbs (1976) detected three esterase-5 alleles in D. pseudoobscura based on their ability to dimerize. Cochrane (1976) and Sampsell (1977) employed heat denaturation techniques to detect the presence of "hidden" alleles at the esterase 5 locus and Adh locus respectively in Drosophila melanogaster.

Of greater interest, however, is the series of papers by Lewontin and his associates, utilizing a combination of electrophoretic, heat and other procedures in a survey of hidden electrophoretic variation in D. pseudoobscura and D. persimilis. These data include the papers of Bernstein, Throckmorton and Hubby (1973), Singh, Hubby and Lewontin (1974), Singh, Hubby and Throckmorton (1975), McDowell and Prakash (1976), Coyne (1976), Singh, Lewontin and Felton (1976), Coyne and Felton (1977), Coyne, Felton and Lewontin (1978) and Singh (1979). The most recent estimates are summarized in Table 1. Consideration of these data indicates a number of striking features. These are discussed more fully by Singh et al. (1976), Coyne et al. (1978) and Singh (1979). Briefly it is apparent that there is a bimodality of polymorphism at several loci. Gene loci such as Xdh, Adh-6 (synonymous with aldehyde oxidase) Est-5 which were polymorphic by a single electrophoretic procedure became more polymorphic, whereas, those which were originally monomorphic; Odh, Mdh, αGpdh and Hk tended to remain so. A similar overall pattern but with a more uniform increase in the number of alleles detected is evident in the results of Singh (1979). In all cases, the data indicate that a single electrohporetic allele is prevalent, the remaining alleles being rare.

Coyne and Felton (1977) have argued that the apparent bimodality of genetic loci with respect to hidden genetic variability is strong evidence against the neutral gene hypothesis. However, as indicated by Watterson and Anderson (1978) such differences may be accounted for by differences in mutation rate. The increase in the number of alleles has increased the genetic divergence between species and populations. For example, the data for Xdh discussed by Coyne (1976)

Table 1. Increase in the number of alleles at enzyme loci with the application of systematic biochemical procedures.

Data Source[2]	Locus	Previous	Current	Increase
Coyne[1]	Est-5	12	30	3.5
	Adh-6	7	16	2
	Xdh	8	37	4.6
	Odh	7	8	-
	Hk-1	2	2	-
	Mdh	2	2	-
	αGpdh	1	1	-
Singh (1979)	Adh-6 (Ao)	4	16	4
	Pt-7	4	9	2
	Pt-8	4	6	1.5
	Pt-10	3	6	2
	Pt-13	3	10	3.3
	Est-5	13	32	2.5
	Est-6	3	9	3
	Xdh	6	37	6

1. compilation of data presented at annual meeting of Evolution Society at Boulder, Colorado, June 1979.

2. D. pseudoobscura only.

indicates a change in Nei's index of genetic identity (Nei 1975) from 0.967 to 0.858, 0.966 to 0.861 and 0.995 to 0.958 for three populations of D. persimilis. The genetic identity for D. pseudoobscura and D. persimilis decreased from 0.156 to 0.033.

The significance of these data with respect to the selectionist-neutralist controversy will be discussed in terms of statistical tests, and linkage disequilibrum studies in their respective sections (see also Singh et al. 1976, Coyne and Felton 1977, Coyne et al. 1978).

The remarkable increase in genetic variability of Xdh and Ao (Adh6), in Drosophilia pseudoobscura is currently the subject of considerable controversy. Finnerty and Johnson (1979) and Finnerty, McCarron and Johnson (1979) have demonstrated the existence of post-translational modification of Xdh and Ao in D. melanogaster and maintain that a considerable proportion of the genetic variation reported by Lewontin and Coyne and Singh is due to such factors (c.f. letters by Coyne, Eanes and Lewontin 1979, Singh 1979b and replies of Johnson and Finnerty 1979a and b). At present it appears that the data of Lewontin, Coyne and Singh are the product of allelic variation. Further, the chemical mechanism(s) leading to the post-translational modification has not been specified, nor has the frequency of such modifier genes in natural populations.

(iii) Determination of Codon Substitution

Recent technological advances in molecular eukaryotic genetics should permit a direct estimate of the number of codons which are substituted at structural and regulatory genes. The use of restriction endonucleases comparing mitochondrial DNA in mice has been successfully

employed by Avise, Lansman and Shade (1979) in an evolutionary context (see also, Nei and Li 1979). The procedures of gene isolation, cloning, and sequencing may eventually permit the direct comparison of DNA sequences in eukaryotes but the apparent complexity of the eukaryotic structural gene and associated, but as yet undefined, regulatory elements (c.f. Siedman, Edgell and Leder 1978, Royal et al. 1979) may preclude simple interpretations concerning the amount and nature of genetic variation. A comparison of the composition of cDNA (copies of mRNA made by reverse transcriptase) may be easier to analyze than the actual structural gene with the use of restriction endonucleases (Crawford et al. 1979). However, the amount of genetic variability present in intervening sequences (in trons) may be of consequence if their structures play a role in the regulation of RNA translation.

iv) Regulatory Genes

For the purposes of this discussion, regulatory genes are defined as those genetic elements which control the transcription, post-transcriptional modification, and translation of other genes. Post-translational modification is omitted from consideration because such alterations modify the phenotypic expression of a gene product and not the gene per se.

The importance of regulatory genes in evolution has been recently discussed in several papers including those by Wallace (1975), Wallace and Kass (1974), Wilson (1976) and King and Wilson (1975). The investigation of genetic variation in regulatory genes has lagged behind the corresponding surveys of structural genes for predominately

two reasons. The first is due to the uncertainty of the actual molecular structure, location and operation of such elements in the eukaryotic genome. The second involves the length of time required to perform the necessary biochemical and genetical studies which unequivocally establish the variation to be regulatory, and not present within the structural element.

Several approaches have been utilized, in attempts to screen regulatory mutants. In protein studies those most commonly employed to date involve determination of the number of molecules present. This involves two factors: synthesis and degredation of the molecule. Synthesis by the definition advanced above is more easily associated with regulation. In the studies of enzymes, caution must be employed in equating enzyme activity with protein concentration. Enzyme activity is a function not only of protein concentration but of rate constant(s) as well. Such rate constants may change without altering other kinetic properties (specifically Km). Other approaches which have been employed involve, the presence or absence of protein in specific cells and tissues or closely related species, and the developmental expression of isoenzymes.

Of all studies initiated to date, only those recently summarized by Chovnick et al. (1976) and McCarron et al. (1979) have successfully demonstrated the presence of genetic variation in a regulatory element. Two genetic variants in D. melanogaster have been localized which lie outside the structural gene at the rosy locus (Xdh) but which evidently modify enzyme activity by a reduction or increase in the number of enzyme molecules. These variants map to a genetic element adjacent to the structural locus. An approach similiar

to that utilized by Chovnick and his group, namely the biochemical characterization and fine structure mapping of electrophoretic and activity variants, has been undertaken by Lubinsky and Bewley (1979) for the catalase gene-enzyme system in Drosophila, by Swhartz and Soper (1976) and O'Donnell et al. (1977) for Adh, amylase by Doane (1977) and dopa decarboxylase by Wright (1977). Synder (1978b) has proposed the existence of regulatory mutations controlling the synthesis of hemoglobins in mice and has undertaken a study utilizing molecular probes to isolate these products. This approach should prove more rapid than the rather tedious recombination analysis conducted by Chovnick et al. Kambysellis (pers. comm.) has begun an investigation of the vitellogenin gene in Hawaiian Drosophila with the construction of cDNA molecular probes.

McDonald and Ayala (1978) and McDonald et al.(1977) have described a possible adaptive response of Adh by a change in gene regulation in D. melanogaster. They maintain that the presence of a variation in a number of regulatory genes located on the third chromosome alters activity of Adh by changing protein concentration. However, the data are equivocal at present because not all possible alterations in the structural gene have been excluded. Differences in "Laurell rockets" could be due to cross-antibody response as a function of structural variation in Adh which was not detected by the criteria employed to assess protein homogeneity. The detection of environmental factors which affect the quantity of Adh in D. melanogaster may also be indicative of a possible flaw in the interpretation of McDonald's data. This aspect is discussed by Clarke et al. (1979).

The possibility of regulatory genes influencing Xdh and acid phosphatase-3 activity in D. pseudoobscura has been discussed by Prakash (1977a) and Norman (1979). Both authors are correct in their qualification that the data at present cannot exclude the possibility of a structural variant.

Powell (1979) and Powell and Lichtenfels (1979) have described the genetics of the differential expression of anylase in the anterior gut of Drosophila pseudoobscura and suggest that the differences in distribution of this enzyme are due to the action of regulatory genes. However, the exact nature of the regulatory elements is not defined at the present time.

Rawls and Lucchesi (1974a,b) have presented evidence which indicates that genetic factors other than those directly associated with an enzymatic locus may regulate the activity of several enzymes in D. melanogaster. It was noted that changes in activity of α-Gpdh was consistent with the premise that control was effected by alteration of the synthesis and/or degredation of the enzyme. The possibility of genetic variation in factors influencing the interaction of the enzyme molecule with its substrate could not be entirely excluded from their explanation.

Dickinson (1979) has reported evidence that enzymes detected by electrophoresis are the result of the differentation of regulatory genes in the evolution of Hawaiian picture-winged Drosophila as have Nair Carson and Sene (1976) for two near sibling species of Hawaiian Drosophila, D. setosimentum and D. ochrobasis.

In summary, although it is apparent and not surprising that variation exists in the regulatory elements of a number of gene-enzyme systems, no evidence is currently available concerning the amount, distribution and significance of such alleles. In fact, any determination of amounts of variation in such elements requires the utilization of painstacking fine gene structure analysis (as by Chovnick), or alternatively the isolation of specific regulatory genes with molecular probes and the utilization of suitable molecular characterizations. For most gene-enzyme systems either approach is not realistic at the present time.

2. Selectionist-neutralist theories

Following a consideration of the amount of genetic variation detected at the biochemical level, it is not surprising that theories which attempt to explain the significance and the maintenace of such variation have become a central theme of population genetic and evolutionary theory. In essence there are two explanations; the neutral gene or mutation-drift hypothesis and the selectionist or adaptative gene hypothesis.

The neutral gene theory stems largely from the mathematical consideration of Kimura and Crow (1964), Kimura (1968, 1969a,b), Crow (1972), Kimura and Ohta (1971a,b,c), Ohta (1974), Yamazaki and Maruyama (1972, 1973, 1974) among others. In its strictist form the theory maintains that the majority of biochemical genetic variants are maintained in natural populations by the interaction of such factors as mutation pressure, linkage relationships to selectable genes, and random genetic drift as a function of effective population size. The

theory does not claim that selective forces are inoperative at the biochemical level but rather that alternate enzyme forms coded by structural genes are equivalent either as a consequence of their physicochemical identity or their interaction with population size.

The selectionist theory proposes that the majority of genes coding allozymes segregating in natural populations are maintained by some form of balancing selection, for example heterotic, frequency-dependent or habitat specific, and are therefore of direct adaptive significance. Although non-selective forces are admitted to play a role, selection is viewed as the "driving force" in determining the frequency and distribution of molecular variants. Selection co-efficients are presumed to be small and variable but of significant magnitude to overcome the effects of random genetic drift in all but the smallest of populations. The main issue between the two theories can be stated as whether most of the genetic variation detected in natural populations at a point in time results from the action of random processes or of selection.

Considering the large amount of data (reviewed in Appendices) it is not surprising that the selectionist-neutralist controversy has remained unresolved for 15 years. This is probably not a consequence of the untestability of either hypothesis but due to the complexity of their predictions. Nevertheless, several points emerge from a consideration of the data.

It is readily apparent that selective forces do and can operate at the biochemical level. In certain instances it has been possible to demonstrate the interaction of selective factors with definable properties of enzyme reactions, and thereby explain the pattern of

variation in natural populations. These cases are perhaps best exemplified by investigations of Adh and α-Gpdh polymorphisms. However, the occasional demonstration of the selective maintenance of enzyme polymorphisms does not disprove the netural gene hypothesis as it claims that a small proportion of enzyme polymorphisms, for a given population size and duration of time will be selectively maintained. The central question is whether the majority of alleles which are segregating at most enzyme loci are selectively maintained or in equilibrium as predicted by the interaction of mutation and drift. The most intensive statistical testing procedures performed to date indicate that for the most part the distribution of the majority of alleles in most species is congruent with the predictions of the neutral hypothesis. Admittedly, the power of several of these procedures is insufficient to be able to discriminate between certain combinations of selective models and the predictions of mutation drift. Attempts to demonstrate the likelihood of selective maintenance of polymorphisms by establishing the relationship between the physiological function or structure of an enzyme and its level of heterozygosity may be misleading in that no generalizable structure-function relationship may be applicable to all enzymes. Thus, it may not be possible to partition enzymes into classes such as regulatory, non-regulatory, multimeric, monomeric and to maintain mutally exclusive categories. For example, if one establishes that regulatory enzymes are more variable, does the causal basis for this correlation relate to the functional role of regulatory enzymes in melabolism or simply imply that it is a monomeric enzyme of a particular molecular size. For the most part, attempts to find correlations between heterozygosity and ecological conditions have not been successful.

Although such correlates might exist, the demonstration of their causal basis remains problematic.

There is little evidence supporting the contention that selective forces create co-adapted gene-enzyme systems. Linkage disequilibrium is rarely found between allozyme loci, and it appears for the most part that genes presented in inversion sequences do not contribute to the fitness of such gene complexes. The lack of linkage disequilibrium is surprising considering the theoretical considerations of Franklin and Lewontin (1970). This suggests that either the allelic forms are neutral, or that selective forces are additive in nature or insufficient to generate significant correlations between loci.

At present, it would appear that properties intrinsic to the protein molecule are most important in accounting for the large amounts of biochemical variation detected in populations and that the role of selective factors in explaining the observed amounts of variation are minimal. In fact it may be argued that protein structure has evolved in such a way as to reduce the perturbations introduced by mutation. Selective neutrality could therefore possibly be viewed as an adaptation at the molecular level.

Nevertheless, a consideration of recent data which indicates the presence of considerable hidden genetic variation and the formalization of the predictions of the neutral gene hypothesis (Nei et al. 1976, Fuerst et al. 1977, Chakraborty et al. 1978) and selection hypothesis (Lewontin et al. 1978, Li 1978) necessitates the modification of both neutralist and selectionist theories if they are to account for the observed amount and distribution of electrophoretically distinguishable

genetic variation.

Thus, the observation of an apparent upper limit to heterozygosity as measured by standard electrophoretic methods, (less of a problem if hidden variation is accounted for) the excess of rare alleles in most population than predicted by the neutral gene theory (worsened by the detection of significant hidden variation), correlations of heterozygosities and gene frequencies in D. willistoni species group (probably will be lessened by the detection of hidden variation, c.f. Coyne 1976), rate of protein divergence in Hawaiian Drosophila (perhaps a consequence of a few regulatory polymorphisms) have lead Ohta (1976) to modify the mutation-drift hypothesis by allowing that the majority of mutations are very slightly deleterious. However, several authors have argued that such modifications are not necessary. Thus Nei (1975) and Chakroborty and Nei (1977) maintain that the reason that observed average heterozygosity is less than that predicted by the neutral gene theory is a consequence of past"bottlenecks" in population size. Such bottlenecks resulting in fixation cannot account for the excess of rare alleles observed in natural populations. Ohta (1976) states that the low frequency alleles are maintained in populations mainly by mutation-selection balance whereas Nei, Mauruyama and Chakraborty (1975) argue that this is the result of recent population expansion. The problem of excess rare alleles has been considered by Li (1978) in the formulation of a theory which combines the joint effects of mutation, selection and random drift. Although heterotic models were found to be inappropriate, models of genic or purifying selection coupled with mutation-drift could account for problems of rare alleles heterozygosities and U shaped distribution of allelic frequencies.

Lewontin, Ginzburg and Tuljapurkar (1978) have also demonstrated that heterosis alone is not a mechanism for maintaining many alleles segregating at a locus even when all heterozygotes are more fit than homozygotes and maintain that alternate theories of selection must be devised if selection is to be viewed as a plausible explanation for the amounts of genetic variation at enzyme loci. Wills (1978) has proposed a model which he terms "rank-order selection' and maintains that this model is capable of maintaining all biochemical polymorphisms. However, it is not clear if this hypothesis will provide the necessary U shaped distribution of gene frequencies which characterizes the observed data. Gillespie (1977) has shown that models incorporating random fluctuations in selection pressures can result in U shaped allelic distributions. Hartl (1977) has proposed a stochastic selection model which he maintains can explain the allelic frequencies which are too high to be explained by mutation and constant selection. However it is not known if this model can account for the excess of rare alleles and the U distribution. Models involving habitat selection or habitat subdivision (c.f. Steiner 1977, Gooch and Hedrick 1979, Hedrick 1976, Taylor 1975, 1977, Taylor and Powell 1977) may be successful in explaining the excess of rare alleles although data indicating such an effect are lacking for the most part. It is conceivable that a combination of heterotic and frequency dependent models may be successful as well. Although no such models are currently described in the literature, it is apparent, that if fitness of rare alleles is maximized when they are expressed with the common electrophoretic allele then selection would explain their maintenance. The fitness of a given rare allele would be frequency dependent because as frequency increased average

fitness would decline due to the association of the allele with other rare alleles.

Li's (1978, 1979b) model which combines purifying or genic selection and mutation drift is perhaps the most realistic to date. Based on his consideration alleles would be effectively neutral as a function of population size if mutations are divided into two classes; those where the selective disadvantage $S \leq 1/N$ (population size) and $S > 1/N$. It is clear that the proportion of non-neutral mutations (alleles) decreases when population size decreases. In addition his model can most likely account for the U shaped distribution of allelic frequencies, with its excess of rare alleles, and the apparent upper limit of heterozygosities. Such models which combine the strict neutralist and selectionist interpretations are more congruent with the known physiological and physicochemical data.

II. Trehalase Gene-Enzyme System

1. Distribution and biosynthesis of trehalose

α,α -trehalose (α-D-glucopyranosyl α-D glucopyranoside) has been isolated from a diverse array of organisms including, algae, bacteria, fungi, insects, invertebrates, yeast, some lower vascular plants, and a few flowering plants (see Elbein 1974 for review). The dissacharide appears to be the predominant sugar in the hemolymph of a number of insect species (Evans and Dethier 1957, Wyatt and Kalf 1957, Wyatt 196la, 1967, Elbein 1974, Bedford 1977, Kramer et al. 1978). It should be noted that trehalose appears in extracellular fluid not in cells. Florkin and Jeuniaux (1964) have suggested that the presence of

trehalose in hemolymph may be a biochemical characteristic of insects. However, it is apparent that the concentration of trehalose varies considerably in relation to developmental stage, interspecific and intraspecific variability (Wyatt 1961b, Bedford 1977). A few examples of hemloymph concentrations of trehalose are presented in Table 2. It appears that trehalose is absent in Dipteran larval hemolymph but appears before pupation in several species (e.g. Phormia regina, and Wimer 1969 and Calliphora erythrocephala Duve 1977). It has been suggested (Duve 1977) that trehalose is stored in larval fat body until release prior to pupation. Analysis of whole larval body and larval fat body support this interpretation (Duve). In C. erythrocephala, the increase in trehalose in whole larva is accompanied by a parallel increase in trehalase activity. Trehalose has been isolated from whole larva of Drosophila repleta (Wyatt and Kalf 1957) but for the most part information concerning the amounts of the dissacarhide in this genus are lacking.

Despite the intraspecific variability in trehalose concentration, it is apparent that its synthesis and degredation are quantitatively regulated by enzymatic and hormonal mechanisms (see below). Figure 1, adapted from Wyatt (1967) shows the metabolic pathway involved in the interconversion of glucose, trehalose, and glycogen in insects. Although the data responsible for this figure have been accumulated from a number of different insect orders, it possibly will serve as a good first approximation for Dipteran insects. Two other enzymes, trehalose phosphorylase and phosphotrehalose which catalyze the reactions;

$$\text{Trehalose} + P_i \xrightleftharpoons{\text{TPase}} \text{glucose-1-phosphate} + \text{glucose}$$

Table 2. Trehalose concentration in a variety of species[1]

Species	life stage	concentration (mM)
Solitary Bee	larva	191
Bombyx mori	5th stage larva	73 - 175
H. crecropia	mature larvae	58
	pupae	18
	diapause pupae	4.4 - 8.8
	adult	increases
C. euphorbia	cessation larval feeding	44
	diapause	29
	adult	<29
P. regina	larvae	not present
	adult	3.5 - 8.8

1 Data are from Wyatt (1961, 1967) Evans and Dethier (1957)

Figure 1. Metabolic pathways linking glucose, trehalose and glycogen in insects. (adapted from Wyatt, 1967)

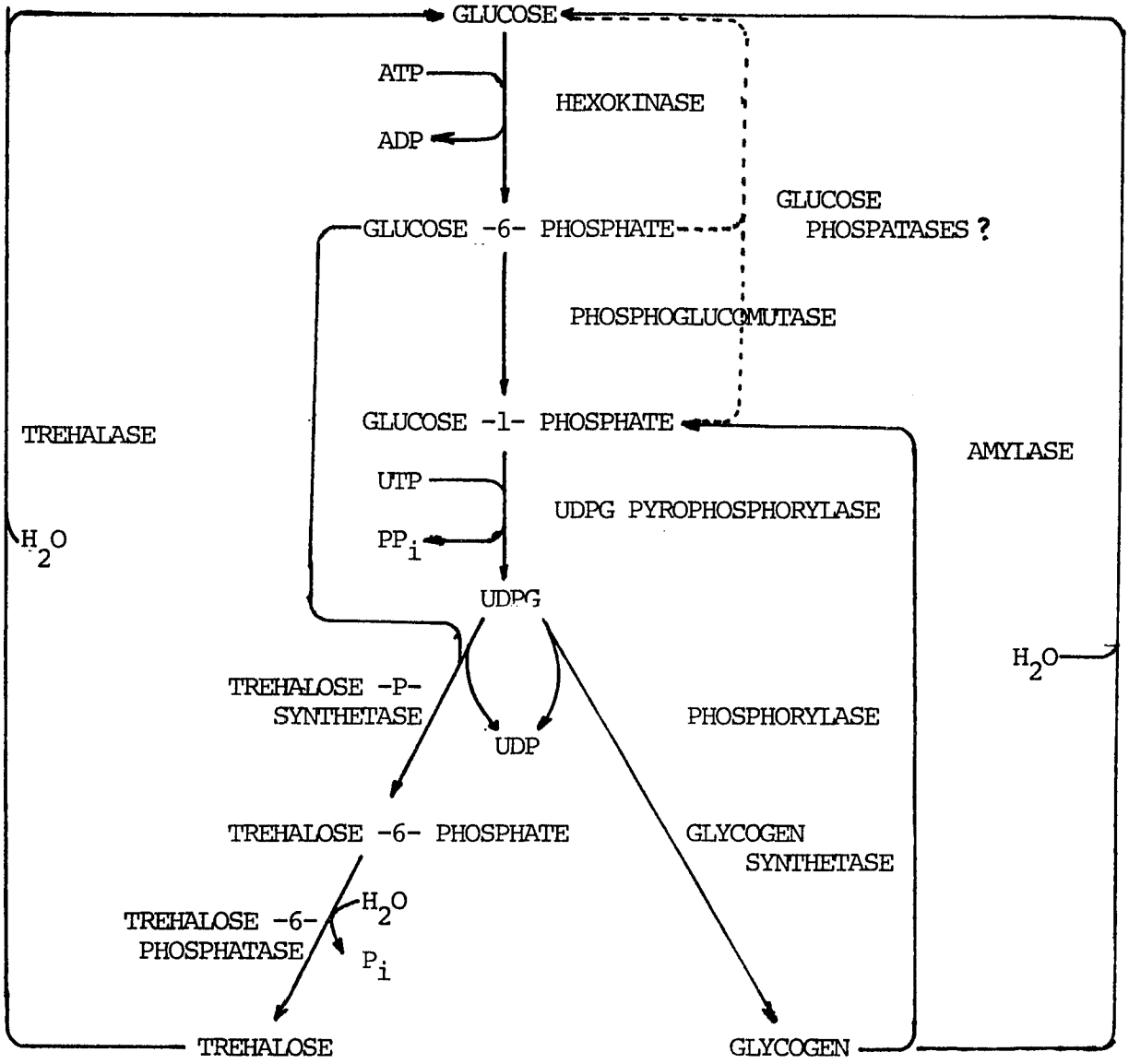

GLUCOSE
ATP
ADP
HEXOKINASE
GLUCOSE -6- PHOSPHATE
GLUCOSE PHOSPATASES ?
PHOSPHOGLUCOMUTASE
GLUCOSE -1- PHOSPHATE
TREHALASE
AMYLASE
UTP
PPi
UDPG PYROPHOSPHORYLASE
H2O
UDPG
H2O
TREHALOSE -P- SYNTHETASE
UDP
PHOSPHORYLASE
TREHALOSE -6- PHOSPHATE
GLYCOGEN SYNTHETASE
H2O
TREHALOSE -6- PHOSPHATASE
Pi
TREHALOSE
GLYCOGEN

Figure 1

and, trehalose-6-phosphate $\overset{\text{PTase}}{\rightleftharpoons}$ glucose-6-phosphate + glucose have been reported in the protozoan, Euglena gracilis (Belocopitow and Marechal 1970) and the bacterium Bacillus popillae (Bhumiratana et al. 1974). The presence of the latter two enzymes has never been established in insects, nor could trehalose phosphorylase be detected in aqueous homogenates of Drosophila melanogaster or D. virilis (Bargello, unpublished) with a phosphoglucomutase-glucose-6-phosphate dehydrogenase couple.

Several of the enzymes shown in Figure 1 have been investigated in a diverse array of species. In Drosophila those characterized include; trehalase (Marzulf 1969, Huber and Lefebvre 1971, Talbot and Huber 1976, Oliver et al. 1978), phsophoglucomutase (Hjorth 1970) herokinases including glucokinase (Jelnes 1971, Knusten et al. 1969) and amylase (McCure 1969, Doane 1965). Of these, phosphoglucomutase, hexokinase, and amylase are reported to be polymorphic in one more Drosophila species.

2. Physiological utilization

The physiological significance of trehalose in insect processes has been reviewed by Wyatt (1967). Of particular interest is the relationship to insect flight (see review of Wyatt 1967, Sacktor 1970). In Phormia regina trehalose has been shown to serve as the predominant energy source for insect flight (Clegg and Evans 1961). Thus, the presence of the enzyme trehalase in flight muscle of P. regina (Reed and Sacktor 1971) and decrease in wingbeat frequency as a function of decreasing trehalose concentration suggests that trehalose is the limiting energy source for flight energy in this species. Sacktor and

Wormser-Shavit (1966) monitored the concentration of several metabolic intermediates including trehalose, glucose, glycogen, and glucose-6-phosphate in thoraces of blowflies during one hour of sustained flight. Glycogen reserves depreciated rapidly over the first 15 min of flight but were maintained at 3-4 μmoles/gram wet weight for the remainder of the test flight. Muscle glycogen was found to be utilized before glycogen from other stores. Trehalose concentration decreased rapidly during the first two min of flight (corresponding to an increase in glucose) and then steadily decreased at a rate similar to that reported by Clegg and Evans (1961). These data were interpreted as indicative of the presence of two kinetically different pools of trehalose. Wigglesworth (1949) reported a decrease in glycogen reserves in flight exhausted individuals of D. melanogaster. Fat reserves were apparently not affected by flight exhaustion in this species. Ten percent trehalose fed to exhausted flies was shown to support continuous flight after 1-1.5 min following ingestion as did glucose, fructose, mannose, sucrose, and maltose. Administration of glycine and alanine decreased the total duration of flight. Sacktor and Wormser-Shavit (1966) report an initial decline, followed by maintenance of steady-state levels of proline and glutamate in Phormia regina during flight. No change was detected in glutamine, asparagine, aspartate, or free NH_4^+. Concentration of alanine increased substantially but decreased to original levels during one hr of sustained flight. Based on theoretical considerations, Weiss-Fogh (1964) suggested that "the very high concentration of trehalose in insect blood can be considered a direct and essential adaptation to flight. Wyatt (1967) implies that trehalose concentration is the

limiting factor in flight muscle energy supply. These conclusions do not apply to all Dipteran or insect species. Thus, Bursell (1975) reported that bloodsucking Diptera which take blood for non-reproductive purposes oxidize proline at a faster or equal rate with respect to pyruvate in flight, whereas those Diptera in which bloodsucking provides a minor dietary or solely a reproductive component do not oxidize proline at a greater rate than pyruvate. Hargrove (1976) reported the ability of the tsetse fly to sustain flight after depletion of thoracic proline was suggestive of the presence of another energy source not involving carbohydrate, direct oxidation of lipids, other Krebs cycle intermediates or amino acids other than proline. Rather, duration of flight in this species resulted from the ability to resynthesize proline at the expense of alanine coupled with an apparant thoracic to abdominal shunt of this proline precursor.

Jutsum and Goldsworthy (1976), Goldsworthy et al. (1979) have demonstrated that trehalose is the predominant energy source for initial flight of the locust (Locusta migratoria) but is replaced by lipid oxidation after approximately 20 min being utilized at only 8-9 percent of the initial rate. In contrast, Van der Horst et al. (1978) report that hemolymph trehalose reaches steady state conditions during sustained flight in male L. migratoria and contributes substantially (23%) to flight muscle metabolism. The remaining energy requirements are presumably derived from diglyceride sources. In the locust Sehistocerea gregaria, long term flight metabolism is supported by diacyl glycerol produced from triacyl glycerol which is stored in the fat body. Interestingly, the fate of the glycerol moeity from diacyl glycerol hydrolysis appears to involve the production of trehalose and diacyl

(Candy et al. 1976). Under restful conditions the ratio of diacyl glycerol to trehalose synthesis is 1, but is increased substantially during flight and following treatment with adipokinetic hormone. Thus, although decreased during flight, this mechanism may provide for the turnover of trehalose reported by van der Horst et al. (1978).

Robinson and Goldsworthy (1976, 1977a,b) indicate that the affect of adipokinetic hormone on flight muscle may be in stimulating the entry of acyl groups into the mitochondria. In addition, they argue that factors other than a decline in trehalose concentration act to reduce carbohydrate utilization in long term flight of S. gregaria. Mechanistically, this may involve the inhibition of trehalose utilization by flight muscle mediated by the action of the adipokinetic hormone. Recently, Candy (1978) reported that the putative neurotransmitter octopamine stimulated the oxidation of glucose, trehalose, butyrate, and diacyl glycerol in perfused flight muscles from S. gregaria but in contrast, Robinson and Goldsworthy could not detect any direct effect of corpus cardiacum extracts on flight muscle.

In summary, although it would appear that a variety of substances may serve as fuels for flight in insects, and the interaction among these and regulatory factors may be complex, it is apparent that trehalose plays a key role in flight metabolism in some Dipteran insects (most probably including Drosophila) and a rather complicated role in Locusta.

In addition to the comparatively intensively investigated role of trehalose in flight metabolism, the dissacharide may play a role in chitin synthesis by serving as a carbon source (Neville, 1967). This may partly explain the increase in blood trehalose concentration

following injury to diapausing Hyalophora cecropia papa, and those of Samia cynthia (Wyatt 1961b). Wyatt (1967) has suggested that the accumulation of trehalose plays a role in conferring cold resistance in some insects.

3. Regulation of trehalose metabolism

It has been established for some time that the primary site of trehalose synthesis from glucose is the insect fat body (see Sacktor 1970) but limited amounts may be synthesized by muscle. Following synthesis, the sugar is rapidly released into hemolymph. The enzymatic process for this conversion has been shown in Figure 1. Regulation of trehalose metabolism in insects can be effected by enzymatic and hormonal control. These are discussed in the following sections:

(i) Enzymatic

Enzymatic regulatory mechanisms within the trehalose pathway have been reviewed for insect species by Wyatt (1967), Sacktor (1970), and Jungries (1976). The metabolic pathway in insects for trehalose synthesis from glucose has been described by Candy and Kilby (1961) and is identical to its biosynthesis in yeast as described by Cabib and Leloir (1958).

Key control mechanisms in the biosynthetic pathway leading to the production of trehalose that have been described involve enzymes which synthesize trehalose and glycogen from UDP-glucose. Silkmoth (Hyalophora cecropia) glycogen synthetase is activated by glucose-6-phosphate and has properties similar to the mammalian enzyme (Murphy and Wyatt 1964, 1965). Thus, the enzyme is presumably activated by

AMP. Trehalose-6-phosphate synthetase from silkmoth is activated by glucose-6-phosphate and magnesium and has shown to be allosterically regulated by trehalose (Murphy and Wyatt 1964, 1965, Jungries et al. 1974). In silkmoth, it is apparent that trehalose would be synthesized under normal conditions at the expense of glycogen due to the greater affinity of trehalose-6- phosphate synthetase for UDP-glucose than glycogen synthetatase. (Km's are 3.0×10^{-4} and 1.6×10^{-3}M for these enzymes, Murphy and Wyatt 1965). This regulatory mechanism is supported by the observations of Spring et al. (1977) concerning the fate of injected glucose into the hemocoel of P. americana. Following injection a period of rapid trehalose synthesis was detected which was followed by a period of glycogen synthesis.

It is also apparent that the synthesis of trehalose is dependent upon readily accessible pools of glucose-6-phosphate and glucose-1-phosphate. Jungries (1977) proposed that because of the inhibitory effect of these sugar phosphates on hexokinase, the enzymes phosphoglucomutase and UPDG-pyrophosphorylase will play key roles in regulating the flow of exogenous and endogenous precursors into synthesized trehalose.

Friedman (1968) has indicated that in the blowfly, trehalose may activate a glucose-6-phosphatase, and in this way, may regulate its own synthesis, but would not immediately affect the synthesis of glycogen (see Figure 1). Therefore, because of the inhibitory effects of trehalose on trehalose-6-phosphate synthetase, and on glucose-6-phosphatase it appears that the rate at which trehalose is removed from sites where it is synthesized will also play a significant role in the regulation of its formation.

More recently, Friedman (1971) has reported that the enzymes glucose-6-phosphatase and trehalose-6-phosphatase are identical proteins in P. regina with molecular weight of 25,000 - 26,000 daltons. The enzyme requires Mg^{2+} for the hydrolysis of either substrate, and apparently a portion of the active site overlaps in the two functions. Glucose-6-phosphate is reported to be a competitive inhibitor of trehalose-6-phosphatase. However, the regulatory significance of this inhibition is uncertain at present.

Although, following the considerations of Statdman (1966) one might expect some form of regulation of the activity of UDPG-pyrophosphorylase in this pathway, there are no reports of such regulation with respect to the trehalose pathway in Diptera.

As mentioned earlier, trehalose appears in extracellular fluids and has not been localized within cells. Although, there is some controversy concerning the localization of the enzyme trehalase (see Sacktor 1970, pp. 298-300) it appears to be present on the plasma membrane. This position and the lack of intracellular trehalase is consistent with the proposed function of the enzyme in transporting sugar into the cell.

In the above context, the report of the simulantaneous existence of trehalose and trehalase in hemolymph of P. regina (Friedman 1961) was surprising. Friedman postulated that hemolymph trehalase in P. regina is inactive due to the presence of a non-dialysable regulatory protein and a metal ion (Mg^{++}, Mn^{++}, Co^{++}).

This inactivation has been utilized as an explanation for the simultaneous occurence of enzyme and substrate in hemolymph. Presumably, the enzyme is activated during exercise and thereby provides a pool of

glucose for glycolysis. There are several difficulties with this interpretation. Van Handel (1978) could not detect the presence of a trehalase inhibitor in undiluted whole hemolymph of the cockroaches Periplaneta americana, P. australasiae, Leucophaea maderae and Nauphoeta cinerea, although substantial trehalase activity could be detected. In contrast, Katagiri (1977) and Matthews et al. (1976) could only detect trehalase activity in hemocytes with no activity present in cell-free hemolymph. Downer and Matthews (1978) reported enhanced trehalase activity in hemolymph of exercised individuals of P. americana when compared to those at rest. Insects rested (isolated) for 48 hr were found to have greater trehalase activity in whole hemolymph than serum. They conclude that hemolymph trehalase activity is confined to the hemocyte fraction but release and activation of the enzyme occurs in response to excitation. The possibility for such a release is supported by the observation of Geiger et al. (1977) that cellular and serum proteins involved in cuticle formation, are freely interchangeable. However, from a physiological viewpoint, release of an active trehalase into hemolymph with the resulting increase in glucose may be counter-productive if the glucose produced diffuses across the gut wall into the intestinal lumnen before it can be utilized in cells. It would seem more reasonable if trehalase were activated on the cell membrane, leading to locally high concentration of glucose which would enter into a specified cell-according to its needs. These considerations indicate that trehalase regulation may be quite complicated and variable in insects.

Several substances have been reported to inhibit trehalase activity (Giebel and Domnas 1976, Agarwal 1976, Labat-Robert et al. 1978). However of these the inhibition of trehalase by glucose and various free amino acids in Sesamia inferens and Culex pipiens quinquefasciatus

by Agarwal (1976) and Giebel and Domnas (1976) may be of physiological significance. The amino acid composition of hemolymph from D. melanogaster and D. subobscura are reported by Collett (1976a,b).

The regulation of several other enzymes within the pathway shown in Figure 1 have been described. These include, glycogen phosphorylase by Stevenson and Wyatt (1964), Childress and Sacktor (1970) hexokinase, glycogen synthetase as reviewed by Wyatt (1967).

(ii) Hormonal regulation

Numerous reports are available which document the hormonal regulation of this pathway in insects. A hyperglycemic hormone first isolated by Steele (1961) from aqueous extracts of the corpus cardiacum of Periplaneta americana, elevates hemolymph trehalose concentrations in insects. Natalize and Fontali (1966) presented evidence which indicates that the hyperglycemic hormone is not species specific. Extracts obtained from locust, housefly, and bee corpora cardiaca produced the same effect in cockroach. The increase in hemolymph trehalose concentration by this hormone appears to lie in the activation of fat body phosphorylase (Hart and Steele1973). This activation of phosphorylase appears to result from an increase in the level of cAMP Hanaoka and Takahashi (1977) indicated that adenylate cyclase activity of P. americana fat body increased three fold following ingestion of hyperglycemic factor resulting in doubled cAMP levels. This second messenger presumably activates the enzyme phosphorylase kinase which mediates the conversion of an inactive phosphorylase b to the active form, phosphorylase a. This would increase the concentration of glucose-1-phosphate which would presumably be shunted into trehalose production through UDPG pyrophos-

phorylase. As described previously, trehalose-6-P synthetase, by virtue of greater affinity for UDPG when glucose-6-P is low, will outcompete glycogen synthetase for UDPG. Therefore, trehalose will be synthesized at the expense of glycogen. Recently, Chen and Friedman (1977a) have indicated that the corpus cardiacum exerts its effect on this and another enzyme site in P. regina. They present evidence which suggests that the corpus cardiacum acts upon trehalose-6-phosphate synthetase resulting in increased trehalose synthesis. In addition, another aspect of the regulation of the interconversion of glycogen and trehalose lies in the apparent ability of the corpus allatum to regulate activity of glycogen synthetase.

Vejberg and Normann (1974) have shown that following cardiacetomy or denervation of the corpus cardiacum, hemolymph trehalose decreases steadily during flight, until the houseflies are exhausted after 45 min. The rate of this decrease appears to be faster than that in controls or in the work reported by Clegg and Evans (1961) which demonstrated the utilization of this sugar for flight in intact blowflies. Jutsum and Goldsworthy (1975, 1976) present evidence which supports the hypothesis that hyperglycemic hormone plays a predominant role in the long term regulation of sugar in the blood of resting locust. The information obtained from Phormia suggests that an important aspect in duration of flight in Diptera involves not only utilization of existing stores of trehalose in hemolymph but also must involve the de novo synthesis of trehalose from fat body glycogen reserves. This mechanism probably explains the decrease in glycogen stores in flight exhausted D. melanogaster.

In addition to the hyperglycemic or hypertrehalosemic hormone, analogous to vertebrate glucagon the existence of insulin and/or a hypotrehalosemic hormone has been established in a diverse array of insect species including Drosophila melanogaster (Seecoff and Dewhurst 1974). Although metabolic studies on intact individuals were not undertaken, results from cells grown in tissue culture indicate that insulin may promote triglyceride storage in Drosophila cells and generally exert anabolic effects similar to those characteristic of the response in vertebrates. Administration of insulin to several invertebrate species including Schistocomatium douthii (Cornford 1974) and Bombyx mori (Weing and Joachim 1936) results in a decrease of blood sugar. Honeybee royal jelly has been shown to contain an insulin like hypoglycemic factor when injected into larvae of Manduca sexta by Kramer et al. (1977). A hypotrehalosemic hormone present in the medial neurosecretory cells of the brain of the blowfly, Calliphora erythrocephala has been reported by Normann (1975). Tager et al. (1969) have reported hypotrehalosemic activity in corpus cardiacum and corpus allatum extracts in adult Manduca sexta. Brain and suboesophageal ganglion of Phormia regina contained hypotrehalosemic activity in a detailed investigation reported by Chen and Freidman (1977b). The possible outcome of interactions between hypertrehalosemic and hypotrehalosemic hormone on regulation of hemolymph trehalse, in resting and flying individuals of P. regina has been described in Chen and Friedman (1977b). Their data suggest that the hypotrehalosemic hormone may have a greater affect in controlling trehalose levels in resting flies but the importance of the hypertrehalosemic hormone

increases in stressful conditions where trehalose is synthesized at the expense of stored glycogen.

In a recent report, Downer (1979a) reported the ability of several known and putative neurotransmitters to produce an hypotrehalosemic response similiar to that produced by exicitation in Periplaneta americana. Octopamine was the most powerful agent. Dopamine, tyramine, and normetanephrine had less pronounced effects. No effect was detectable with synephrine, 5-hydroxytryptamine or isoproterenol. The response was inhibited with β-blocking agents, propranolol and dichloroisoproterenol, but monoamine oxidase inhibitors iproniazid phosphate and nialamide were without effect. However, Downer (1979a) suggested that the ability to generate the response in head ligated individuals is indicative of the non-involvement of the corpus cardiaca and presents evidence (Downer 1979b) that treatment of isolated fat body from Periplaneta americana with 10^{-4} M octopamine, 10^{-4} dopamine increases the concentration of trehalose at the expense of glycogen as measured by a trehalogenic index. This effect is identical to that produced by extracts of corpora cardiaca. Therefore, it is evident that octopamine acts directly on the fat body to stimulate trehalose production. The mechanism involved may be due to an increase in glycogen phosphorylase activity as indicated by Robertson and Steele (1974) through an octopamine sensitive adenyl-cyclase system. Candy (1977) has shown that octapamine is capable of stimulating the oxidation of glucose, trehalose, butyrate and diacyl glycerol by perfused locust flight muscle. However, in contrast to the report of Robinson and Goldsworthy (1977a) coropora cardiaca extracts had no effect on muscle metabolism. It is interesting to note that the hyperglycemic and adipokinetic hormone

extracted from locust corpus cardiacum may be idential proteins of eight amino acids (Holwerda et al. 1977a). However, Holwerda, Weeda and Van Doorn (1977) have separated hyperglycemic and adipokinetic hormones which are not identical to the locust hormone.

Carbohydrate metabolism in insects may also be influenced by diapause hormone (Yamashita et al. 1972). These authors suggested that diapause hormone may affect the de novo synthesis of trehalases in the ovaries of Bombyx mori. Yamashita and Hasegawa (1976) report that synthesis of glycogen in ovaries of B. mori is entirely at the expense of hemolymph trehalose and therefore depends upon activity of trehalase. However, the relevance of the action of this hormone on other physiological manifestations of trehalose is uncertain at present.

4. Summary

In summary, the regulation of trehalase metabolism in insects can be effected by a number of enzymatic and hormonal mechanisms and the interaction of these two processes. Of note is the inhibitory effect of glucose on trehalase, trehalose on trehalose-6-phosphate synthetase which is activated by glucose-6-phosphate. Glycogen synthetase is activated by AMP and glucose-6-phosphate. Trehalose regulates the concentration of glucose-6-phosphate by affecting activity of enzyme concerned with the hydrolysis of this sugar phosphate. A key element in the regulation of trehalose synthesis is the relationship between the Km of trehalose-6-P synthetase and glycogen synthetase for UDP-glucose. It appears that under normal conditions trehalose synthesis is favored but this can be regulated by the action of

hypertrehalosemic hormone and a factor present in the corpus allatum from Phormia. Three hormones, hyperglycemic (hypertrehalosemic), hypotrehalosemic, and diapause have been shown to influence the synthesis of the enzyme trehalase. None are reported to affect its degredation at this time. Hyperglycemic hormone persumably acts through the effects glycogen phosphorylase via cAMP formed by the adenylate cydase system. The mechanism of action of hypotrehalosemic hormone and diapause hormone is unknown. In addition, several neurotransmitters, and putative transmitters are effective in regulating the synthesis of trehalose at the expense of glycogen. Of particular note, is the effect of octopamine which acts via cAMP on phosphorylase.

At present it would appear that trehalose production by the insect fat body is influenced by several factors, which may be species specific. The interrelationship of these factors is undoubtedly a key in understanding the mechanism by which trehalose is regulated and its role in physiological processes.

CHAPTER 1

EVOLUTIONARY DYNAMICS OF BIOCHEMICAL POLYMORPHISMS

I. INTRODUCTION

The significance of large amounts of biochemical variation that are maintained in natural populations is currently a central theme of population genetics. In essence there are two opposing views which attempt to explain the evolutionary implications of these data; the neutral gene theory and the selectionist theory. To reiterate briefly, the main issue between the two theories can be stated as whether most of the genetic variation in proteins which occurs in natural populations at a point in time results from the action of random processes or from selection. The dichotomy between the neutralist and selectionists interpretations can be illustrated in Figure 1-1. This figure illustrates a continuum of fitness values for alleles at gene loci coding for soluble proteins. The dotted line in this figure is consistent with selectionist interpretation, whereas the solid line reflects the strict neutralist position. The fitness values shown in this graph represent the average fitness of an allele over all possible genetic backgrounds or, in other terms, the average effect of substituting one allele for another at a particular gene locus averaged over all possible genetic backgrounds. The utilization of the average effect does not preclude that allelic fitness is variable when expressed on a number of different genetic backgrounds, but this facet has been overlooked in the selectionist - neutralist controversy.

Figure 1-1. Selectionist ------ versus neutralist ———— estimation of the fitness distribution of biochemical alleles.

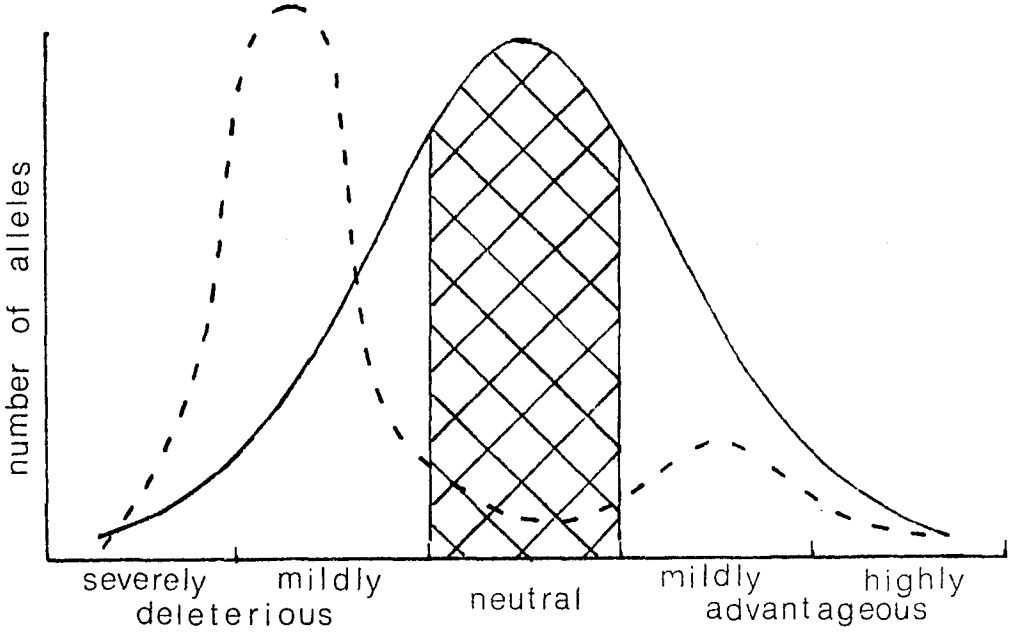

number of alleles
severely deleterious
mildly
neutral
mildly advantageous
highly
average effect

Figure 1-1

It is becoming apparent that both neutral and selected alleles can co-exist in natural populations (Yamazaki and Maruyama, 1974, among others). Neutral alleles may exist as a consequence of several factors. These include an effective population size sufficiently small to increase the importance of stochastic factors relative to selective ones. This would hold even if selectable differences exist between these forms. It is also apparent that selectively neutral alleles independent of population size may result from the functional equivalence of their gene products. If one simply considers the average effect of allelic substitutions, such functional monomorphism may arise in at least two ways: from the physicochemical identity of the allelic products in question, and/or by some form of compensation either inherent in protein structure or due to biochemical and/or physiological processes. This "compensation" would minimize the biological differences between the allelic products and thereby shield such genes from selective constraints even if their effects may be advantageous or deleterious to the individual's fitness. Thus, in the case where such physiological differences between allozymes are manifested, selection can be expected to play a major role in the evolutionary dynamics of the genes producing these allozymic forms. This would hold true for all but small populations.

Although both selected and neutral alleles may coexist in natural populations, discussions concerning the relative proportion of neutral and selected genes are also misleading. Such considerations are synonymous with attempts to determine the position of alleles

along a fitness continuum ranging from deleterious through neutral to advantageous. The difficulty with such attempts lies in the implication that allelic fitness is a static parameter. This contradicts the considerations of Kojima (1971) and Wright (1970) which stress the dynamic properties of genotypic fitness. Thus, the fitness conferred by an allele cannot be assumed to be constant over time or across individuals. This latter point is illustrated in Figure 1-2. It is more probable that biochemical alleles can, in a manner analogous to Wright's "Shifting Balance Theory of Evolution" (Wright, 1970), enter into and oscillate between neutral and selectable phases of evolution depending on a number of interacting factors. In the case of allozymes the following interacting factors may be involved; the structural properties of the enzymes produced by the alleles, the nature of the physiological processes of which they are a part, and finally changes in the overall genetic background upon which these allozymes are expressed.

If one adopts the view that the fitness conferred by allozymes can be variable, then discussions concerning the factors contributing to variability of "allozyme fitness" are perhaps more pertinent to an understanding of molecular evolution. The concept of conditional neutrality was introduced by Bargiello et al. (1977) as an illustration of the possible biochemical factors which may lead to a change in the apparent fitness of alleles coding enzymes when different levels of biological organization are considered. The definition and recognition of the "unit of selection" is a critical component of this concept. For the present purposes, the "unit of selection" is

Figure 1-2. Relative fitness of an allele across individuals (assuming average effect is zero, i.e. neutral).

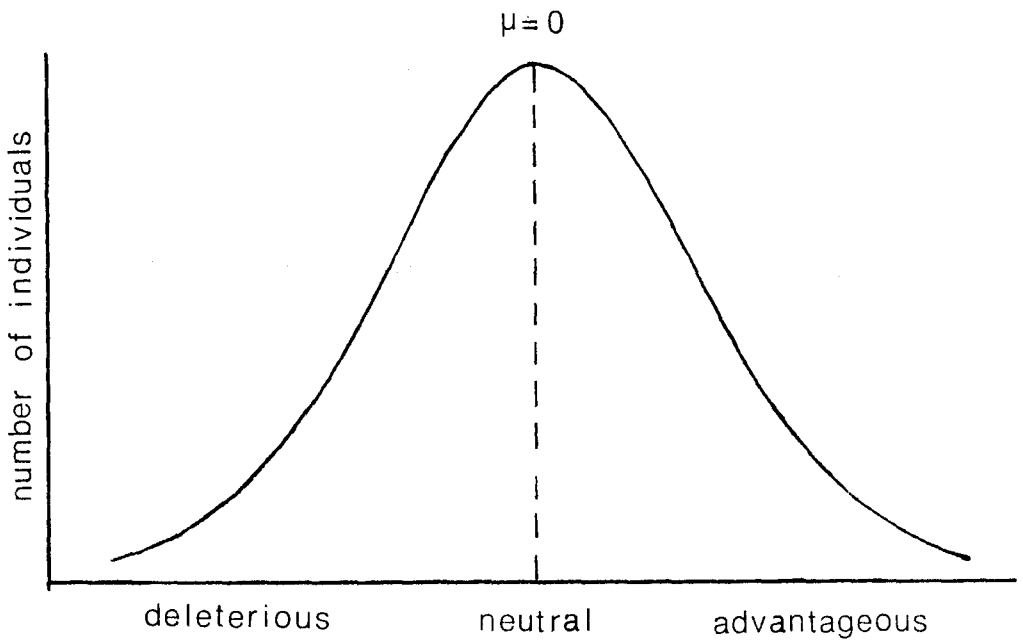

μ = 0
number of individuals
deleterious
neutral
advantageous
relative fitness

Figure 1-2

defined as the smallest set of genes responsible for the production of a given phenotype which may be directly affected by the action of natural selection. The "selective units" envisioned by this definition are variable in size and in organizational complexity, and may be arranged in a hierarchical and interactive fashion reflecting different levels of biological organization. Total organismal fitness would be equal to the summation of the individual components and all possible interactions between them. This definition stems in part from the theoretical considerations of Franklin and Lewontin (1970) which indicated that the genotype rather than individual genes is the unit of selection. This conclusion arose as a consequence of the integration of the genome by genic interactions such as linkage and epistasis. In contrast, the definition utilized in this thesis reflects an attempt to integrate a number of interconnecting biological processes, and suggests the possible existence of a number of levels of selective units rather than the entire genome. In effect this constitutes an attempt to partition the genomic unit of selection into components recognizable at the level of the individual biochemical and physiological processes.

The interrelationship among the physicochemical properties of allozymes, the possibility of biological compensation for differences in these properties, and the operational nature of the "unit of selection" forms the basis for the hypothesis of conditional neutrality. In this paper we wish to expand the discussion of the nature of conditional neutrality in a qualitative manner.

II. CONDITIONAL NEUTRALITY

1. Enzyme multiplicity

The concept of conditional neutrality was originally introduced as a possible explanation for the positive correlation between enzyme multiplicity and genetic variability (Bargiello et al., 1977). The context of the original discussion centered on the evolutionary dynamics of multiple enzyme forms such as vertebrate esterases. These esterases are characterized by a number of electrophoretic bands with overlapping substrate and inhibitor properties. The genetic basis underlying this multiplicity is complex, but appears to generally involve a number of distinct genetic loci. The evolutionary argument for the correlation can be summarized in the following manner.

Consider an enzyme characterized by several multiple forms in a given tissue. We assume that any one of the isoenzyme forms in this multiple series is likely to exhibit optimal catalytic function with a given substrate. For example, enzyme E_1 may optimally act on substrate S_1 but less efficiently on substrate S_2, which in turn is metabolized optimally by enzyme E_2. Enzymes E_1 and E_2 may share subunits or may differ entirely in their underlying genetic determination. The metabolic significance of such multiplicity lies partly in the increased functional metabolic range of the entire enzyme series (Vessel, 1972). This relationship between isoenzyme function and metabolism can be defined as biochemical plasticity. Thus, from a functional viewpoint, or that of individual fitness, E_1 is

complemented by enzyme E_2 and vice versa.

The existence of multiple enzyme forms with these properties in a particular tissue may result in compensation for the physiological effects of genetic variants at one or more of the structural loci underlying the protein multiplicity. Thus, if a mutation affects the structure of E_1, resulting in altered catalytic activity of this form, the deleterious or advantageous effects of this mutation with respect to a particular substrate could be effectively shielded from selection due to the presence of E_2 even if the variant was in a homozygous state. This shielding, which results from the compensation for the alterations in the physicochemical properties of part of the multiple enzyme series by the remaining unaltered forms exemplifies one possible case resulting in conditional neutrality. For the period of time during which this compensation is operational, the frequency and distribution of the two alleles coding allozymic forms of E_1 would be determined by any of a number of nonselective forces. The physicochemical properties of genetic variants which would disrupt the physiological response of the entire enzyme together with compensatory properties intrinsic to the components of this series defines the limits of the conditionally neutral allozymes. Once outside these limits the allozymes would be subject to selective constraints and would remain in this state until the relationship between multiple forms was reestablished to provide the necessary compensatory properties. This reorganized and balanced enzymatic complex could arise as a consequence of new mutation, recombination, or some other genetic mechanism.

2. Biochemical pathways

The argument formulated for multiple enzyme systems can be modified to include an aspect of genetic variability at particular loci within biochemical pathways. Consider a simple biochemical pathway of the following form: $A \xrightarrow{\alpha n} B \xrightarrow{\beta n} C \xrightarrow{\gamma n} D$ where A, B, C and D are the concentrations of metabolic intermediates, and enzymes α_n, β_n, and γ_n catalyze each successive stage. Any given enzyme e.g. α_n can exist in a number of allelic forms (allozymes), α_1, α_2, ..., α_n. Each allelic form can be characterized by a number of physicochemical properties which determine the rate at which one metabolic intermediate is converted to a successive form. If from a physiological viewpoint, the concentration and/or the rate of formation of the end product D must fall within some optimal range, then this pathway may be critical to individual fitness. Alterations in the physicochemical properties of any one enzyme α_n, β_n, or γ_n which would result in some change in D would alter fitness.

It two alleles at any one locus coding an enzyme within this pathway did not differ in their physicochemical properties, then the concentration of D, and its rate of formation would remain constant. By definition, the two alleles would be functionally equivalent and hence selectively neutral. However, it is conceivable that different combinations of alleles at these enzyme loci would result in effectively the same concentration and rate of formation of the end product of D. For example, allelic combinations: α_1, β_6, γ_3; α_3, β_4, γ_1; α_2, β_2, γ_3; may result in equivalent pathways in that they all produce the optimal concentration of end product D. Therefore, in these combinations, the alleles α_1, α_2, α_3 are effectively neutral,

even though they may differ in their physicochemical properties. Likewise allelic group β_2, β_4, β_6, and γ_1, γ_3 would be functionally identical. Thus, by analogy to Wright's shifting balance theory (Wright, 1970), the allelic combinations listed above would rest on an adaptive peak, in that they optimize the physiological effect of the biochemical pathway. As long as these alleles remain in the listed combinations, they will be selectively neutral and their distribution and frequency in populations could be determined by non- selective forces. Unless such beneficial combinations are linked together in some way (e.g. inversions in D. pseudoobscura), they will be broken up and randomly reassorted in each generation. The suboptimal combinations generated would be eliminated by selection and those alleles that remain would be selectively equivalent.

Bewley and Lucceshi (1977) have suggested that the lack of lethality of α Gpdh null alleles is due to compensation within the coresponding pathway. Although such flies are flightless, the alleles are effectively neutral with respect to other fitness components.

3. Unit of selection

The reason that conditional neutrality has arisen in the preceeding considerations is a direct consequence of the fact that the gene locus determining the primary structure of the enzyme is no longer the unit of selection. In the section considering enzyme multiplicity, the isoenzyme forms in a particular tissue have been defined as the selective unit. In the example illustrated in the preceeding section, the biochemical pathway is the unit of selection.

The discussion presented in the preceding sections although quite simplistic and at two different levels of biological organization, may reflect a more general argument concerning the evolutionary significance of biochemical variation. If one accepts that the fitness of an individual is the interactive effect of a number of "selectable units" such as the physiological and biochemical processes described above, and that these processes have evolved in such a manner as to maximize physiological homeostasis (i.e. stability to perturbations) of the individual, then it is reasonable to assume that, providing the physicochemical properties of allozymes that comprise a "selectable unit" do not differ radically, the perturbations caused by allelic substitutions should be absorbed by the homeostatic mechanisms. This leads to the suggestion that, after their initial establishment, biological systems may have evolved further in such a manner as to minimize perturbations introduced by allozyme substitutions. Thus, the relative importance of selection in determining the distribution and frequency of allozymes ultimately depends upon the relative importance of the protein to the selective unit of which it forms a part. If the role of an enzyme is of sufficient magnitude that modifications in its physicochemical properties result in a disruption of the homeostasis of the physiological process, then the genes coding this enzyme form may be selectively constrained. Should the altered properties be insufficient to perturb the system then the genes coding these allozymes will be conditionally neutral.

These cases may be exemplified as follows. For example, the majority of alleles involved in the synthesis of alcohol dehydrogenase in

Drosophila may be selectively constained if they differ physicochemically because they play a significant role in the detoxification of environmental alcohol. However, conditional neutrality may arise in this system in species such as D. pseudoobscura (Singh, 1976) which possess multiple non-allelic forms of Adh by an argument similar to that invoked for esterase multiplicity (Bargiello et al., 1977 and above).

Genes coding enzymes such as hexokinase, trehalase, etc. which play a role in intermediary metabolism may by virtue of compensatory mechanisms possible in such pathways (e.g. allosteric feedback loops) be tolerant of a high proportion of genetic variants. Thus, although differences in the physical chemistry of these allozymes are evident and are selectable in principle, they may be shielded from selection due to the nature of the "actual unit of selection".

The preceeding discussion concerning the interaction of an allozyme and its selective unit are analogous and possible extensions in theme of the hypothesis advanced by Conrad (1977) concerning molecular adaptability. If one establishes that the evolution of primary protein structure followed a course which assures a minimal change in the functional properties of proteins following mutation, then the incidence of conditionally neutral alleles would be increased significantly because the intrinsic compensatory mechanisms described above would be perturbed to a lesser extent if the effects of most mutations result in proteins with only slightly altered physicochemical properties.

4. Hitch-hiking of neutral alleles (conditional selection)

In the preceeding sections, the discussion has centered upon the possible shielding of "selectable" allozymes from selective constraints. The same theme but resulting in selection between allozymes can nevertheless be applied to alleles which in theory cannot be selected in that changes in their primary structure have no, or at best, little effect on their chemical properties. These possibilities arise if one considers the genotype as the unit of selection and the effects of the background genotype on the apparent contribution to fitness of an individual allele.

Consider a single gene locus with two alleles A_1 and A_2. If one assumes that the fitness contribution of the allozymes coded by this locus are not only variable across time, but also depend to a large degree on the genetic background, then one might construct the following fitness continuum (Figure 1-3).

As shown in Figure 1-3 the average contribution to fitness of allele A_1 is zero (solid line) and because of the physicochemical identity, one would predict that the allele, A_2, would demonstrate the identical fitness continuum. However, this need not be the case if for example, the distribution of the allele A_2 was restricted to a subset of all possible genetic backgrounds. As shown in the figure (dotted line), the average fitness need not equal zero. This condition may arise as a consequence of linkage, such that the allele A_2 was located within an inversion, or alternatively, if the frequency of A_2 was substantially lower and therefore present on a non-random sample of genetic backgrounds as a consequence of sampling error.

Figure 1-3. Fitness of allele A_1 with respect to A_2 ——— and A_2 to A_1 --------, with respect to the genetic background.

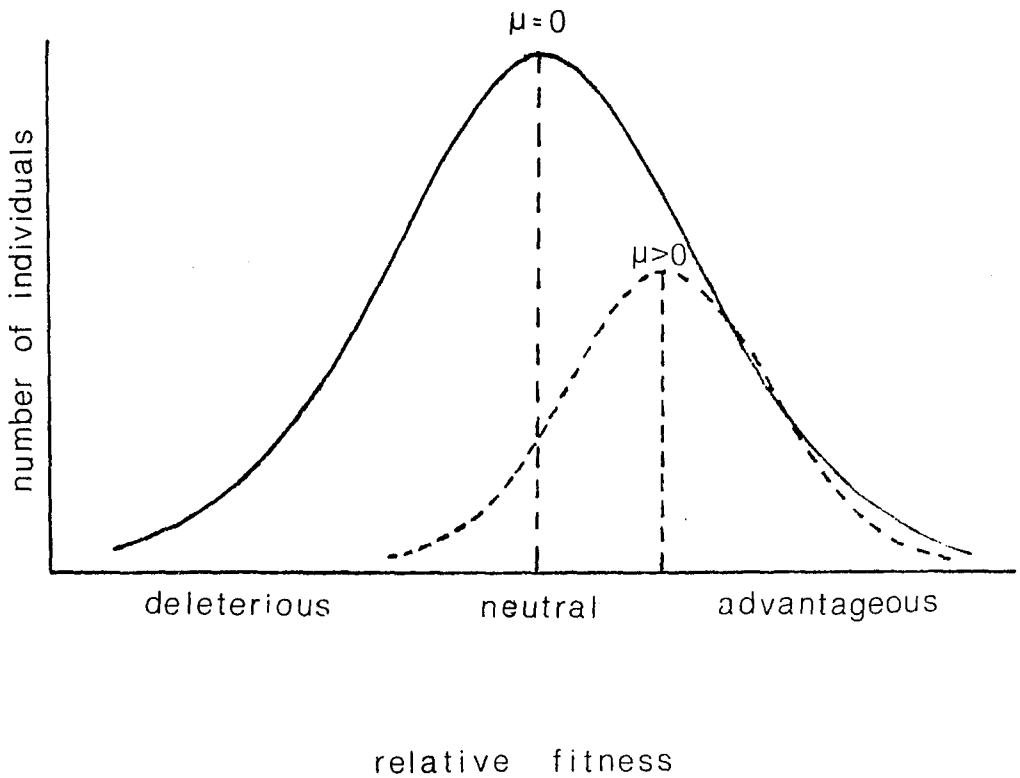

μ = 0
μ>0
number of individuals
deleterious
neutral
advantageous
relative fitness

Figure 1-3

This second case is somewhat artificial, since it would not be expected to persist in natural populations because of the reshuffling of the genetic backgrounds during sexual reproduction (recombination and/or segregation).

The influence of inversions on biochemical polymorphisms is more significant. Correlations between inversions and biochemical polymorphisms have been observed in natural populations by Kojima et al. (1970) for three loci, Adh, Odh, and Est-C and three inversions, In2LB, In3AG, and In3RG respectively in D. melanogaster (Katsunuma populations). However, the existence of such correlation does not necessarily imply a difference in the physiological value of alleles, and is most often interpreted as reflecting the "hitch-hiking" of a functionally monomorphic locus in an inversion. This behavior has been described mathematically by several authors including Ohta and Kimura (1975), Maynard Smith and Haigh (1974) and Thomson (1977).

The concept of hitch-hiking, as described above, implies that the gene locus under consideration does not contribute to the fitness of the inversion. Therefore, any possible differences in the selective value of the two alleles (A_1 and A_2) is a consequence of their association with a selectable inversion. For example, allele A_2 could be trapped within a selectable inversion and if for some reason, historical or chance sampling, allele A_1 was excluded from this inversion, an apparent difference in the selective value of the two alleles would be evident. This observation would lead to a prediction of differences in the frequency and distribution of the two alleles in natural population even though the physicochemical and physiological properties of the two alleles were identical. If there

was no knowledge of the association of one of the alleles with an inversion, the two alleles would in all probability not be considered selectively equivalent. Therefore, the net result of the association of the allele and the inversion would be the creation of an apparent selectable difference between functionally equivalent alleles. This effect would be due entirely to the genetic background.

If the rigorous definition of "hitch-hiking" is relaxed in such a manner as to allow that the gene locus A contribute to the fitness of the inversion, then the selective neutrality of alleles A_1 and A_2 implies that the two alleles contribute equally to the fitness of the inversion. The extent of the contribution to the fitness of the inversion by gene locus A would depend to some degree upon the genetic constitution of the inversion (i.e. what other alleles are present at other genetic loci within the inversion). Given this assumption, the opportunity arises for the differential interaction of the alleles at locus A with the other genetic loci contained in the inversion.

Therefore, as shown in Figure 1-3, the relative fitness of A_2 would become greater than that of A_1 because of its association with a suitable array of gene loci. But unlike "classical hitch-hiking", where the difference in fitness of the two alleles results completely from differences in the fitness of the inversions, in the present case the allele A_2 is to some extent responsible for the increase in fitness of the inversion. This result could arise from the interaction of this allele with a particular array of other gene loci also present within the inversion. Because of the equivalence in the physicochemical

and physiological properties of the alleles A_1 and A_2, the selective advantage described above for allele A_2 would occur in an identical manner for allele A_1. Thus, the apparent deviation from selective neutrality of the two alleles arises as a consequence of the non-random distribution of the two alleles on the genetic background rather than due to differences in the properties of the alleles. The "selective unit" is therefore comprised of the interactive effects of the biochemical locus of interest and the genetic background rather than the independent effects of either. This results in the potential selective differentiation between the two alleles.

The preceeding discussion can be illustrated in the following manner: Assume that there are three other variable genetic loci, B, C, and D which are present in the same inversion system as A, and that the interaction among the four loci results in the production of a selectable phenotype. The argument presented in the preceeding paragraph maintains that the fitness of alleles A_1 and A_2 are identical when expressed with any identical combination of loci B, C and D, but assumes further that the genotypes A_1 or A_2 in combination with alleles B_1 C_3 D_5 result in an adaptive peak and that part of this increase in fitness is due to the presence of alleles A_1 or A_2. Thus, if another allele A_3 were present at the A locus, it would be characterized by properties which would not maximize the fitness of genotype A_3 B_1 C_3 D_5. The evolutionary significance of these assumptions arises if one of the selectively equivalent alleles, A_1 or A_2 is excluded from the optimal combination. Then, despite the biochemical equivalence of the gene products of A_1 and A_2, the two alleles are capable of being

discriminated by selection. The nature of the interaction between loci A and B, C and D plays a contributing but misleading role to this discrimination as it would appear that there was some functional distinction in the interaction between A_1 and A_2 with B_1 C_3 D_5 rather than merely a non-random distribution.

A number of interesting possibilities may arise if one considers the third allele, A_3 to have the fitness continuum shown in Figure 1-4. As shown in this figure, the average effect of this allele is less than the selectively equivalent alleles A_1 and A_2 but its range is greater. One possibility of this relationship in fitness values between alleles A_1, A_2 and A_3 arises if allele A_3 becomes trapped in an inversion which raises its fitness value to an extent so that it exceeds the average of A_1 and A_2 (shaded portion in Figure 1-4). Thus, despite its average selective disadvantage with respect to alleles A_1 and A_2, its frequency would increase in the population as long as it remained isolated in the favorable inversion. This "conditional selective advantage" would persist for the duration of this association, and as previously explained, the increase in fitness of this complex would not necessarily exclude the contribution of allele A_3.

It is not known whether the mechanisms described in the preceeding section do indeed exist in biological systems. Nevertheless, these considerations stress the importance of establishing the "unit of selection" before one attempts to ascertain whether the functional products of alleles at a genetic locus are selectable or neutral.

Figure 1-4. Fitness continuum of alleles A_1, A_2, and A_3 such that the average effect of $A_1 = A_2 > A_3$, but variation in fitness of $A_3 > A_1$ and A_2.

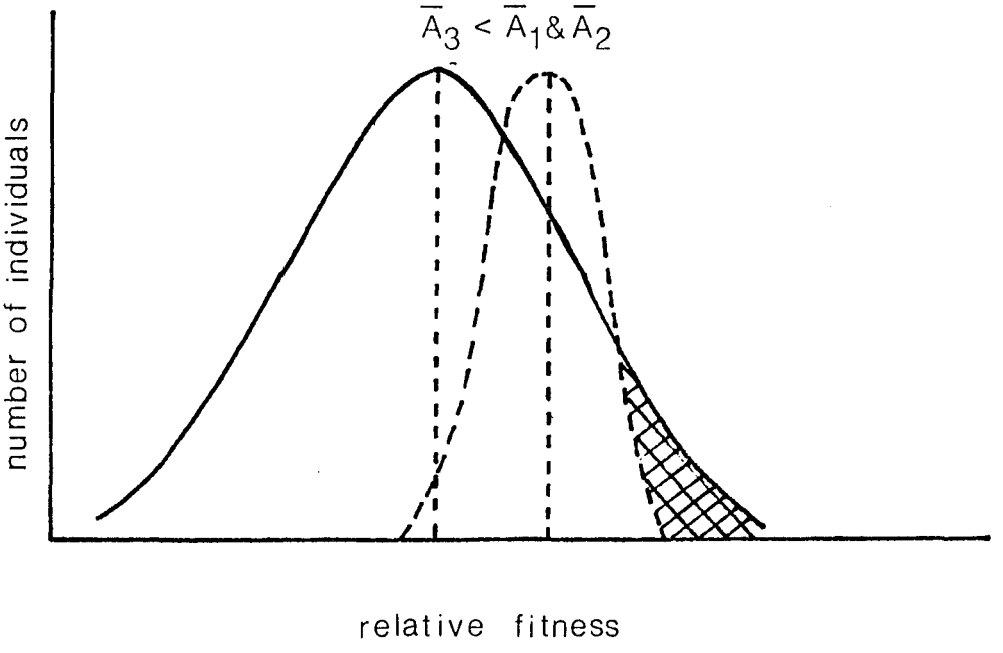


Figure 1-4

III. CONCLUSIONS

This section of chapter 2 has examined the dynamic aspects of the fitness of genetic variants in soluble proteins (allozymes) in the context of the unit of selection. It is argued that an organism is composed of a number of selective units whose interaction results in total organismal fitness. A unit of selection may involve a single enzyme in the detoxification of a specific environmental substance, or a multiple isoenzyme series, or a biochemical pathway involved in some aspect of intermediary metabolism. Thus, the possible contribution of an allozyme to total fitness depends upon its relative contribution to a particular selective unit and, in turn, the contribution of the selective unit to total fitness. Each of these parameters is variable in its importance depending upon the genetic background (other selective units) and the environment to which the individual is exposed.

The concept of conditional neutrality has been incorporated into this framework of the "selective unit" to include those mechanisms which result in compensation for alterations in the physicochemical properties of an allozyme. Possible compensatory mechanisms have been suggested for single proteins, multiple enzyme systems, and biochemical pathways. They arise as a consequence of intrinsic "homeostatic mechanisms" within a selective unit which would effectively shield enzyme variants from selective constraints. It is suggested that these homeostatic mechanisms have evolved as a result of selective pressures operating at the level of the selective unit.

Thus, the relative contribution of an allozyme locus to the unit of selection will ultimately determine its "selectability". For the period of time during which the compensatory mechanism, is operational the distribution and frequency of the allozyme will be determined by non-selective forces.

Selection will begin to play a major role in molecular evolution when the physicochemical properties of the allozyme in question result in a situation where the limits of applicable homeostatic mechanisms are exceeded. This may arise in two ways; firstly, from new mutations resulting in greatly altered physicochemical properties in the enzymes coded by the gene locus under consideration, or secondly, from changes in the homeostatic mechanisms affecting the selective unit. These latter changes may arise either as a consequence of altered selective pressures acting upon the homeostatic unit, or simply from the occurrence of a new mutation which disrupts the homeostatic mechanism. Once the compensatory mechanism is ineffective, selective forces will play a major role in the evolutionary dynamics of allozymes.

In this way, the molecular evolution of proteins can be viewed in a manner analogous to Wright's shifting balance theory of evolution, in that it would involve both neutral and selective phases.

Finally, it can be demonstrated, that differences in the physicochemical properties of enzymes are a precondition for molecular evolution by selective processes. Although physicochemically identical alleles (selectively neutral alleles) can have apparent differences in fitness, and hence "evolve", this can result only by a mechanism of "hitch-hiking" with a co-adapted gene complex. Although the enzyme

locus in question may be part of this co-adapted gene complex from a functional viewpoint, the presence of physicochemically identical alleles cannot alter the fitness and hence the evolutionary course of the complexes in question.

It is unlikely that the mechanisms described in any of the preceeding sections will entirely answer all questions concerning molecular variation in natural populations. Conditional neutrality should not be invoked as an explanation for all protein polymorphism. One must also consider the regulatory function of the molecule (Johnson, 1974), its molecular size (Koehn and Eanes, 1977) and its electrophoretic mutation rate (Zouros, 1976). It is most probable that there is no one explanation which will adequately explain all cases of molecular evolution. Undoubtedly, the more information that is available on all possible aspects of the biology of an allozyme, the more definite the prediction which can be made concerning its evolutionary course.

However, we can begin to view the mechanisms involved in molecular evolution from a more functional point of view by attempting to integrate both molecular and population data bases with putative effects on the fitness of individual organisms. In this respect, information from biochemical, cellular, organismal, and population levels converge on a general notion that homeostatic features of biological systems are often overlooked. It is the development of such homeostatic aspects of biochemical systems that may result in a hierarchy of "selective units" which are important factors in ascertaining the role of allozymes in molecular evolution.

IV. MODIFICATION OF ENZYME FUNCTION BY SELECTION

1. Introduction

Although numerous investigations have attempted to discuss whether polymorphisms at enzyme loci are selectively constrained or effectively neutral, few (e.g. Clarke and Allendorf, 1979) have suggested mechanisms whereby selection may act at the molecular level. This section of Chapter 1 considers the effects of two quantifiable enzyme properties, Michaelis constant (Km) and maximum catalytic activity (Vmax) on enzyme function and consequently, their role in the determination of fitness.

The model which is proposed is semi-quantitative and is based upon the utilization of trehalose as a substrate for flight. Several assumptions and simplifications have been made in the biochemistry of insect flight and in the interpretation of Km and Vmax. These are described below.

(i) Kinetics of enzyme reactions

The definition of Km and Vmax to be utilized in this study are derived from the simplest representation of a one substrate reaction:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_p} E + P \qquad (1)$$

where K_1 denotes the rate constant for the formation of the enzyme substrate complex, K_{-1} is the rate constant for the reversible dissociation of the enzyme complex, and K_p is the rate constant for the breakdown of ES to enzyme and product. In accordance with the

Briggs-Haldane steady-state approach, Km = $K_{-1} + K_p/K_1$ and Vmax = K_p [E]t where [E]t is the total concentration of catalytic sites (i.e. proportional to enzyme concentration).

It should be apparent that when $K_p << K_{-1}$, Km is a measure of the dissociation constant of the ES complex, and in this sense is a measure of the affinity between enzyme and substrate, when $K_p >> K_{-1}$, then Km $\simeq K_p/K_1$ and is a kinetic constant. However, when as in most cases, K_p is the same order of magnitude as K_{-1}, the physical significance of K_m cannot be stated with certainty in the absence of other data concerning the relative magnitude of the rate constants, K_1, K_{-1}, and K_p. The interpretation of Vmax is somewhat simpler. Vmax may be altered by either a change affecting the rate constant K_p, or [E]t. Km and Vmax may change independently if for example alterations in K_p are compensated by changes in K_{-1} and/or K_1. Changes in [E]t would not alter Km as there would be no single effect on any single rate constant as long as [S] >> [E]t.

From a viewpoint of selection, Km could be altered by changes in the physical structure of the enzyme which would result in differences in one or more of the rate constants. Thus, for example an increase in enzyme-substrate affinity measured by the ratio of K_1/K_{-1} would not necessarily affect Vmax unless K_p were altered. Km could also be modified in a physiological system without changes in enzyme structure, by alterations in the concentration or structure of a regulatory molecule which could act as a competitive inhibitor.

The parameter Vmax could be selectively modified in two ways; by changes in enzyme structure which would alter the rate constant

Kp or by changes in [E]t. The latter may be accomplished in two ways, but alterations in the specificity of a regulatory molecule or by regulatory mutations, affecting the production (i.e. transcription, translation, activation) of the enzyme, or its subsequent catabolism.

The interactions of Km and Vmax on product formation can be expressed by the following equation on acceptance of certain assumptions (Briggs-Haldane steady-state approach, see Segel, 1975, pp. 25-29).

$$V = [S]\ Vmax/Km + [S] \qquad (2)$$

It is apparent that components of biochemical fitness and hence alterations of Km and Vmax must be mediated through the physiological expression of this rate equation. The interaction of Km and Vmax in the determination of product formation can best be illustrated by a consideration of Table 1-1. In this table, Vmax has been assumed to equal 1 μM of product/min. Therefore, alterations in v under the influence of Vmax can be obtained by multiplying the value in the table by the corresponding alternate value of Vmax. Obviously doubling Vmax, doubles v, halfing Vmax halfs v. The enzymatic reaction described by 1 and 2 is termed first order when $[S] << Km$ and zero order when $[S] >> Km$. Therefore in a first order reaction v is proportional to Vmax/Km, whereas in zero order phase v = Vmax, i.e. directly proportional to substrate concentration. The relationship between substrate utilization and time (c.f. Segel, 1975, pp. 39-44) for first order reactions is:

$$\log [S] = K/2.3t + \log [S_o] \qquad (3)$$

Table 1-1. Velocity of reaction as a function of substrate concentration and Km.

Substrate concentration mM	V_1* Km=5.0mM	V_2 Km=1.0mM	V_3 Km=0.5mM	V_4 Km=0.1mM
0.50	0.0919	0.3333	0.5000	0.8333
0.75	0.1304	0.4286	0.6000	0.8824
1.00	0.1667	0.5000	0.6667	0.9091
1.25	0.200	0.5556	0.7143	0.9259
1.50	0.2308	0.6000	0.7500	0.9375
2.00	0.2857	0.6667	0.8000	0.9524
6.00	0.5455	0.8571	0.9231	0.9836
10.00	0.6667	0.9091	0.9524	0.9901
20.00	0.8000	0.9524	0.9756	0.9950
30.00	0.9091	0.9804	0.9900	0.9980

* velocity: $V = \frac{[S]}{Km + [S]} \quad Vmax$

where S is the final substrate concentration, S_o is the initial concentration, K is a rate constant equal to Vmax/Km and t is time. For zero order reactions, velocity, and hence the relationship between substrate utilization and time is proportional to Vmax, and when [S] = Km, then velocity is Vmax/2.

A consideration of these equations, and the numerical values in Table 1-1 indicates that in the absence of inhibitors, enzyme reactions are most sensitive to control when [S] ≃ Km. Regulatory molecules which affect Km, would be relatively ineffective compared to those affecting Vmax in the regulation of zero order reactions, whereas both would be effective in regulating v when [S] ≃ Km; the reaction proceeds as if it were uncatalyzed, therefore the effect of inhibitors would not be a factor.

(ii) Duration of flight

The model to be investigated concerns a simplification of the utilization of trehalose as a substrate for insect flight. If one assumes that duration of flight in Drosophila is directly related to hemolymph trehalose concentration as indicated in other Dipteran species, it is apparent from the literature review that two interrelated aspects must be investigated for a complete model of insect flight; the catabolism of hemolymph trehalose and the anabolic synthesis of trehalose from glycogen reserves as a response to utilization. With respect to these factors, the establishment of the limits to duration of flight can be modeled as follows. The information utilized has been referenced in the literature review on trehalases.

At the onset of flight, energy is derived from two sources, hemolymph trehalose and muscle glycogen. Muscle glycogen is presumed to be utilized rapidly. Two factors control the concentration of trehalose, the rate of catabolism by trehalase and the rate of de novo synthesis at the expense of non-muscular glycogen stores. The de novo synthesis depends in part on the mobilization of hypertrehalosemic hormone, which would activate glycogen phosphorylase by the action of a second messanger (cAMP in this case). The glucose -1-phosphate produced in this reaction would be converted to UDP-glucose and subsequently to trehalose-6-phosphate (Fig. 1). Glycogen synthesis would be repressed by virtue of the lower Km of trehalose-6-phosphate synthetase for UDP glucose compared with glycogen synthetase. Trehalose-6-phosphate would subsequently be hydrolyzed by fat body phosphatase and trehalose would be released into the hemolymph. The rate of catabolism of trehalose to glucose is assumed to be the mechanism limiting flight. Thus when the rate of trehalose velocity declined below a present critical level flight would terminate due to the inability of the enzyme to produce sufficient glucose from the available substrate concentration.

The model to be investigated with respect to the interaction of Km, Vmax and selection, makes the following simpliying assumption. First it assumes, that all glycogen stores are expended, thus trehalose is the only substrate and its concentration steadily declines with flight. Second, it assumes that the availability of trehalose is rate limiting, and that any excess glucose produced by the enzyme which cannot be immediately utilized is lost to flight metabolism.

Two separate cases will be explored. The first proposes that the enzyme trehalase is unregulated, the second deals with the presence of a regulated enzyme. Such regulation could involve the presence of regulatory molecules which act as competitive inhibitors (alter Km but not Vmax) or non-competitively (alter Vmax but not Km) or by a mixed type of inhibition. It is further assumed that the degree of inhibition is sensitive to product formation, therefore, the enzyme is progressively deregulated as [S] decreases. This could be accomplished by alteration in Ki (inhibition constant) or [I]. Duration of flight is estimated by the time required for the enzyme to decrease hemolymph trehalose concentration to a level where a sufficient rate of glucose production is no longer possible. Two other factors are also considered, the amount of trehalose utilized, and the efficiency of this utilization, as measured by trehalose consumption per time unit of flight. Fitness is viewed to be determined by two components: duration of flight and efficiency of utilization.

Although it is not feasible to analyze all possible cases, the outcome of several simulations of either hypothesis will be described and hopefully will illustrate the interaction of Vmax, Km and selection. In each case (i.e. regulated vs. unregulated enzyme) the effects of alteration of Km with constant Vmax, and altered Vmax with constant Km are considered in terms of both components of fitness.

2. Case 1: Unregulated enzyme, identical Vmax, variable Km

From a consideration of the velocities presented in Table 1-1, it is tempting to conclude that an enzyme form with the lowest Km would support flight for the greatest duration of time. However, for the case of an unregulated enzyme this may not be the case as demonstrated by the following illustration. On the assumption that initial substrate concentration is 2.0 mM and that flight requires a minimum of 0.200 μM glucose/time unit, then an enzyme, E_1, with Km of 5.0 mM and Vmax = 1 μM/time unit can support flight until substrate concentration approaches 1.25 mM as the velocity of the enzyme reaction at this substrate concentration is 0.200 μM glucose/min (Table 1-1). In comparison, an enzyme E_2 with Km of 1.0 mM could support flight at substrate concentrations approaching 25 mM (not shown in Table 1-1, but V_2 at 25 mM, Vmax 1 μM/time unit = 0.200 μM/time unit). Assuming that a first order reaction exists when [S] > Km, the time required for E_1 to convert 2.0 mM of trehalose to 1.25 mM is 2.35×10^3 time units, compared with 2.88×10^3 time units for reaction E_2 (i.e. approximately 1.5×10^3 time units of zero order kinetics [S] > Km and 1.38×10^3 time units of first order kinetics). Thus, under these conditions, the duration of flight for enzyme E_2 would be longer. However, the amount of glucose utilized by E_1 is 470 μM of glucose at the expense of 0.75 mM of trehalose (i.e. 3.19×10^{-5} mM trehalose/time unit), whereas the amount of glucose utilized by E_2 is 576 μM of glucose at the expense of 1.75 mM of trehalose or 6.08×10^{-4} mM trehalose/time unit. Therefore, although under the conditions selected, E_2 results in longer duration of flight, than E_1 the expenditure of energy as measured by trehalose consumption is 19 x

greater per unit time. Hence in this case, if individual fitness was determined predominantly by duration of flight than enzyme E_2 would have a slight relative advantage, but if the efficiency of trehalose utilization played a relatively important role then E_1 would be favored. Perhaps surprisingly, if the initial starting conditions and critical glucose production rate were lowered to 0.000125 M trehalose, and 0.0909 μM glucose/time unit respectively, enzyme E_1 could support flight for a longer period of time and more efficiently than enzyme E_2. In this case if individual fitness was determined predominantly by flight duration than obviously E_1 is favored and the selective differential would increase as efficiency of utilization assumed a greater proportion of fitness.

3. Case 2. Unregulated enzyme, identical Km variable Vmax, first order reaction.

In this case the following assumptions are made; 0.200 μM of glucose/time unit are needed to sustain flight, Km of $E_1 = E_2 =$ 5.0 mM, Vmax $E_1 =$ 1 μM/time unit, $E_2 = 2$ μM/time unit, and the initial substrate concentration is 0.002 M substrate. Based on these assumptions, the limiting substrate concentration for enzyme E_1 is 0.00125 M because v at S = 0.00125 is 0.200 μM glucose/time unit, and for E_2 the limiting substrate concentration is 0.000555 M because v at this substrate concentration is 0.1998 μM glucose/time unit. Under these conditions the time required for E_1 to utilize 0.00075 M trehalose is 2.35×10^3 time units, whereas for E_2, the time to utilize 0.001445 M trehalose is 3.20×10^3 units. Therefore under thse conditions a fly with enzyme E_2 would fly 1.36 times longer than a fly with enzyme E_2. However, the

the energy expenditure of fly with E_1 is 3.19×10^{-7} M trehalose/time unit, compared with 4.52×10^{-7} M/time unit for E_2, or an increase of energy required of 1.40. In this case if overall fitness were determined by duration flight, obviously E_2 would be selectively favored. Because the increase in duration of flight, 1.36 x, closely approximates the increase in energy utilization 1.40 x, E_2 would still retain its potential selective advantage if efficiency of utilization was the predominant fitness component. Therefore, selection would favor the enzyme with the higher Vmax.

4. Case 3. Regulated enzyme.

In the case that the hydrolysis of trehalose was regulated by another molecule, and that this regulation was sensitive to the rate of glucose formation, then it is readily apparent that of two enzymes with identical Vmax, but with different Km, the enzyme with lower Km would be favored. This is a consequence of its ability to produce glucose at the required rate at a lower substrate concentration (c.f. Table 1.1). Efficiency of substrate utilization would not be a factor in this case, because both reactions would proceed as zero order reactions (i.e. constant amount of glucose produced independent of substrate concentration). Similarly for two enzymes with identical Km, but different Vmax, the enzyme with higher Vmax would be favored. The implication of these calculations are that, enzymes with lower Km and highest Vmax would be selectively favored. However, the limits on Km and Vmax would be imposed by the relationship of these kinetic parameters with the inhibitor constant. This can be illustrated with the following example for a competitive inhibitor. An enzyme with Km

0.1 mM, Vmax 1 μM/time unit would produce 0.9524 μM glucose/time unit if the substrate concentration were 0.002 mM (c.f. equation 3 above). If the amount of glucose needed to sustain flight was 0.200 μM/time unit, then the enzyme activity must be inhibited by approximately 80%, then the relationship between % inhibition, inhibitor concentration [I], inhibitor constant Ki, [S] and Km, % inhibition = $[I]/[I] + Ki\,(1 + [S]/Km)$, could be used to illustrate the necessary inhibitor constants and concentrations required to maintain zero order reaction kinetics at a preset level. Clearly as Km decreased either [I] would increase or Ki decrease. If such changes were not possible, then the efficiency of substrate utilization would decrease and consequently such forms with low Km would be at a selective disadvantage, if efficiency of utilization were a component of fitness. A similar optimization argument could be formulated for the interaction of Vmax and an inhibitor.

V. Conclusions

A consideration of the selected cases presented above may indicate a number of evolutionary principles concerning biochemical reactions, although admittedly a greater variety of cases must be examined.

It is apparent that for the case of an unregulated enzyme, the kinetic parameters, Km and Vmax and steady-state substrate concentration would be expected to evolve in response to selective pressures acting upon the rate of product utilization. It is clear that an optimum Km and Vmax would exist and that substrate concentration is the only effective means of regulating enzyme activity. Hence selection (acting

through fitness components duration of flight, and efficiency of utilization) might act in such a way as to fix the rate of product utilization which in turn would define the optimum steady-state substrate concentration with respect to both Km and Vmax simultaneously. The actual physicochemical method which would alter Vmax would be unimportant in this case.

It is also apparent that a regulated enzyme system could evolve greater fitness relative to an unregulated one as a consequence of the possibility of more efficient substrate utilization, hence greater steady-state concentrations and longer flight duration. In contrast to unregulated systems, the evolution of enzyme function as measured by Km and Vmax would depend upon the properties of the regulatory molecule in relation to the demands imposed by product utilization. In this case, the physicochemical methods used to alter Vmax (i.e. either modification of enzyme structure affecting Kp and enzyme turnover, or enzyme quantity) would depend upon the properties and amenability of the regulatory molecule to co-evolution. Thus, depending on the system in which an enzyme exerts its catalytic activity, either one or both kinetic parameters would be amenable to selection. By the same argument one of the properties may vary in a fashion undetectable by selection.

CHAPTER 2

DETERMINATION OF PHYSICOCHEMICAL PROPERTIES OF *DROSOPHILA* TREHALASE

I. INTRODUCTION

The enzyme trehalase, E.C. 3.2.1.28 catalyzes the hydrolysis of the dissacharide α, α-trehalose into its constituent α-D-glucose moieties. Labat et al. (1973) have indicated that the product of this reaction is the α-D isomer of glucose. Therefore, presumably this isomer must be converted to the β-D form prior to its biological utilization.

Much of the recent work on this enzyme in insects has centered on two separate but somewhat related problems; the regulation of trehalase activity in relation to the physiological utilization of the dissacharide, and secondly, the possible presence, distribution and function of trehalase isoenzymes in insect tissues. Interest in the regulation of activity arises partly from reports of the simultaneous occurrence of the enzyme and its substrate trehalose in a number of insect species (Van Handel 1978, Katagiri 1977, Matthews et al. 1976, Friedman 1961) as well as the established physiological significance of trehalose which derives from its apparent predominance in the hemolymph of most insect species. As a consequence, the enzyme trehalase is expected to play a key role in those physiological processes which are dependent upon glucose reserves. These include, duration of flight (Clegg and Evans 1961, Ford and Candy 1972), moulting cycles (Candy and Kilby 1962) where it is activated to provide glucose needed for chitin synthesis, and possibly a role in conferring cold resistance (Wyatt, 1967).

Multiple forms of insect trehalases have been reported in several species including Apis mellifera (Lefebvre and Huber 1970), Vespa orientalis (Fischl et al. 1976). Distinct tissue specific forms have been reported in Phormia regina (Friedman and Alexander 1971,

Friedman 1975) and Blaberus discoidalis (Gilby et al. 1967). Multiple forms of trehalase were reported in D. melanogaster by Talbot and Huber (1976) but more recently, the same laboratory (Oliver et al. 1977) has indicated that trehalase in this species is present as a single form coded by a single structural gene locus which was mapped with segmented aneuoploids to the region 2R 55 B-E.

For the most part, trehalase appears to be a membrane bound enzyme which may be solubilized with detergents (Triton X, SDS, deoxycholate) or by repeated freeze-thaw cycles and sonication. It is unclear whether the forms of this enzyme which are soluble in aqueous extracts are distinct or merely represent a rather loose association of the enzyme with the membrane. The apparent function of trehalase most likely involves the transport of glucose across the plasma membrane of metabolically active cells because the dissacharide trehalose has not been detected intracellularly, and the cytological localization of the enzyme. More recently however, Brandt and Huber (1979) have indicated that trehalase in honey bee thorax is contained entirely in mitochondria and is most likely attached to the inner mitochondrial membrane. This report implies that trehalose can pass through cellular membranes and challenges the assumed transport function of the enzyme. However, the inability of investigators to detect trehalose intracellularly becomes problematic to this interpretation.

The biochemical properties of insect trehalases have been reported in a number of insect species. It is apparent that the insect enzyme is remarkably specific for α,α trehalose as neither α,β-trehalose or β,β-trehalose are hydrolyzed (Labat-Robert et al. 1978). However, several other sugars are hydrolyzed in varying degrees. Labat-Robert

et al. (1978) report the Km of trehalase from Melolontha vulgaris for α,α trehalose, α-D-allopyranosyl-α-D-glucopyranodise, α-D-galacto-pyranosyl α-D-glucopyranoside, α-D-glucopyranosyl-α-D-xylopyranoside and 6'-deoxy-α, α-trehalose as 0.617 mM, 3.57 mM, 3.33 mM, 27.7 mM and 2.00 mM respectively. Ballario et al. (1978) have indicated that a purified trehalase from thebrine shrimp Artemia salina will hydrolyze cellobiose, lactose, and mellobiose at 23, 14, and 6 percent respectively of the relative rate of trehalose.

Labat-Robert et al. (1978) have interpreted their results as indicative of the importance of the primary alcohol group at C-6 for the formation of the enzyme substrate complex. Thus, although 6'-deoxy-α,α-trehalose is hydrolyzed, 6',6'-dideoxytrehalöse is neither a substrate or inhibitor. Hydroxyl groups at C-3 and C-4 were proposed to participate in forcing a half-chair conformation which is most favorable for the subsequent hydrolysis. In addition, the C-2 hydroxyl moiety was suggested to play an important role in binding the substrate to the active site.

Amino, carboxyl, and imidazole groups have been proposed to occur in the active site of trehalase (Talbot and Huber 1975, Labat et al. 1974, and Avigad et al. 1965), whereas sulfhydryl groups seem to be absent from the active site of trehalase (Huber and Lefebvre 1971, Labat et al. 1974). Terra et al. (1977) present thermodynamic evidence which suggests that both acid and basic groups in the active site of Rhynchosciara americana trehalase are carboxgl groups and speculate that one acts as an acid to protonate the glycozyl oxygen and the other acts as a base to stabilize a developing carbonium ion. An analysis of

inhibitor action by Labat-Robert et al. (1978) indicated the involvement of "general acid-base catalyzed steps in the rate-controlling process" of trehalase activity and possibly the "formation of an oxocarbonium ion in the mechanism of action of trehalase". Therefore, it appears that the hydrolysis of trehalase can be viewed mechanistically as a nucleophilic substitution, with water as the nucleophile. The enzyme presumably lowers the energy of activation by making the bond linking the two glucose moieties more accessible to attack by inducing some stress into the molecule, and/or by stabilizing the transition complex - in this case the carbonium ion.

Several studies have reported physicochemical properties of insect trehalases. Some of these data are summarized in Table 2-1. In general, the pH optima of insect trehalase lies between 5.0 and 6.0. Trehalase from eggs of Spodoptera littoralis and adults of Periplaneta americana are exceptions with a pH optima of 3.5 (Issahaya and Yablonski 1976, Matthews et al. 1976). The Km of the enzyme ranges from 30 mM in S. littoralis eggs to 0.50 mM in midgut of Blaberus discoidalis (Gilby et al. 1967). Similarly, the energy of activation estimated from Arrhenius plots ranges from 9.15 to 28.9 Kcal/mole for Bombyx mori larvae at onset of spining (Shimada 1976) and Culex pipiens quinque-fasciatus (Giebel and Domnas 1976) respectively. Estimates of molecular weight range from 63,000 for the muscle enzyme from Blaberus discoidalis to 122,000 for the intestinal enzyme isolated from R. americana. A large number of inhibitors of trehalase activity have been reported, a sample of which are presented in Table 2-2. Generally, it appears that most insect trehalases are inhibited somewhat by TRIS, the Ki varying

Table 2-1. Summary of physicochemical constants for trehalases from insect sources.

Species	pH optimum	temperature stability	Km(mM) @ °C		Arrhenius Constant Kcal/mole	pI	M.W.	Reference
Drosophila melanogaster								
Oregon R	5.2	50%@46°C-5min	1.80	37	-	-	-	Marzulf (1969)
	5.6	55%@50°C-10min	1.36	30	-	-	100,000	Huber and Lefebvre (1971)
thorax	5.0	-	-	-	-	-	-	Talbot and Huber (1976)
abdomen	6.0	-	-	-	-	-	-	Talbot and Huber (1976)
	-	-	-	-	-	4.73	-	Oliver et al. (1976)
Culex pipiens quinquefasciatus	5.5-6.5	20%@40°C-20min no substrate 77%40°C-20min	1.21	30	28.9	-	-	Grebel and Domas (1976)
Phormia regina								
midgut and blood	4.5	-	1.5-1.9	32			115-117,000	Friedman (1965)
Head, muscle, rectal papilla	5.0-5.5	-	3.1-3.5	32			78-90,000	
Saissetia olea	5.5	-	-	-	-	-	-	Ishaaya and Swirski (1976)
Hyalophora ceeropia								
muscle	6.5	-	3.6	30				Gussin and Wyatt (1965)
larval gut	5.7	-	0.4	30				

...cont'd.

Table 2-1. (cont'd.)

Species	pH optimum	temperature stability	Km(mM) @ °C		Arrhenius Constant Kcal/mole	pI	M.W.	Reference
Bombyx mori								
fifth instar	6.5	-	10.00	30	17.36	-	-	Shimada (1976)
onset of spining	6.0	-	0.91	30	9.38	-	-	
end of spining	5.5	-	0.71	30	9.15	-	-	
10hr after end	4.0	-	2.86	30	11.90	-	-	
Spodoptera littoralis								
eggs	3.5	30%@65°C	30	37	-	-	-	Ishaaya and Yablonski (1978)
Periplaneta ameneana	3.5	-	-	-	-	-	-	Matthews *et al.* (1976)
Blaberus discoidalis								
unactivated muscle form	6.0	-	3.3	30	15.0		63-80,000	Gilby *et al.* (1967)
activated mucle form	6.0	-	1.7	30				
midgut	5.0	-	0.5	30				
Rhynchoscaria americana	6.0	-	0.67	30	16.7	4.6	122,000	Terra *et al.* (1978)
Artemia salina	5.6	50%@60°-10min	4.20	25	-	6.2	75,000	Balerio *et al.* (1978)

from 29.2 to 74 mM at pH 6.0 and a number of dissacharides including sucrose, turanose, and a number of substituted glycosides including α-D-glucopyranosyl-α-D mannopyranoside and 2'-trehalosamine. In the moth M. vulgaris these latter two carbohydrates are competitive inhibitors, Ki, 5.7×10^{-6} M and 2.31×10^{-4} M respectively (Labat-Robert et al. 1978). Dihydrochalcones were reported to be mixed inhibitors of porcine trehalases but did not inhibit the moth enzyme (Labat-Robert et al. 1978). Several insect trehalases have been reported to be inhibited by heavy metal ions such as Hg^{+} but this effect does not appear to be a general phenomena. The enzyme from D. melanogaster is not inhibited by PHMB (p-hydroxymercuribenzoate) (Huber and Lefebvre 1971).

The developmental expression of trehalase from D. melanogaster has been reported by Marzulf (1969). Low levels of trehalase activity were observed in 0-4 day larvae (1-3rd instar) increasing somewhat in 0-4 day pupae, and increasing markedly in early adult life, remaining constant for 0-2 days. There is no information available concerning the physicochemical properties of the larval and pupal trehalases in Drosophila. Shimada (1976) has shown that Bombyx mori trehalases from various life cycle stages varies in Km and energy of activation (Table 2-1).

This chapter investigates the physicochemical properties of trehalases from species of Drosophila. These data are a prerequisite to the evolutionary interpretations of Chapter 3.

Table 2-2. Inhibitors of trehalase activity

Species	Inhibitors (Ki or percentage inhibition)	References
Drosophila melanogaster	TRIS (29.2 mM), Sucrose (0.54 mM) pHMB (18.5%) Concentrated phosphate buffers	Marzulf (1969) and Lefebvre (1971)
Hylaphora cecropia (muscle)	glucosamine (90%), maltose (29%) cellobiose (23%) Gentiobiose (23%) sucrose (77%), turanose (41%) methylα-D glucoside (30%), methyl-α-D glucoside (23%) methyl α-D-mannoside (46%) phenyl α-D-glucoside (39%) Salicin (possible but interefers with glucose oxidase)	Gussin and Wyatt (1965)
Bombyx mori		
fifth instar	Calcium, Manganase, Copper, Zinc	Shimada (1976)
onset of spining	Calcium, Manganase, Copper, Zinc	
end of spining	Magnesium, Manganase, Copper, Zinc	
10 hr. of end	all of above	
Rhynchoscaria americana	Sucrose (1.4mM), p-nitrophenyl β-D glycoside (2.5 mM) turanose (5.9mM) phenyl-β-D glucoside (6.5mM) p-nitrophenyl β-D-glucoside (7.1mM) Tris (74mM@pH6.0 competitive; 182 mM @pH 9.2 non-competitive)	Terra et al. (1978)
Culex pipiens quinquefasciatus	glucose 5mM (100%), glucosamine 5mM (30%) α-D-glucose-1-phosphate 5mM (20%), D-glucose-6-phosphate 5mM (26%), gluconic acid 5mM (25%) glucuronic acid 5mM (22%), β-methyl glucoside 5mM (18%),β phenyl glucoside 5mM (20%) N -α-D-glucoside 5mM (15%) PNP-α-D-glucoside 5mM (15%) sucrose 5mM (98%), maltose 5mM (11%), raffinose 6mM (20%), AMP 1mM (12%) ADP 1mM (18%) ATP 1mM (13%) CTP 0.68 mM (12%) UMP 0.6mM (15%) UTP 0.65 mM (12%) $HgCl_2$ 0.5 mM (100%) Pb acetate 0.5 mM (100%) $CuCl_2$ 0.5mM (80%) $AgNO_3$ 0.5mM (100%) 1-phenyl-2-thiourea 0.5mM (100%) D-hydroxymercuribenzeate 0.5mM (100%) pHMB + 1mgm cysteine (100%) pHMB + 100 mgm cysteine (100%)	Giebel and Domnas (1976)

II. MATERIALS AND METHODS

1. Culture conditions

Stocks of Drosophila were maintained on standard cornmeal or raisin food in half-pint bottles. Gram quantities of flies were harvested from plexiglass cages 36"x36"x24" containing 6-8 dishes of food. For age and developmental studies, flies were grown at 25°C, collected daily on emergence, placed on fresh, lightly yeasted food and allowed to age. Developmental investigations of trehalase in larvae and pupae were accomplished by size and "time from hatching" criteria.

2. Sample preparation

(i) Individual flies were homogenized in 100 µl of appropriate upper electrophoretic buffer (phase α) containing 10% (w/v) trehalose and a small volume of 0.04% bromophenol blue, and centrifuged in homogenization tubes (3 ml) in a Beckman Microfuge B (8500 g). The available supernatant ≃ 80 µl was applied directly to appropriate gels.

(ii) Mass homogenates; 10% (w/v) of Drosophila was homogenized in distilled, deionized water and centrifuged at 48,200 g at 4°C in a Sorval RC-2B model centrifuge for 1 hr. The supernatant was filtered on a coarse glass funnel and subsequently desalted with either Sephadex G25 fine or G-25 medium (Pharmacia Fine Chemicals) equilibrated with 0.1 M phosphate buffer pH 6.0. For electrophoretic investigations, samples were desalted on columns equilibrated with the appropriate upper electrophoretic buffer.

3. Electrophoresis

The procedure of polyacrylamide gel electrophoresis (PAGE) in multiphasic buffer systems (MBS) which is employed in this study has been developed and described by Jovin (1973a,b,c) and Chrambach et al. (1976). MBS catalogue has been compiled by Jovin et al. (1970) and is available on microfiche from National Technical Information Service, Springfield Va., 22161, Publication No.'s PB 259309-259312.

Briefly, the design of MBS in PAGE involves two co-ordinated stages. The "stacking phase" provides a thin starting zone for each sample component (s) and is accomplished when the electrophoretic mobility of buffer constituents 1 and 2 have the following relationship with respect to the electrophoretic mobility of the sample in the specified electrophoretic phase (eg. α, ζ, β, τ etc.) - $|\bar{r}^{\zeta}| < |\bar{r}_3^{\zeta}| < |\bar{r}_2^{\beta}|$ (these phases are described in Table 2-3). The second phase, termed "unstacking" results in the resolution of sample components by their sequential removal from the original stack and is achieved when $|\bar{r}_3^{\pi}| < |\bar{r}_1^{\pi}|$. Resolved sample components may be "restacked", that is migrate at the interface of the π/Ψ boundary, if the Ψ electrophoretic phase is formed. This is accomplished by the removal of the original phase α and overlayering the gel containing resolved proteins with phase ζ buffer. Sample components will restack if their mobility in phase Ψ exceeds the mobility of constituent > in this phase, and if their mobility in phase π is less than that of component 1. This is identical to the stacking condition and can be expressed as $|\bar{r}_7^{\Psi}| < |\bar{r}_3^{\Psi}| < |\bar{r}_1^{\pi}|$.

The MBS employed in these investigations are 1420, 3017, and 4014. The consituent concentrations of these systems and some subsystems

are described in Tables 2-3, 2-4, 2-5, 2-6. A subsystem of 4014, designated 4014.10 has been employed. The modification involved the alteration of the ion mobilities in the operative stacking phase (phase ζ) of this system. The modified constituent concentrations of phase β (Table 2-3) result in a change of mobility of constituent 1 in phase ζ ($\bar{r}_1^{\zeta} = -0.193$) and changes the constituent concentrations for phase ζ, α, τ, to those listed in Table 2-3. The modification of phase ζ ensured that soluble trehalase from Drosophila would stack in this electrophoretic phase, but that proteins with electrophoretic mobility less than -0.193 at pH 7.98 would not be contained within the stack. Unstacking and restacking parameters for eight subsystems of 4014.10 are presented in Table 2-4. Restacking protein components is not feasible for all subsystems since the steady-state boundary $\phi_7^{\Psi}/\phi_1^{\Psi}$ and/or $\phi_7^{\pi}/\phi_1^{\pi} < 1.5$ (equation 65 Jovin 1973a) cannot be met (c.f. Table 2-4). Included in this table is the ratio $\bar{r}_7^{\Psi}/\bar{r}_1^{\pi}$. This value defines approximately the minimum Rm of a protein which may be restacked in that particular gel system. For example, in 4014.10 X III ($r_1^{\pi} = -0.256$), proteins with Rm > 0.902 can be restacked, those with Rm < 0.902 will unstack in phase Ψ because the mobility of $\bar{r}_7^{\Psi} = -0.231$ (i.e. $-0.256 \times 0.902 = -0.231$) will exceed that of the protein. These data are useful in the establishment of preparative procedures.

4. Polyacrylamide gels

Two types of polyacrylamide gels were employed in these investigations - restrictive gels (i.e. low %C) and non-restrictive gels (high %C). Percent T is defined as total acrylamide (i.e. monomer + crosslinking reagent in grams per unit volume; percent C is defined

Table 2-3. Final Phase Concentrations (M) 4014.10

Constituent	Electrophoretic Phase				
	alpha (α)[1]	zeta (ζ)[2]	beta (β)[3]	epsilon (ε)[4]	tau (τ)[5]
C_1, tricine	0.0400	0.0400			
C_2, cacodylic acid			0.0431		
C_3, HCL				0.0500	
C_6, triethanolamine	0.0463	0.0463	0.0493	0.0625	0.0374
C_7, Hepes					0.0312

1. Upper electrophoretic buffer
2. Operative stacking phase
3. Stacking gel prior to electrophoresis
4. Lower electrophoretic buffer
5. Restacking phase prior to electrophoresis (restacking buffer)

Table 2-4. Unstacking and restacking parameters of system 4014.10

Phase π				Phase γ			Phase Ψ						
r_1^{π}	c_1^{π}	c_6^{π}	pH^{π}	c_3^{γ}	c_6^{γ}	pH^{γ}	r_7^{Ψ}	c_7^{Ψ}	c_5^{Ψ}	pH^{Ψ}	R_m 7/1	ϕ_7/ϕ_1	$\phi_7^{\pi}/\phi_1^{\pi}$
-0.193	0.0326	0.0377	7.98	0.0556	0.0607	6.76	-0.180	0.0254	0.0305	7.74	0.933	1.96*	1.65*
-0.214	0.0295	0.0428	8.07	0.0502	0.0635	7.23	-0.201	0.230	0.0363	7.78	0.939	1.77*	1.56*
-0.235	0.0269	0.0490	8.15	0.0457	0.0678	7.49	-0.217	0.0210	0.0431	8.02	0.923	1.61*	1.48
-0.256	0.0246	0.0568	8.24	0.0419	0.0741	7.68	-0.231	0.0192	0.0514	8.15	0.902	1.48	1.40
-0.277	0.0228	0.0668	8.34	0.0388	0.0828	7.86	-0.242	0.0178	0.0618	8.28	0.874	1.37	1.33
-0.298	0.0211	0.0803	8.44	0.0360	0.0952	8.02	-0.251	0.0164	0.0756	8.42	0.842	1.28	1.27
-0.319	0.0200	0.0995	8.55	0.0337	0.1134	8.17	-0.258	0.0156	0.0951	8.55	0.801	1.26	1.21
-0.340	0.0186	0.1228	8.68	0.0316	0.1418	8.34	-0.264	0.0145	0.1247	8.71	0.777	1.15	1.16

Phase π - operational separation phase

Phase γ - separation phase prior to electrophoresis

Phase Ψ - operational restacked separation gel

r_j^{α} - relative constituent mobility of constituent j in phase α

c_j^{α} - constituent concentration of j in phase α

R_m 7/1 - ratio of r_7^{Ψ}/r_1^{Ψ} - approximately defines the minimum Rm of sample which may be restacked

ϕ_j^{α} - fraction of constituent j dissociation into ion species of phase α

* - does not meet steady state boundary condition - see text.

Table 2-5. Final Phase Concentrations (M) MBS 1420 (pH 8.00)

Constitiuent	Electrophoretic Phase[1] alpha (α)	zeta (ζ)	beta (β)	gamma (γ)
C_1, triethanolamine	0.0400	0.0400		
C_2, NH_4OH			0.0567	
C_3, NH_4OH				0.0549
C_6, Cacodylic Acid	0.0008	0.0008	0.0174	0.0314

1. See Tables 2-3 and 2-4 for explanation of phases.

Table 2-6. Final Phase Concentrations (M) 3017 (pH 4.75)

	Electrophoretic Phase[1]			
Constituent	alpha (α)	zeta (β)	beta (ζ)	gamma (γ)
C_1 , Cacodylic acid	0.0400	0.0400		
C_2 , Acetic acid			0.0509	
C_3 , Acetic acid				0.0500
C_2 , Triethanolamine	0.0008	0.0008	0.0116	0.0259

1. See Tables 2-3 and 2-4 for explanation of phases.

by the ratio of crosslinker/monomer + crosslinker. Restrictive gels (6, 7, 8, 9, 10, 11%T, 2%C Bisacrylamide crosslinked) formed in 9cm x 4mm l.d. pyrex glass tubes were overlayered by a non-restrictive stacking gel (5% T, 15%C DATD, N,N'-Diallytartdiamide crosslinked, Bauman and Chrambach 1976). The preceeding combination of restrictive and non-restrictive gels were employed in molecular weight determinations, species comparisons, and routine electrophoretic screening procedures, which utilized the molecular sieving capacity of such restrictive gels. Non-restrictive gels were employed to calculate the charge of a protein with respect to Na^+ and in the preparative electrophoretic system.

Polymerization of gels from 1420 and 3017 was accomplished at 25°C by a procedure described by Chrambach et al. (1976) using suggested concentrations of TEMED, (N,N,N,N-tetramethylethylenediamine, Kodak Chemical), RN (riboflavin) and AP (ammonium persulphate) as initiators and catalysts. Polyacrylamide gels used with MBS 4014.10 were polymerized by the addition of 2mM AP and 2mM TEMED when necessary. It should be noted that concentrations of triethanolamine less than 0.05 M are insufficient to result in satisfactory gelation without the addition of TEMED, whereas concentrations of triethanolamine greater than 0.12 M are inhibitory and exacerbated by the addition of TEMED. Stacking gels for 4014.10 were polymerized with the addition of 5.0 mM TEMED and 2.5 mM AP.

5. Electrophoresis

Electrophoresis of non-preparative gels for MBS 4014.10 and 1420 was performed at 100 V constant, 25 mA maximum current on 16 gels (7 cm x 4 mm dia, 25 µl sample/gel) until the tracking dye had

migrated 6.0 cm. Gels from MBS 3017 were subjected to 200 v constant 25 mA maximum current.

6. Localization of trehalase on polyacrylamide gels

Zones of enzyme activity were visualized on polyacrylamide gels by placing each gel in 6 ml of a "glucostat" reagent containing 100 ml of 0.2 M phosphate buffer pH 6.0, 5 ml glucose oxidase containing 40 U/ml (Worthington Biochemicals), 5 ml horseradish peroxidase 50 U/ml (Boehringer-Mannheim) and 1 ml of 1% o-dianisidine (Sigma Chemical Co.) and 0.2 M trehalose (Sigma Chemical Col). Staining time varied with sample activity but was generally between 30 and 90 min at 25^{o}C for mass homogenate and individual samples.

7. Purification

(i) Electrophoretic purification rationale

The electrophoretic purification procedure involves two sequential electrophoretic operations. The first is performed under conditions chosen to ensure that the protein of interest is just maintained within the protein stack. That is, the electrophetic mobility of that sample component in the operative separation gel (phase π) is greater than the mobility of the trailing ion (constituent 1) in this electrophoretic phase, but less than the mobility of the leading ion (constituent 2) in the electrophoretic phase (phase λ) which forms after the migration of the leading ion (constituent 2) into the separation phase (phase γ which exists prior to the onset of electrophoresis). The fraction collected after this operation is free from all proteins which unstack under these electrophoretic conditions). The collected fraction is subjected to a second electro-

phoretic operation which is designed to selectively unstack the protein component of interest. Proteins which remain stacked are allowed to migrate out of the resolving gel. Protein components which are unstacked in this stage of the purification are subsequently selectively restacked if the appropriate conditions are possible or simply electrophoretically eluted from the supporting gel into a suitable collection device if no restacking conditions are available.

(ii) Electrophoretic purification procedure

Three ml of prepared sample containing 10% w/v trehalose and 0.04% bromphenol blue was subjected to electrophoresis at 100 V constant 35 mA maximum at 25°C on system 4014.10 X. Typically, electrophoresis was performed on two 9 cm x 4 mm gels simultaneously, resulting in a maximum current of 8.71 mA/cm^2. Proteins migrating with the tracking dye (stacked proteins) were collected in a dialysis sack (Suzuki et al. 1973) attached to the bottom of the electrophoretic column (approximately after 230 min). These proteins were re-equilibrated with phase α buffer on PD-10 columns containing Sephadex G-25M (Pharmacia Fine Chemicals). Three ml of this sample (containing 10% w/v trehalose and 1 drop 0.04% bromphenol blue) was subjected to electrophoresis (100 V constant, 35 mA maximum, 25°C) in 4014.10 XII on a single gel. Proteins migrating 1 cm behind the tracking dye were eluted electrophoretically from the column, collected in a dialysis sack and analyzed for trehalase activity.

(iii) Affinity ligands

Salicin and trehalose were attached to Epoxy activated Sepharose 6-B (Pharmacia Fine Chemicals) by the manufactures suggested

procedure. Briefly, 300 mg of salicin; or 1000 mg of trehalose were incubated with 4 g of washed Epoxy activated Sepharase 6B suspended in 20 ml of 0.1 N NaOH for 12-16 hr at 45°C. The unbound ligand was removed by successive washes with 0.1 M NaOH borate buffer pH 8.0 containing 0.5 M NaCl and 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl. Residual reactive sites present in the activated sepharox resin were blocked by incubation with 1.0 M ethanolamine for 12-16 hr. p-aminophenyl-α-D-glucopyranoside (paαD-g) was coupled to Affigel 10 (Bio Rad Lab. Inc.) by the manufacturers procedure. Four hundred and fifty mg of (paαD-g was dissolved in 25 ml of 0.1 M bicarbonate buffer pH 8.5 and incubated with constant agitation at 4°C until the concentration of displaced N-hydroxysuccinimide (measured at 260 nM) was constant. Residual reactive sites were blocked by incubation with 1.0 M ethanolamine pH 9.0. Con A sepharose gel was purchased from (Pharmacia Fine Chemicals).

Affinity residues were utilized in K-9 column (Pharmacia Fine Chemicals) total volume ≃ 7.0 ml. Columns were equilibrated with 0.1 M phosphate buffer pH 6.0. One ml of desalted sample was applied to column. Trehalase activity in the crude sample and in samples eluted from columns was determined by either the "glucostal reagent" or by hexokinase glucose-6-phosphate dehyrogenase couple (see below). Columns which retained trehalase activity were washed successively with 0.1 M phosphate buffer pH 7.5 containing 0.25 M NaCl, 0.1 M TRIS-HCL buffer pH 7.5 containing 0.25 M NaCl, 0.1 M phosphate buffer pH 6.0, and 0.1 M phosphate buffer pH 6.0 containing 0.1 M glucose.

8. Biochemical characterizations

(i) Definition of Unit

One unit of trehalase is defined as the amount of enzyme which results in the hydrolysis of 1 μm of trehalose/min under the assay conditions specified in the determination of Michaelis constants.

(ii) Determination of pH optima

Each sample (50 μl) was incubated at 25°C for 30 minutes with 2.95 ml of 0.1 M phosphate buffer (phosphoric acid, sodium phosphate monobasic, and dibasic), pH 4.5 to 7.5 in 0.5 intervals, containing 3.2 mM trehalose. The reaction was terminated by placing the sample in a boiling water bath for 10 minutes. The amount of glucose produced in 100 μl during the thirty minute interval was determined with the addition of 2.9 ml of "Glucostat" reagent containing: 100 ml of 0.1 M phosphate buffer pH 6.0, 5 ml glucose oxidase containing 40 U/ml (Worthington Biochemicals), 5 ml horseradish peroxidase, 50 U/ml (Boehringer-Mannheim) and 1 ml of 1% σ-dianisidine (Sigma Chemical Co.). The "glucostat" reaction was terminated after 15 min by the addition of 50 μl of 4 M HCL. Absorbance was read at 460 nm on a Beckman Acta CII spectrophotometer.

(iii) Determination of Michaelis Constants

Michaelis constants were estimated from Lineweaver-Burke plots, (1/v vs. 1/[S]) Woolf-Augustinsson-Hofstee plots (V vs. V/[S])

and Hanes-Woolf plots ([S]/V vs. [S]). Enzyme activity was determined by coupling the formation of glucose by trehalase with 28 units of hexokinase (Boehringer-Mannheim(and 55 units glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (Boehringer-Mannheim) in 0.1 M phosphate buffer pH 6.0 at 25^{O}C. The rate of NADH formation was recorded for 6 min at 340 nm on a Beckman Acata CII spectrophotometer. It should be noted that even at maximal activity, the assay curve remained J shaped, a possible consequence of the time lag in the interconversion of α-D-glucose to the β-D-glucose isomer (LABAT et al. 1973). Consequently the change in absorbance was calculated between 1 and 6 min. The reaction velocity was approximately linear over this time course. The Lineweaver-Burke plots obtained by this procedure were identical to those obtained by an end point method utilizing the "glucostat" reagent described in the pH optima reaction.

(iv) Inhibitor properties

The inhibitory potential of a number of compounds was determined by either preincubation of the putative inhibitor in the presence of enzyme and substrate, followed by the determination of the amount of glucose produced by this reagent mixture, or by direct measurement of the change in reaction velocity with the hexokinase-glucose-6-phosphate dehydrogenase couple. The potential inhibitory effects of 10^{-4}M DFP (diisopropylphosphoflouridate) 10^{-4} M paraoxon (Diethyl p - nitrophenyl phosphate) 10^{-4} BNPP (bis-nitrophenyl phosphate) and 10^{-2} M pHMB (p-hydroxymercuribenzoate) were determined by incubating trehalases separated on electrophoretic gels at 25^{O}C for 30 min in 25 ml of each

compound in a suitable buffer (0.2 M phosphate pH 6.0 for organophosphates, aqueous solution of pHMB). In all cases, gels were subsequently rinsed with distilled water and stained with "glucostat" reagent to determine residual activity.

(v) Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using crystalline Bovine Serum Albumin (BSA) as standard, or by the procedure supplied by Bio Rad Laboratories using beta globulin as protein reference.

(vi) Molecular Weight Determination

Molecular weight was estimated by the procedure outlined by Rodbard (1976) from estimates of Kr (retardation coefficient) obtained from the Ferguson relationship (log M = log Mo-KrT; where M = the electrophoretic mobility and is related to Rm; Mo = the free electrophoretic mobility and T = total acrylamide concentration). Protein standards, myoglobin, chymotrypsin, ovalbumin (monomer), bovine serum albumin (monomer, dimer, tetramer), catalase, ferritin and trehalase samples were prepared for electrophoresis by the procedure described above. A minimum of three replicates were analyzed at each of the gel concentrations, 6, 7, 8, 9, 10%T 2% C employed. R_1 values were determined by dried measurement of gels.

III RESULTS

1. Purification of trehalase from D. melanogaster

Of the several purification techniques attempted, preparative electrophoresis proved most successful. Trehalase from D. melanogaster was not bound in significant amounts to affinity chromatography columns using conclavin A, salicin, and trehalose as ligands. Only 56-66% of total trehalase activity could be recovered in the void volume when a crude enzyme preparation was passed through an Affigel 10 column with p-aminophenyl α-D glucopyranoside as ligand. However, despite the release of bound protein from the column with a 0.1 M phosphate buffer pH 7.5 containing 0.25 M NaCl wash, no trehalase activity was recovered in this fraction.

It is interesting to note that a small percentage of trehalase was absorbed to a Sephacryl S-200 column equilibrated with 0.1 M phosphate buffer pH 7.5 containing 0.25 M NaCl. Thus, in addition to the major peak obtained at elution volume, Ve = 188-194 ml, a second trehalase peak was obtained at Ve 340-350 ml. The void volumn, Vo, of this column was approximately 130 ml total volume, V_t was equal to 375 ml. However, the amount of enzyme absorbed to this column was insufficient to utilize as a routine purification procedure. An attempt to shift the trehalase peak to the void volume of this Sephacryl column by preincubation of the enzyme with Blue Dextran 200 was unsuccessful. Therefore, it would appear that trehalase is not significantly bound to any free glucose residues contained in this polysaccharide.

Table 2-7 presents a survey of electrophoretic mobility of trehalase in a variety of 4014.10 subsystems on non-restrictive gels. These data are presented to illustrate the method of selecting the appropriate gel systems in the protein purification scheme. As shown in the table, trehalase from D. melanogaster just unstacks in system 4014.10 XI but remains stacked in 4014.10 X. System 4014.10 XII was chosen for the second purification stage because the mobility of trehalase in 4014.10 XI was insufficient to prevent contamination of "stacked proteins" during elution. As shown in Table 2-4 no restacking conditions are possible for either system because the steady state moving boundary conditions are violated.

Table 2-8 presents a purification summary of soluble trehalase from D. melanogaster. As shown in this table, the specific activity of the final sample is 18.48 U/mg. This represents a ten-fold enzyme purification with a yield of 17%. However, only 1.6% of the original protein was recovered. These data are further analyzed in the discussion.

Lineweaver-Burke (L-B) and Woolf-Augustinsson-Hofstee (W-A-H) plots for crude and purified enzyme preparations are presented in Figure 2-1. Km values estimated from linear regressions of these data are 0.650 mM and 0.660 mM for the crude and purified preparations respectively (L-B plots); and 0.654 mM and 0.642 mM for crude and pure preparations by W-A-H plots. Statistically there is no significant difference ($p < 0.05$) between Km values for crude and purified preparations obtained from W-A-H plots (comparison of slopes, -Km).

Table 2-9 presents Rm values of trehalases from a number of species of Drosophila. These species are arranged into four groups, A, B, C and D, based on electrophoretic mobility. The electrophoretic

Table 2-7. Relative mobilities of trehalase to bromphenol blue on 5% T; 15% C DATD crosslinked gels.

Subsystem 4014.10	X	XI	XII	XIII	XV	XVI
$\bar{r}_1^{\pi}$	-0.193	-0.214	-0.235	-0.256	-0.298	-0.319
$\bar{r}_m$ trehalase	0.99	0.95	0.89	0.84	0.73	0.64

Table 2-8. Summary of electrophoretic purification of soluble trehalase from D. melanogaster.

Step	Total Protein (mg)	Protein Concentration (mg/ml)	Volume Activity (U/ml)	Specific Activity (U/mg)	Yield %	Purification factor fold
Crude desalted sample	10.01	1.16	17.32	1.73	-	-
0.193 gel	0.88	0.10	7.99	9.04	46.12	5.23
0.235 gel	0.11	0.05	2.01	18.49	11.60	10.69

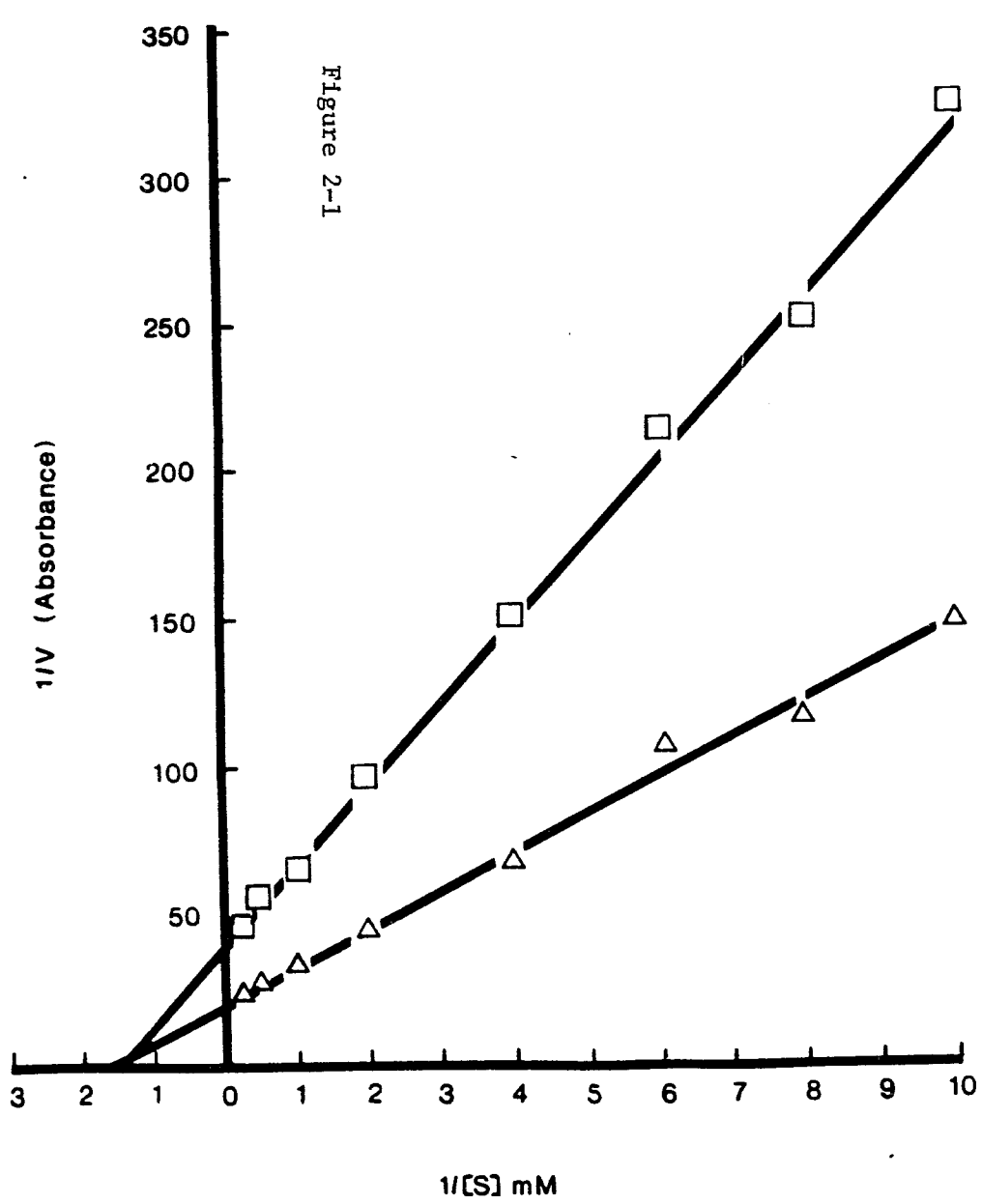
Table 2-9. Relative mobilities of trehalases from Drosophila species.

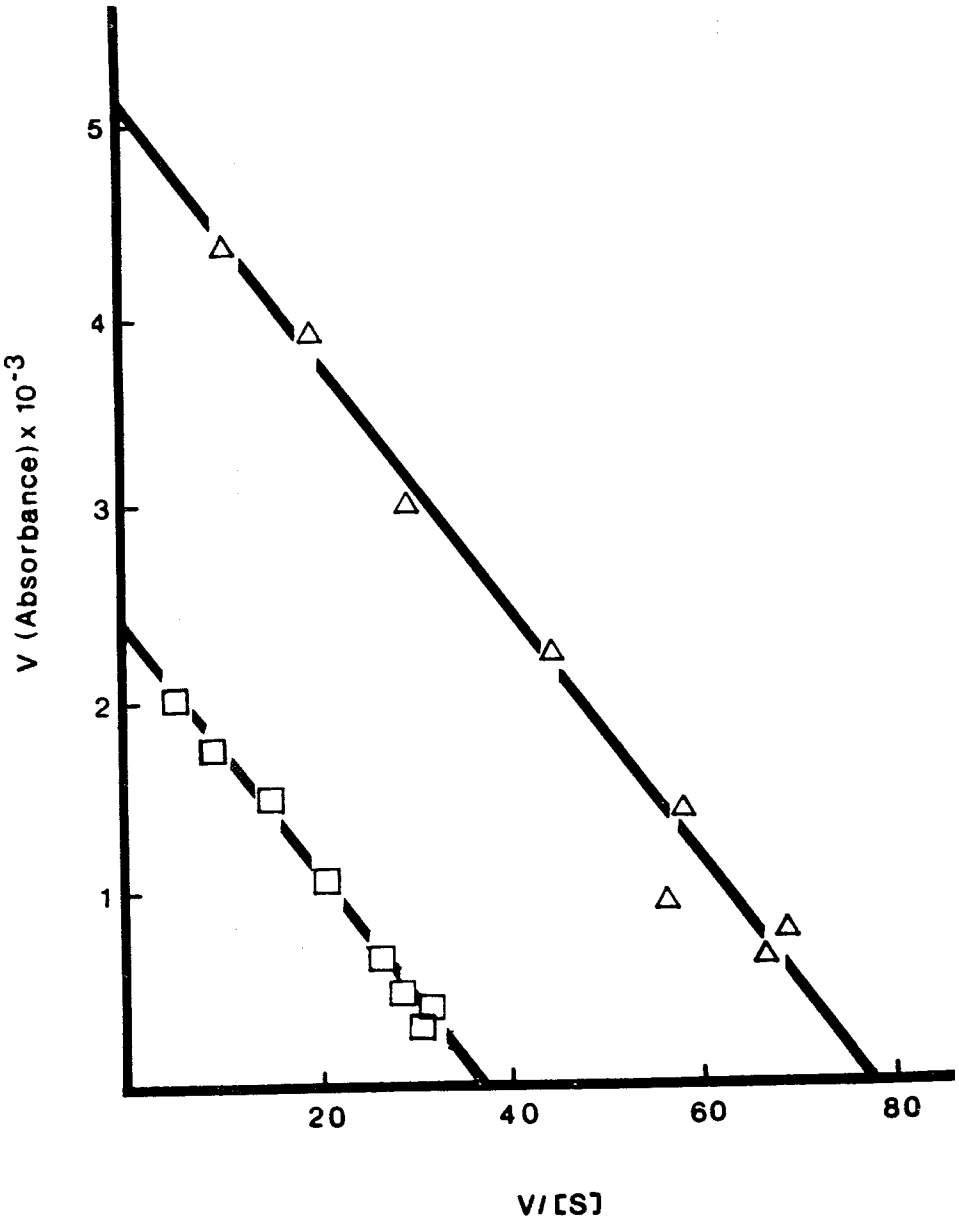
Group[1]	Species	Rm[2]	SEM[3]	Rm^{Na+4}
A	D. arizonensis	0.95	± 0.02	-0.190
	D. mojavensis	1.03	± 0.05	-0.206
B	D. mulleri	1.04	± 0.04	-0.202
	D. quadralineata	1.04	± 0.08	-0.208
	D. nitidithorax	1.08	± 0.02	-0.216
C	D. americana	1.15	± 0.04	-0.230
	D. funebris	1.17	± 0.01	-0.234
	D. robusta	1.17	± 0.01	-0.234
	D. pseudoobscura	1.18	± 0.02	-0.236
	D. montana	1.19	± 0.01	-0.238
	D. argentostriata	1.20	± 0.02	-0.240
D	D. virilis	1.27	± 0.002	-0.254
	D. hypocosta	1.27	± 0.01	-0.254
	D. pararubida	1.29	± 0.002	-0.258
	D. lebanonensis	1.32	± 0.001	-0.264
	D. lebanonensis castelli	1.34	± 0.01	-0.268
none	D. simulans	0.78	± 0.01	-0.156
	D. quadralineata (slow form)	0.72	± 0.04	-0.144

1. Group - see text
2. Relative mobility with respect to D. melanogaster
3. Standard error of mean
4. Assumes that trehalase from D. melanogaster has mobility of -0.200 with respect to sodium.

Figure 2-1. Lineweaver-Burke plot (left) and Woolf-Augustinsoon-Hofstee (right) Kinetic plots. △ crude enzyme preparation; □ purified enzyme.

Figure 2-1


350
300
250
200
150
100
50
1/V (Absorbance)
3
2
1
0
1
2
3
4
5
6
7
8
9
10
1/[S] mM


5
4
3
2
1
V (Absorbance) x 10-3
20
40
60
80
V/[S]

conditions required for the first electrophoretic separation for each group corresponds to the subsystems presented in Table 2-4. Thus, species from group A will migrate within or close to the stack in subsystem 4014.10 X. Trehalase from D. simulans and the slow form from D. quadralineata cannot be stacked in any of these systems. It should be feasible to restack trehalases with HEPES from species in groups C and D. The data presented in this table cannot be used to establish the homology of trehalases from these species of Drosophila, as no attempt has been made to standardize sample conditions prior to electrophoresis. Therefore, the differences noted may be due to differences in protein concentration or ionic strength of individual samples, and may not represent differences in the primary structure of the enzyme.

2. Inhibitors of trehalase activity

Table 2-10 illustrates the effects of dilution on enzyme activity. Dilution of the crude aqueous extract of trehalase does not produce a corresponding linear decrease in activity. Dilution does reduce activity in a sample which was desalted on Sephadex G-25M and concentrated on a Minion B-15 concentrator (Amicon). Thus, 1/2 and 1/3 dilution of the crude enzyme preparation decreased activity by 60 and 45% respectively, whereas activity was reduced to 47% and 32% by the corresponding dilution of the desalted sample. These results are consistent with an interpretation which postulates the presence of trehalase inhibitors in the crude extract. As a means of investigating this possibility, 1 ml fractions were collected from the effluent of the columns used for desalting the crude preparations and tested for their ability to inhibit trehalase activity. The results from two such independent

Table 2-10. Effects of sample dilution on trehalase activity

Preparation	Dilution	ΔA/min	% Activity of Control
Crude	undiluted	0.0429	100%
	50%	0.0255	59.5
	66%	0.0195	45.5
Desalted	undiluted	0.0443	100
	50%	0.0207	46.7
	66%	0.0143	32.3

experiments are summarized in Table 2-11. It is evident from a consideration of these data that factors inhibiting trehalase activity are present in the crude extract. However, the reason for the variability in degree of inhibition in the two experiments is uncertain. Preincubation of each fraction at 100^{o}C for 5 min failed to significantly alter the amount of inhibition induced. The apparent increase in activity relative to the control by fractions 1-4 is due to the presence of glucose in these fractions and the employment of the glucostat reagent.

Preliminary determinations of the potential inhibitory properties of a variety of substances are presented in Table 2-12. Of the amino acids investigated, only tryptophan appears to inhibit trehalase when incubated with equimolar concentrations of substrate. The Ki determined for phenylalanine (assuming competitive inhibition) undoubtedly results from experimental error in the construction of kinetic plots and is exacerbated by the low concentration of the amino acid employed. The inhibition constant in this case reflects a decrease of $\simeq$ 5% in activity when equimolar concentrations of subtrate are employed. Similarly, the discrepancy in the Ki determined for TRIS inhibition by Lineweaver Burke and Hofstee plots is also subject to the same error. However, it is apparent that high concentrations of TRIS are inhibitory. Assuming a Km for trehalase of 0.7 mM, the decrease in activity of trehalase at 0.1 and 0.5 M corresponds to a Ki ranging from 8.0 - 11.0 mM. Of all the substances employed, the competitive inhibition of trehalase by salicin is most consistent, although, at best it is moderate with Ki in the range 0.4 - 0.55 mM. A number of

Table 2-11. Effect of intrinsic molecules on the inhibition of trehalase

	Fraction	ΔA/min	% of control
Experiment 1	Control	0.0127	
	I1	0.0150	118.11
	I4	0.0135	106.29
	I7	0.0036	28.35
	I10	0.0075	59.06
	I13	0.0110	86.66
Experiment 2	Control	0.0352	
	I5	0.0312	88.64
	I5H	0.0305	86.65
	I6	0.0268	76.14
	I6H	0.0275	78.13
	I7	0.0279	79.26
	I7H	0.0272	77.27
	I8	0.0302	85.80
	I8H	0.0270	76.70
	I9	0.0305	86.65
	I9H	0.0318	90.34

Table 2-12. Potential inhibitors of Drosophila trehalase

Amino Acids	% Activity of control	Ki (mM)	
		Lineweaver Burke	Hofstee
glycine (0.0133M)	94.50±8.53	n.d.	n.d.
glycylglycine (0.0133M)	106.49±5.51	n.d.	n.d.
alanine (0.0133M)	106.28±6.17	n.d.	n.d.
aspartic acid (0.0133M)	58.79 -	n.d.	n.d.
tryptophan (0.0133M)	36.86±9.39	n.d.	n.d.
lysine (0.001M)	n.d.	-	-
phenylalanine (0.001M)	n.d.	1.93	-
Carbohydrates			
Salicin (0.1M)	36.05	n.d.	n.d.
Salicin (0.001M)	n.d.	0.419	0.552
para-aminophenyl α-D-glucopyranoside	n.d.	-	-
turanose	100%	-	-
$MgCl_2$ (0.066M)	93.36±3.49	n.d.	n.d.
$MgCl_2$ (0.133M)	94.13±4.03	n.d.	n.d.
EDTA (0.0133M)	112.45 -		
TRIS (0.001M)	n.d.	-	4.70
TRIS (0.1M)	55.54±7.60	n.d.	n.d.
TRIS (0.5M)	26.72±1.53	n.d.	n.d.
p-hyroxymercuribenzoate (10^{-2}M)	100%		
di-isopropyl phospho-flouridate (DFP 10^{-4}M)	100%		
diethyl p-nitrophenyl phosphate (paraoxon 10^{-4}M)	100%		
bis nitrophenyl phosphate (BNPP, 10^{-4}M)	100%		

n.d. = not determined.

organophosphates failed to inhibit trehalase activity when gels were preincubated in buffered solutions of these inhibitors for 30 min, rinsed and stained for residual activity.

3. Molecular Weight Determination

Estimates of molecular weight for trehalases from seven species of Drosophila calculated from the data presented in Table 2-13 are reported in Table 2-14. The average molecular weight of trehalase from the D. melanogaster subgroup (D. melanogaster, D. simulans, D. mauritiania, D. teisseri, D. yakuba and D. erecta is 72,300 ± 5,300. Although not shown in table, trehalases from the remaining members of D. obscura subgroup (D. subobscura, D. pseudoobscrua, D. persimilis and D. miranda have identical electrophoretic mobility and hence Kr and M.W. as the thoracic form of D. obscura. It should be noted that there is no statistically significant difference between Kr for thoracic and abdominal forms of trehalase from D. erecta and D. obscura respectively. These data are $F_{0.05[1,6]} = 5.99 > F_s$ D. erecta $= 1.59$ and $> F_s$ D. obscura $= 1.60$.

4. Developmental

The developmental progression of trehalases in D. melanogaster, D. simulans, D. mauritiania, D. erecta, D. teisseri, and D. yakuba has been investigated qualitatively by activity in electrophoretic gels. In all cases, the number and electrophoretic position of bands was invariant when 1st instar, 2nd instar, 3rd instar larvae early and pharate pupae and adult forms were compared. Qualitatively (as judged by staining time and intensity), enzyme activity remains at low levels, throughout

Table 2-13. Molecular Weight Determination - Protein standard parameters.

Protein	$\bar{R}$[1]	Kr[2] ± Sb[3]	Kr	M.W.
Myoglobin	1.73	-0.0360±0.0071	0.1897	17,800
Chymotrypsin	1.94	-0.0373±0.0027	0.1931	25,100
Ovalbumin (Monomer)	2.33	-0.0422±0.0017	0.2054	43,500
Bovine Serum Albumin (Monomer)	2.69	-0.0543±0.0033	0.2330	67,000
Transferrin (Human)	2.97	-0.0567±0.0029	0.2380	90,000
Bovine Serum Albumin (Dimer)	3.40	-0.0812±0.0034	0.2849	134,000
Catalase	4.08	-0.0887±0.0037	0.2979	232,000
Bovine Serum Albumin (Tetramer)	4.16	-0.1133±0.0066	0.3366	268,000
Ferritin	5.09	-0.2166±0.0183	0.4654	450,000

1. $\bar{R}$ mean geometric radius values from Rodbard and Chrambach (1971).
2. Kr experimentally defined retardation coefficient obtained from Ferguson relation ($\log M = \log M_o - Kr\ T$) where M = the electrophoretic mobility; M_o = free electrophoretic mobility; Kr = retardation coefficient; T = percent total acrylamide).
3. Sb standard error of the slope of the regression coefficient (Kr) obtained from Ferguson plot.
4. M.W. molecular weight values from Rodbard and Chrambach (1971).

Table 2-14. Summary of molecular weights of Drosophila species.

Drosophila spp	Kr ± Sb[2]	Kr	R^3	M.W.[4]
D. melanogaster	-0.0558±0.0011	0.2362	2.7254	69,000
D. simulans and D. mauritiania	-0.0571±0.0023	0.2390	2.7594	71,600
D. teissieri and D. yakuba	-0.0562±0.0023	0.2371	2.7363	69,800
D. erecta (abdomen)	-0.0626±0.0011	0.2502	2.8953	82,700
D. erecta (thorax)	-0.0554±0.0019	0.2354	2.7157	68,300
D. obscura (abdomen)	-0.0589±0.0034	0.2408	2.7812	73,300
D. obscura (thorax)	-0.0523±0.0021	0.2286	2.6331	62,200

1. Kr retardation coefficient determined experimentally from Ferguson plots.

2. Sb standard error of regression coefficient (Kr).

3. $\bar{R}$ Mean geometrical radius determined by estimation from the standard curve determined from regression of Kr vs. $\bar{R}$ (known standards) $\bar{R}$ = 12.1360 Kr - 0.1411: Correlation coefficient r = .9545.

4. M.W. Estimated from the relationship:

$$\bar{R} = [3(MW)\bar{\nu} / 4\pi\Pi]^{1/3}$$

where $\bar{\nu}$ = partial specific volume (assumed to be 0.74) and Π = Avogadro's number (assumes zero hydration of the protein molecule).

larval and early pupal stages, but increases substantially in pharate pupae and adults. In D. erecta the relative staining intensity of the abdominal form of the enzyme decreases with respect to the thoracic form in late pupal stages and adult stages when entire individuals are assayed for activity.

A detailed electrophoretic and physicochemical investigation of the properties of trehalases from adult D. melanogaster age 0-3 days and 12-15 days and D. simulans adult, age 0-4 days and 12-15 days was undertaken. These data are presented in the following sections.

5. Properties of trehalases in D. melanogaster and D. simulans

i) Electrophoresis of D. melanogaster and D. simulans

Figure 2-2 demonstrates the regions of enzyme activity localized by the "glucostat" reagent (see Materials and Methods) in D. melanogaster, D. simulans and their hybrids following electrophoresis on 9% T, 2% C acrylamide gels (4014.10 XII). Two distinct bands are present in both species, the more cathodal of which have similar electrophoretic mobility, 0.082 ± 0.010 (s.d.) in D. melanogaster and 0.087 ± 0.012 in D. simulans ($t = 0.294$, $p < 0.01$; 13 d.f.). The two anodal bands differ significantly in electrophoretic mobility from each other in both species. D. melanogaster is characterized by a band with mobility 0.411 ± 0.012 (s.d.) whereas the form present in D. simulans has mobility 0.324 ± 0.009 (s.d.) ($t = 14.99$, $p > 0.01$; 13 d.f.). No additional enzyme forms could be detected with gel system 1420 (cathodal migration).

Ferguson plots of soluble trehalases from D. melanogaster (0-3 days) and D. simulans (0-4 days) are presented in Figure 2-3.

Figure 2-2. Schematic of 9% polyacrylamide gel showing regions of enzyme activity following incubation with glucostat reagent.

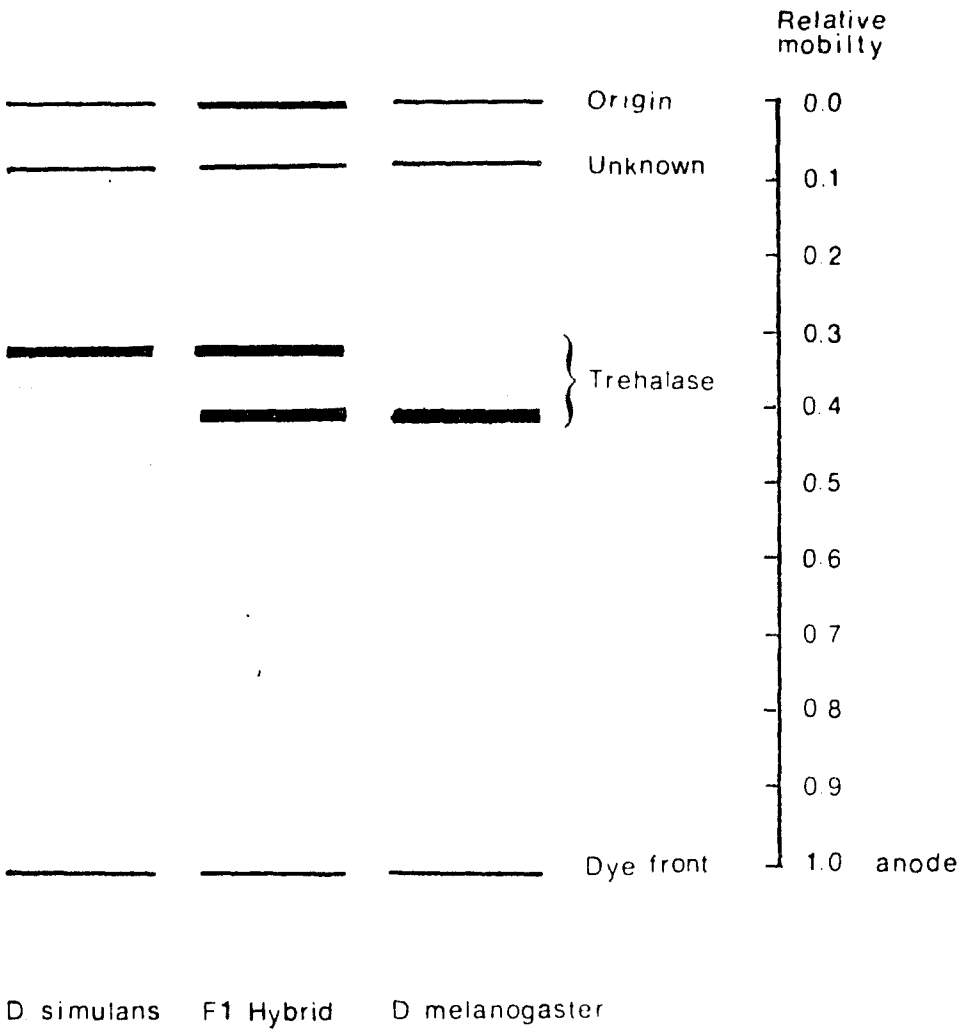

Relative mobility
Origin
Unknown
Trehalase
Dye front
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
anode
D simulans
F1 Hybrid
D melanogaster

Figure 2-2

Figure 2-3. Ferguson Plot of trehalases: ● D. simulans (r = 0.9996), ▲ D. melanogaster (r = 0.9979) (each point represents mean of a minimum of three determinations, s.d. $\leq$ 0.005 for all points).

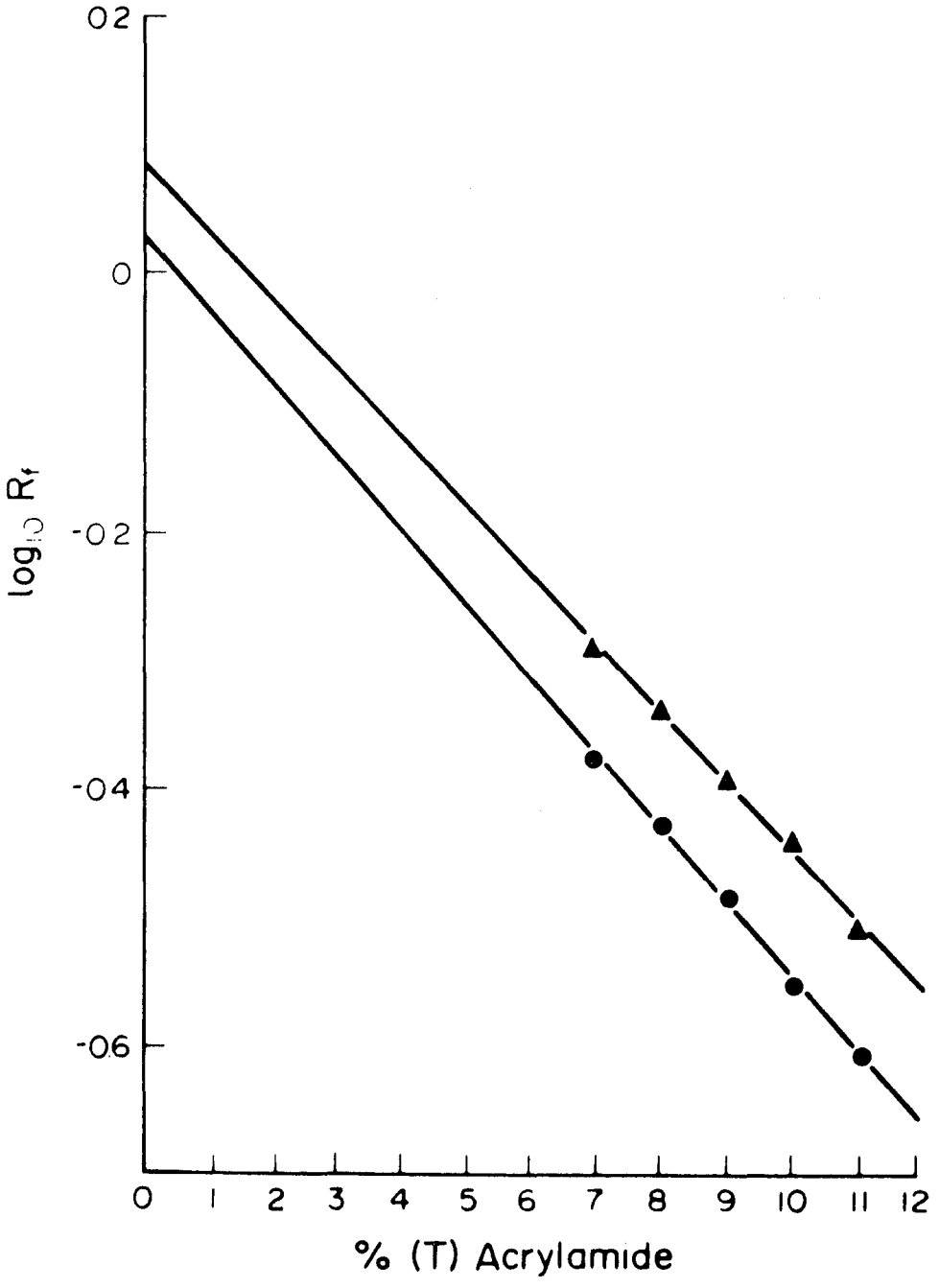


Figure 2-3

Analysis of these data indicate that there is no significant difference in the retardation coefficient (K_r, absolute value of the slope of the regression) of the two enzyme forms. These values are $K_r = 0.0540$ for D. melanogaster and $K_r = 0.0567$ for D. simulans ($F_{0.05[1,6]} = 5.99 > F_s = 1.43$).

ii) pH optima

The response of trehalases to variation in pH is shown in Figure 2-4. The pH optima of both enzyme forms in both age categories are similar, lying between 5.5 and 6.0. This result is in agreement with the pH optima reported by Marzulf (1969) and Huber and Lefebvre (1971) for D. melanogaster. It is interesting to note that the pH optima for the two allozyme forms are identical. Thus, the amino acid substitutions which result in different electrophoretic mobility have no apparent effect on the pH optima of the two enzyme forms.

iii) Michaelis constants

Lineweaver-Burke (L-B) plots for the two age classes of D. melanogaster and D. simulans utilized in this study are presented in Figure 2-5. Estimates of the Michaelis constants determined from the least square regression of these data are presented in Table 2-15. Included in this Table are Michaelis constants calculated from Woolf-Augustinsson-Hofstee (W-A-H) plots (V versus V/[S]) and Hanes-Woolf (H-W) plots ([S]/V vs. [S]). A qualitative comparison of these results indicates that the K_m values of three of the four samples remain similar regardless of plotting method. The apparent departure of K_m in 0-4 days D. simulans is not statistically significant in W-A-H plots, i.e. the

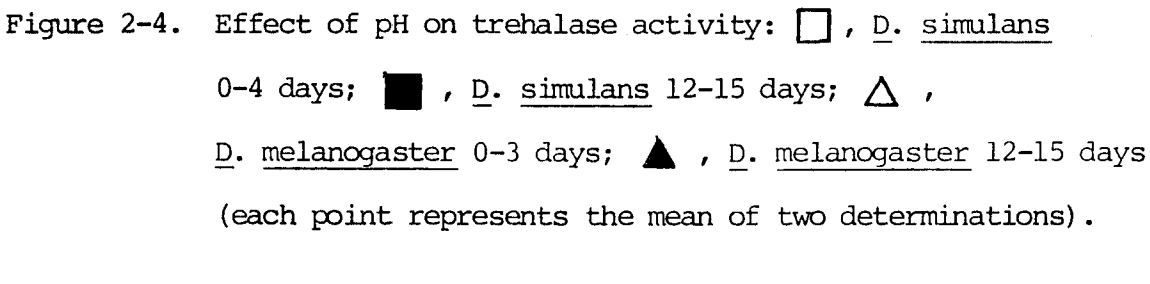


Figure 2-4. Effect of pH on trehalase activity: □, D. simulans 0-4 days; ■, D. simulans 12-15 days; △, D. melanogaster 0-3 days; ▲, D. melanogaster 12-15 days (each point represents the mean of two determinations).

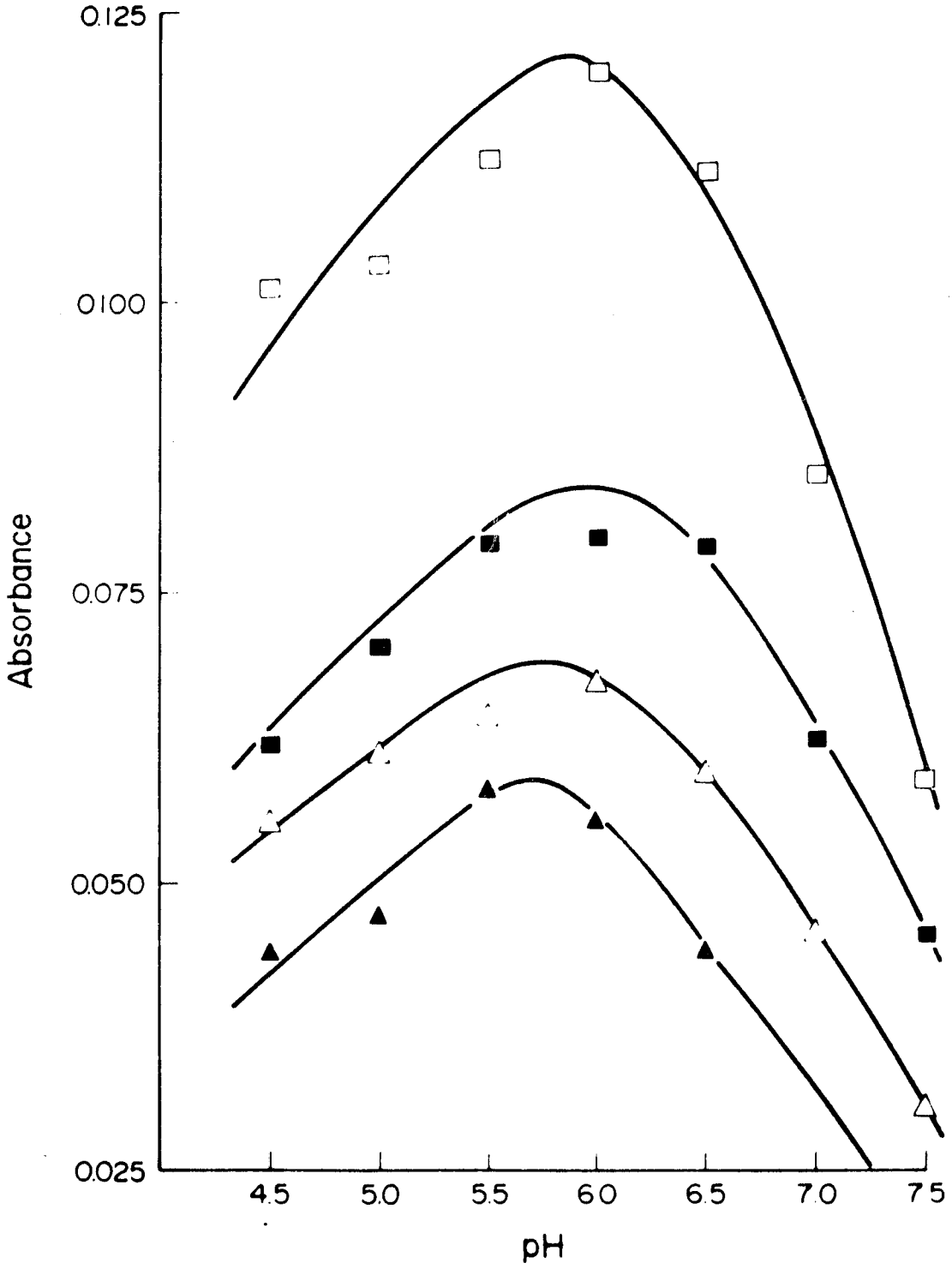


Figure 2-4

Figure 2-5. Lineweaver-Burke plots: □ , D. melanogaster 12-15 days; ■ , D. melanogaster 0-3 days; ○ , D. simulans 12-15 days; ● , D. simulans 0-4 days (all points represent mean of two determinations).

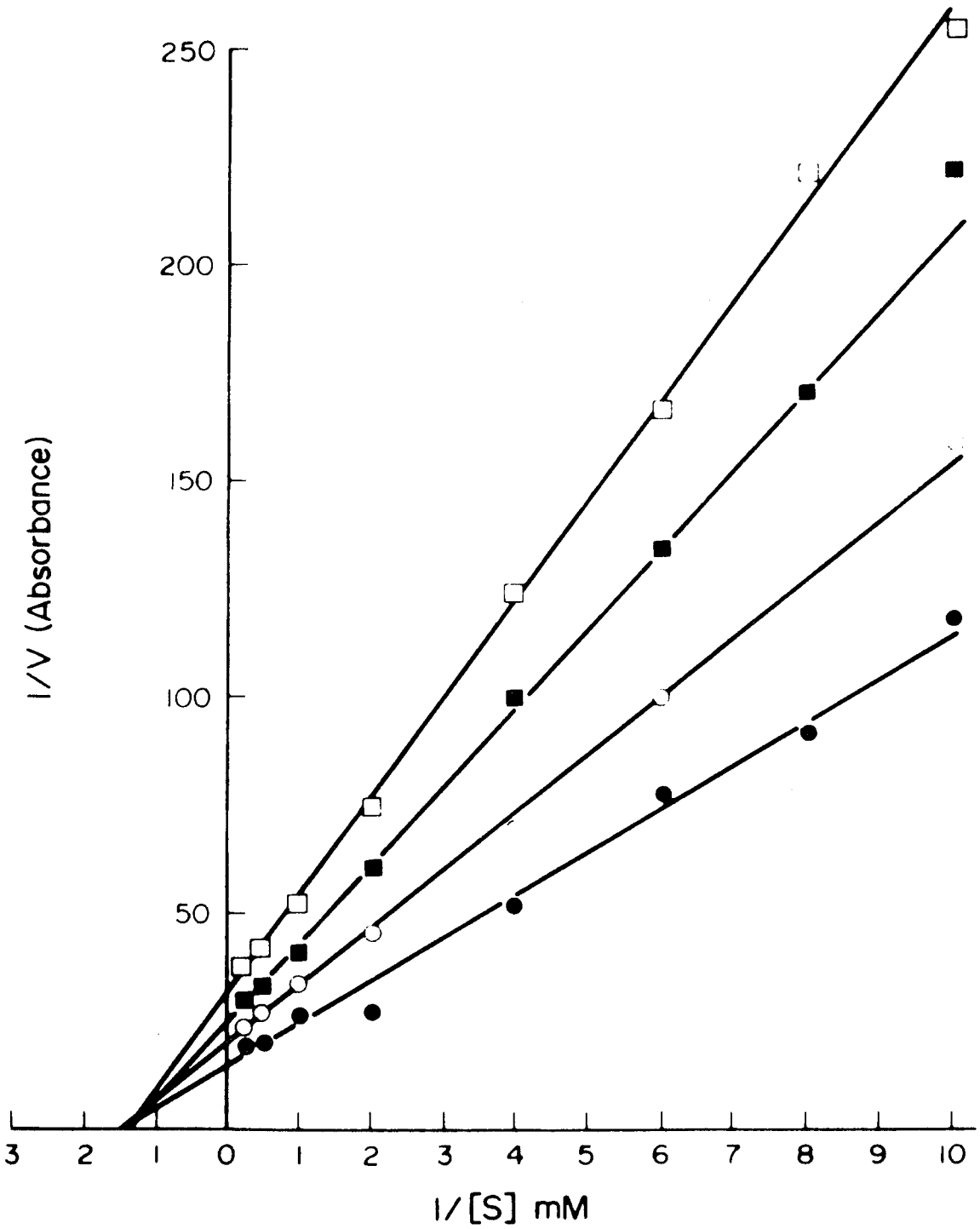


Figure 2-5

Table 2-15. Summary of kinetic constants

Source of enzyme	Lineweaver-Burke			Woolf-Augustinsson-Hofstee			Hanes-Woolf		
	K_m (mM)	V_{max} (ΔE/min)	r	K_m (mM)	V_{max} (ΔE/min)	r	K_m (mM)	V_{max} (ΔE/min)	r
D. melanogaster									
0-3 days	0.752	0.0412	0.989	0.671	0.0383	0.976	0.658	0.0380	0.999
12-15 days	0.748	0.0327	0.999	0.733	0.0323	0.996	0.702	0.0315	0.999
D. simulans									
0-4 days	0.703	0.0693	0.995	0.512	0.0581	0.923	0.480	0.0564	0.996
12-15 days	0.701	0.0517	0.998	0.627	0.0480	0.994	0.619	0.0477	0.999

r = correlation coefficient

ΔE = change in absorbance

slopes ($-K_m$) are homogeneous ($F_{0.05[3,22]} = 3.02 > F_s = 1.53$), and may be due to error associated with clumping [S] points near the [S]/V axis in the H-W plot. This latter feature accounts for the highly significant coefficient obtained in this analysis. Nevertheless, it is uncertain whether the K_m for 0-4 days D. simulans is correctly estimated by the latter two plots, or merely reflects some unexpected distortion caused by the transformation of the data points obtained for this sample.

iv. Maximal catalytic activity

Despite the similarity in apparent K_m (L-B plots) among the four samples, there are marked differences in the maximal catalytic activity of soluble trehalases if comparisons are based per gram of wet weight. These differences are only changed slightly by the different plotting techniques employed. In all cases, the activities of the four samples can be arranged in the following decreasing sequence; 0-4 days, D. simulans > 12-15 days D. simulans > 0-3 days D. melanogaster > 12-15 days D. melanogaster. On average, the activity of D. simulans samples exceed those of D. melanogaster by a factor of 1.5. This difference is accentuated if one considers the specific activity (units/mg) of the samples. However, this latter treatment may be misleading as the differences in protein concentration in crude preparations (Table 2-16) may be unrelated to quantities of the enzyme trehalase.

Table 2-16. Estimates of maximal catalytic activity

Source of enzyme	Maximal Catalytic Activity[1]			
	Lineweaver-Burke	Woolf-Augustinsson-Hofstee	Hanes-Woolf	Protein (mg/ml) Concentration
D. melanogaster				
0 - 3 days	0.198±0.050[2] (0.319)[3]	0.185 (0.298)	0.183 (0.295)	0.62
12 - 15 days	0.158±0.011 (0.158)	0.156 (0.156)	0.152 (0.156)	1.00
D. simulans				
0 - 4 days	0.334±0.052 (0.458)	0.280 (0.384)	0.272 (0.373)	0.73
12 - 15 days	0.249±0.022 (0.566)	0.232 (0.527)	0.230 (0.523)	0.44

(1) Maximal catalytic activity - μM trehalose/min/ml. Assumes that hydrolysis of 1 μM trehalose results in the reduction of 2 μM of NAD when coupled with hexokinase and glucose-6-phosphate dehydrogenase.

(2) ± standard error (calculated for Lineweaver-Burke estimate only)

(3) Specific activity (U/mg)

IV. DISCUSSION

1. Purification

The inability of salicin and trehalose substituted Sepharose 6-B affinity residues to bind a substantial proportion of trehalase from D. melanogaster is somewhat surprising considering the apparent affinity of the enzyme for these carbohydrates (salicin Ki ≃ 0.5 mM, trehalose Km 0.65 mM). Perhaps the lack of expected specific binding in these cases is due to the unavailability of key recognition sites between the enzyme and carbohydrate. Such sites may no longer be exposed on the carbohydrate as a consequence of the nature of the covalent linkage between these ligands and the epoxy activated Sepharose-6B. It should be noted that any of the available hydroxyl groups would be bound by the linking mechanism. The importance of the C-6, C-4, C-3, and C-2 hydroxyl groups of trehalose as recognition sites have been described in the introduction (Section II-1). In addition, the lack of binding may have resulted from the hydrolysis of salicin and trehalose at the high pH (12.8) and temperature (45^{o}C) employed to effect coupling.

Surprisingly, despite the apparent lack of inhibition of trehalase by p-aminophenyl α-D glucopyranoside, there was an indication that trehalose was bound to this carbohydrate when it was attached to Affigel 10 resin by its amino group. This method of binding may have made the glucoside residue available for binding to trehalase. This possibility is supported by the observation that trehalase is inhibited by α-D-glucose and its derivatives (Table 2-2). The inability to

remove trehalase from this column either indicates an extremely tight coupling of the enzyme and ligand, or more likely indicates that the presumed binding was artefactual or non-specific. Howerver, there is no single factor which would readily account for the loss of 33-44% of trehalase activity following interaction with this column.

The absence of interaction between trehalase and Con A Sepharose suggests that trehalase does not contain carbohydrate residues bearing terminal α-D-glucosyl or related residues, or at least, these residues are not free to interact with concanavalin A. It is interesting to note that the substrate trehalose has a modest affinity for Con A (K_4 = 5.38 x 10^{-3} liters/mole) Wallach (1974). However, this interaction proved ineffective as a purification tool when crude enzyme, substrate and Con A resin were simultaneously present. Several explanations may be suggested, the most likely being the possibility that the binding of trehalose by the enzyme occupies all available α-D-glucosyl residues and therefy counteracts the affinity between trehalose and Con A. Alternatively, Con A may bind the substrate in such a manner to prevent recognition between enzyme and substrate. Either interpretation is consistent with the observation of Labat-Robert et al. (1978) that all free hydroxyl groups are important for binding enzyme and substrate.

The low purification factor obtained for trehalase (Table 2-8) following electrophoretic purification by selective unstacking appears to stem largely from a substantial decrease in yield of trehalase in each successive electrophoretic stage. Thus, despite the recovery of only 1.6% of the initital protein contained in the crude extract, only 17% of the original enzyme activity could be recovered in the final product. One or more of the following considerations may explain this

low yield. The first two may reflect deficiencies in any system based on preparative PAGE and are discussed more extensively by Chrambach et al. (1976, pp. 14, 106, 138).

The first consideration concerns the load capacity of the gels utilized. The amount of protein recovered in the trehalase fraction after each electrophoretic operation remains remarkably constant, approximately 25 $\mu g/cm^2$ of gel. That is, 101 $\mu g/4.02\ cm^2$ from the first operation (two 2.01 cm^2 gels were employed) and 47 $\mu g/2.01\ cm^2$ from the second. These values are substantially lower than the 50 $\mu g/cm^2$ per component recovery estimated by Chrambach et al. (1976, page 30).

A second consideration involves the underestimation of the bandwidth of trehalase in the non-restrictive gels employed. If bandwidth is greater than suggested by the direct colorimetric staining procedure, then a substantial amount of protein could be lost due to the associated difficulties in the calculation of appropriate collection times. Clearly, the error would be greater in preparative systems which collect unstacked proteins. Utilizing longer collection times may increase yield but would result in the contamination of the component of interest with adjacent proteins.

As expected, the fraction obtained from the second electrophoretic operation (unstacked band collected) can be shown to be contaminated by two or three minor bands when electrophoresis is performed on 10% T, 2% C BIS-crosslinked gels. These impurities may have been introduced by the collection of unstacked enzyme. However, this interpretation is somewhat complicated because such restrictive gels may be expected to resolve protein components which have identical mobility in the non-restrictive gels employed in the preparative scheme. Surprisingly, the

amount of protein recovered from the second operation (unstacked enzyme collected) approximates that from the first (stacked enzyme collected). The significance of these data are uncertain and may be coincidental. Nevertheless, the implication is clear that the collection of unstacked samples necessarily compromises either enzyme purity or yield within the confines of load capacity. Clearly, it is of advantage to collect stacked or selectively restacked proteins.

Several other factors which may have led to the poor yield of trehalase include the possibility of enzyme denaturation during electrophoresis, enzyme aggregation, or adhesion of the enzyme to reagent impurities which are contained in the polymerized gel (Chrambach et al. 1976, page 52). Denaturation as a consequence of heating is unlikely in this case as the critical temperature is 50-55^{o}C (Chapter 3). The possibility of aggregation and/or adhesion is suggested by the detection of enzyme activity on the surface of the stacking gel (5% T, 15% C DATD-crosslinked) following colorimetric assay of the gel employed in the purification. However, it is possible that this residual activity results in part by exceeding the gel load capacity as described above.

Another factor which may decrease the purification factor is the possibility that the final protein concentration is overestimated due to the interference of the Bio Rad protein reagent with triethanolamine. This interference limits the detection of protein to concentrations exceeding 0.034 mg/ml. Although admittedly, a soft argument, the preceeding factor, coupled with the results of electrophoresis on restrictive gels which indicated the presence of 2 or 3 minor bands in addition to a major zone containing trehalase, are consistent with an interpretation that the final preparation is of greater purity than

suggested by the data presented in Table 2-8.

Although it is apparent that the protein preparation is not homogeneous, the similarity of Km between the crude and partially purified preparations suggests that desalting aqueous homogenates on Sephadex G-25 may be sufficient to accurately estimate the Km of soluble trehalase from D. melanogaster.

2. Inhibition of trehalase

Of the data presented in the results which indicate the inhibitory effect of several compounds, the possibility of a natural inhibition of trehalase activity contained in crude enzyme extracts is most interesting. Such data would support the contention of Friedman (1961) that trehalase and trehalose co-exist in the hemolymph of Phormia regina due to the presence of a regulatory molecule which affects trehalase activity. Friedman has postulated that this molecule is a large (non-dialysable) heat-labile protein which requires the presence of divalent ions (Mg, Mn, Co) for effective action.

The results presented in Tables 2-10 and 2-11 suggest that trehalase present in crude aqueous extracts of D. melanogaster is inhibited by native factors which are not excluded by Sephadex G-25 M ($\therefore$ MW < 5,000 daltons). However, these factors are not heat labile. Although both $MgCl_2$ and EDTA have no apparent effect on enzyme activity (Table 2-12), these data do not necessarily apply because they were obtained on desalted (Sephadex G-25 M) preparations. Such preparations would only contain an inhibitor with M.W. > 5,000 daltons.

The similarity of Km values of desalted and electrophoretically purified trehalase cast further doubt upon the presence of a large

proteinaceous (> 5,000 daltons) molecule which would regulate the activity of trehalase by alterations in Km (i.e. competitive or uncompetitive inhibition) as suggested by Friedman. However, these data are equivocal for reasons of protein purity, and also, the possibility exists that the regulatory molecule is more tightly bound to the enzyme than suggested by Friedman, and hence is not removed by either desalting or electrophoretic purification. However, the lack of effect of Mg and EDTA on enzyme activity in this case would argue against this possibility.

The apparent co-existence of enzyme and substrate in the hemolymph of insect species may better be explained by Katagiri (1977) who has shown that trehalase in cockroach hemolymph is not free but localized in hemocytes. Therefore, the increase in trehalase activity obtained by dilution of hemolymph can be explained by cell lysis and the consequent release of trehalase rather than by the effects of dilution on a regulatory molecule.

The reduction of activity in crude extracts of D. melanogaster can be explained by the presence of small molecules. Following the considerations of Agarwal (1976), these molecules are most likely free amino acids, or sugar. Of the amino acids tested (Table 2-12) only aspartic acid, tryptophan, and phenylalanine had an inhibitory effect, although the Ki determined for phenylalanine most likely reflects a small experimental error. It is rather unlikely that these substances account for the entire extent of inhibition observed in crude extracts, as aspartic acid accounts for only 5.2% (1.25 µM), phenylalanine for < 0.05% (0.12 µM) of the total amino acid composition of hemolymph from D. melanogaster (Collett 1976,a,b). Tryptophan is apparently not

present as a free amino acid.

The inhibition of trehalase activity by TRIS reported here (Ki = 8-11 mM) agrees with the reports of inhibition in R. americana (Ki = 74 mM, Terra et al. 1978) and D. melanogaster (Ki = 29.2 mM, Huber and Lefebvre 1971). This inhibition explains in part the inability to detect trehalase on electrophoretic gels using TRIS (eg. Davis (1964) system contains 0.3696 M TRIS in the separation phase at pH 9.43, Jovin 1971, page 896). The influence of high pH causing irreversible inactivation of trehalase during electrophoresis may also play a role.

The failure of organophosphates to inhibit trehalase activity is interesting. Of particular note is the inability of DFP to inhibit trehalase activity. This suggests that a DFP reactive serine residue is not involved in the catalytic activity of trehalase. The retention of catalytic activity by trehalase from D. melanogaster following pHMB treatment suggests that sulphdryl groups are not involved in the hydrolysis of trehalose. However, as noted earlier, trehalases from larva of mosquito Culex pipiens are inhibited by heavy metals (Hg, Pb) and by pHMB + cysteine. The significance of this discrepancy with respect to the composition of the active site of the enzyme from these sources is uncertain.

3. Comparison of age samples of D. melanogaster and D. simulans

Of the enzyme forms illustrated in Figure 2-2, only the two anodal bands (R_f = 0.411 and 0.324 in D. melanogaster and D. simulans respectively) have an absolute requirement for trehalose and are undoubtedly soluble trehalases. The two more cathodal bands can be

visualized by the addition of glucose as well as trehalose and appear without the presence of glucose oxidase in the "glucostat" reagent. Therefore, these two forms most likely represent glucose oxidase activity or are some unexplained artifacts of the staining procedure. Unlike the reports of Marzulf (1969) and Huber and Lefebvre (1971) there is no sucrase activity associated with the trehalase band in either species.

The similarity of the retardation coefficients (K_r, Figure 2-3 and Table 2-14) indicates that the two enzyme forms in the two species are charge isomers.

Although not shown in Figure 2-3, the Ferguson plots of 12-15 day D. simulans and 12-15 day D. melanogaster are identical to the plots obtained for 0-4 day D. simulans and 0-3 day D. melanogaster. This similarity is consistent with the interpretation that the structure of the soluble trehalase enzyme molecule is not appreciably modified with age in either species.

The K_m values obtained from Lineweaver-Burke (L-B) plots in this study (Table 2-15) may be compared with those reported from a variety of other species (Table 2-1). The Michaelis constant obtained in the present study for D. melanogaster (crica 0.70 mM) is substantially lower than that reported by Huber and Lefebvre (1971) - 1.36 mM and Marzulf (1969) - 1.8 mM. However, these data sets are not directly comparable due to differences in assay temperature and purity of enzyme preparations.

It is interesting to note that no comparable changes in K_m occur as a function of age in adult Drosophila as have been reported in larvae of Bombyx mori by Shimada (1976). Despite the consistent

difference in K_m (L-B) for soluble trehalases from D. simulans and D. melanogaster (0.703 mM and 0.701 mM; 0.748 mM and 0.752 mM respectively) it is unlikely that these differences are biologically significant. There is some indication of a decrease in K_m as a function of age in both Drosophila species when Woolf-Augustinsson-Hofstee and Hanes-Woolf plots are considered (Table 2-15). However, the differences between age classes (W-A-H) are not statistically significant ($F_s = 0.504$; $p > 0.05$, D. melanogaster; $F_s = 0.11$; $p > 0.05$, D. simulans). If the differences observed are biologically significant, it is apparent that these changes in K_m are not related to alterations of sufficient magnitude to change the electrophoretic properties of the enzyme molecules.

The reasons underlying the differences in maximal catalytic activity (Table 2-16) are uncertain at present. Although they may represent real differences in V_{max} of the enzymes (i.e. concentration of catalytic sites, eg. enzyme turnover or number of catalytic sites), the species difference most probably involves an alteration in the concentration of the enzyme in the crude preparation. This may reflect differential rates of synthesis or regulation of this enzyme in the highly inbred strains of Drosophila utilized in this investigation. If one accepts that the similarity of retardation coefficients indicates the absence of structural alterations in the enzyme molecule, then it is unlikely that the possible age differences reported reflect changes in enzyme structure. The differences in maximal catalytic activity between age groups most likely represent a decrease in the quantity of enzyme as a result of expected protein catabolism and decreased protein synthesis in older flies.

V. SUMMARY

The data presented and discussed in the preceding chapter are required and facilitate biochemical comparisons of trehalases forms from intra and interspecific sources. Of particular note is the similarity of Km in desalted and crude preparations and in age samples of adult D. melanogaster and D. simulans. These data indicate that extensive purification procedures and careful ageing of flies are not necessary to accurately estimate Km for this enzyme. Although not discussed per se, the presence of two bands in hybrids of D. melanogaster and D. simulans and the molecular weight estimate of 70,000 daltons for the enzyme from these species is consistent with a monomeric interpretation of enzyme structure.

The similarity of Kr, Km, and pH optima for samples from D. melanogaster and D. simulans indicate that the enzyme forms are identical physicochemically. This aspect is discussed in detail in the following chapter.

Data from inhibition studies indicate that trehalase from D. melanogaster can be inhibited by a variety of natural occurring substances. However, the presence of a large proteinaceous, divalent metal ion dependent regulatory molecule similar to that reported by Friedman (1961) is not supported by the data presented in this chapter. A consideration of the inhibition, lack of inhibition, and lack of binding of trehalase to a variety of affinity residues, corroborates the recognition sites in the enzyme substrate complex reported by Labat-Robert et al. (1978) and Giebel and Domnas (1976).

With these data serving as a basis, it should be possible to interpret the evolutionary implications of inter and intraspecific differences in trehalase expression in species of Drosophila. This is the theme of the following chapter.

CHAPTER 3

EVOLUTIONARY BIOCHEMICAL GENETICS OF THE TREHALASE ENZYME LOCUS

I. INTRODUCTION

It has been recognized for some time that many enzymes which appear to be homogeneous under a single electrophoretic condition are in fact heterogeneous when other criteria are utilized. The term electromorph has been proposed by King and Ohta (1975) to designate different allelic products which are "hidden" within a single electrophoretic band. Numerous biochemical procedures are currently available which when utilized in systematic combinations may resolve the number of alleles represented by a single electromorph (Sing, Lewontin, and Felton 1976). Those commonly employed in population studies involve: systematic alteration of gel concentration (eg. gel sieving as adapted by Johnson 1977) and variation of separation pH (eg. Singh et al. 1976), heat denaturation studies (Bernstein, Throckmorton and Hubby 1973) and isoelectric focusing (Synder 1978).

The application of such systematic techniques by investigators concerned with reassessing the amount and distribution of genetical variation in populations of Drosophila have revealed a bimodality of polymorphism at several loci. Coyne, Felton and Lewontin (1978) report that loci (Xdh, Adh-6 or Ao, and Est-5 in D. pseudoobscura) which were polymorphic when assayed by a single electrophoretic procedure became more polymorphic when other techniques were simultaneously employed, whereas those originally monomorphic (Odh, Mah, Hk, and α-Gpdh) tended to remain so. Singh (1979a) has reported a similar bimodality of genetic variants, but reports a more uniform increase in the number of "hidden" alleles at both monomorphic and polymorphic loci. Each

electrophoretic band was shown to contain an average of three additional alleles.

Coyne and Felton (1977) interpret this bimodality as suggestive of the differential action of natural selection on the two apparent classes of enzyme loci, but as indicated by Watterson and Anderson (1978) this may reflect differences in the mutation rate of this loci or at least differences in the mutation rate to biochemically discernable products (cf. Zouros 1979). Bernstein, Throckmorton and Hubby (1973) suggest that the presence of a substantial proportion of heat sensitive alleles within an electromorph is indicative of the differential ability of selection to distinguish electrophoretic and heat-sensitive alleles. This suggestion is strengthened by the observation of Finnerty and Johnson (1979) that a clear relationship exists between the degree of shape modification and the extent of alteration of both thermal stability and enzyme activity.

The question concerning the selective maintenance of alleles detected by systematic techniques can be approached by two methodologues; the stastistical approach as exemplified by the papers of Fuerst, Chakraborty and Nei (1977) and Chakraborty, Fuerst and Nei (1978) or the experimental approach which investigates the physicochemical and physiological properties of allozymes and thereby establishes the possibility of their discrimination by selective forces. The second approach is to be utilized here.

This chapter reports the results of a genetic and biochemical investigation of the trehalase gene-enzyme system in Drosophila. The enzyme trehalase, D.C. 3.2.1.28, catalyzes the hydrolysis of the dissacharide trehalose into its two constituent α-D-glucose molecules.

The substrate, trehalose, is known to be the principle carbohydrate in the hemolymph of taxonmically higher insects and to play an important role in physiological processes which are dependent upon sugar reserves (see Wyatt 1967 for review). Thus, on the basis of the apparent relationship between the enzyme and the physiological utilization of the substrate, it can be argued that the enzyme locus is selectable. From an evolutionary viewpoint, this relationship should allow the quantification of the "unit of selection" and consequently the role of the alleles present at the trehalase locus in evolutionary processes (c.f. Chapter 1).

II. MATERIALS AND METHODS

1. Stock Cultures

Drosophila cultures were maintained on raisin-molasses media at 25°C in pint milk bottles. Strains of *D. melanogaster*, *D. simulans*, *D. teissieri* and *D. yakuba* were obtained from either the Midamerica *Drosophila* Stock Culture Center, Bowling Green, Ohio, or from the University of Texas Stock Center at Austin, Texas. Laboratory strains utilized in this investigation were *D. melanogaster*; Agana Guam, Amherst, Canton-S, Crimea, Florida-9, Hikone-R, Ken Ting Taiwan, Kuala Lumpur, Lausanne-S, Mysore India, Oregon-R, Samarkand, Seto Japan, Swedish-b-6, Swedish-C, Urbana-S, Wageningen,Victoria and Queensland Australia, mutant strain "held-out" plus 14 additional wild type strains; *D. simulans*, California-1, Cal-4, Cal-5, Cook Islands, Georgetown Buyana, Lima Peru, two other unspecified laboratory strains. Strains of *D. erecta* and *D. mauritiana* were generously provided by Drs. M. Ashburner and J. David, *Drosophila obscura* subgroup species include, *D. pseudoobscura*, *D. persimilis*, and *D. miranda*, all isofemale lines from McDonald Ranch, *D. subobscura* Spain and *D. obscura* Norway.

2. Electrophoresis

The electrophoretic procedures utilized have been described in Chapter 2.

3. Thermal denaturation

For mass homogenates, samples prepared by the procedure described in Chapter 2 were incubated for 10 min at $0^{o}C$ (control), $35^{o}C$, $40^{o}C$, $45^{o}C$ $50^{o}C$ and $55^{o}C$, then immersed immediately in crushed ice. Residual activity was determined with the glucostat reagent (Chapter 2). Individual flies were homogenized in 100 µl of distilled deionized H_2O in Beckman Microfuge tubes and desalted on Bio Rad Econo-column 0.5 cm i.d.x 10 cm containing Bio-Gel P-6 200-400 mesh polyacrylamide resin, equilibrated with 0.2 M Phosphate buffer pH 6.0. The final sample volume, Vo - 200 µl, was divided into two 100 µl, aliquots, one of which was incubated at $50^{o}C$ for 10 min, the second at $0^{o}C$ served as the control. Residual activity was determined by incubating 80 µl of sample with 0.82 ml of 0.2 M phosphate buffer pH 6.0 containing 100 µl of 0.2 M trehalose for 45 min at $25^{o}C$. The amount of glucose formed was determined in 0.75 ml of the reaction mixture with 0.75 ml glucostate reagent.

4. Kinetics

Kinetic constants were determined from Lineweaver-Burke ($1/\underline{V}$ vs. $1/\underline{S}$) and Woolfe, Augustinsson, Hofstee ($\underline{V}/\underline{S}$ vs $\underline{V}$) kinetic plots. Enzyme activity was determined by the procedure outlined in Chapter 2.

5. Protein determination

Protein concentration was routinely determined with Bio Rad protein assay kit (500-0001; acidic solution of Coomassie Brilliant Blue R250 with bovine plasma gama globulin as standard).

III. RESULTS

1. Species comparisons

(i) Electrophoresis

The electrophoretic banding pattern of trehalases for species from the melanogaster and obscura subgroups of Drosophila are shown in Figure 3-1 as they appear on 8% T, 2% C polyacrylamide gels utilizing multiphasic buffer systems (MBS) 4014.10 XVI at pH 8.24. The electrophoretic pattern at pH 5.76 (MBS 3017.0 VI), remains the same although each band differs in relative mobility with respect to separation at pH 8.24. Trehalase enzyme forms in D. obscura and D. erecta which are marked with asterisks in Figure 3-1 are present in the abdomen of these species. The more anodal forms are located in head, thorax, and abdomen but are designated as the thoracic form in this report. Individuals from hybrids of D. simulans by D. mauritiana and D. persimilis by D. pseudoobscura do not express any additional forms of the enzyme.

The developmental expression of the banding pattern of trehalases from the melanogaster and obscura subgroups remain constant. Thus, the same trehalase form present in adults is present in all preadult stages. In D. erecta both enzyme forms are expressed in early pupae, however the relative staining intensity of the abdominal form decreases in late pupal stages.

(ii) Ferguson Plots

Feguson plots of melanogaster and obscura subgroup trehalases are presented in Figures 2-3, 3-3, and 3-4. Estimates of Kr (retardation

Figure 3.1. Electrophoretic mobility of trehalase on MBS 4014.10.XVI pH 8.24: 1) D. simulans and D. mauritiana; 2) D. simulans by D. melanogaster hybrids; 3) D. melanogaster; 4) D. erecta; 5) D. teissieri and D. yakuba; 6) D. obscura; 7) D. subobscura, D. persimilis, D. psuedoobscura, and D. miranda.

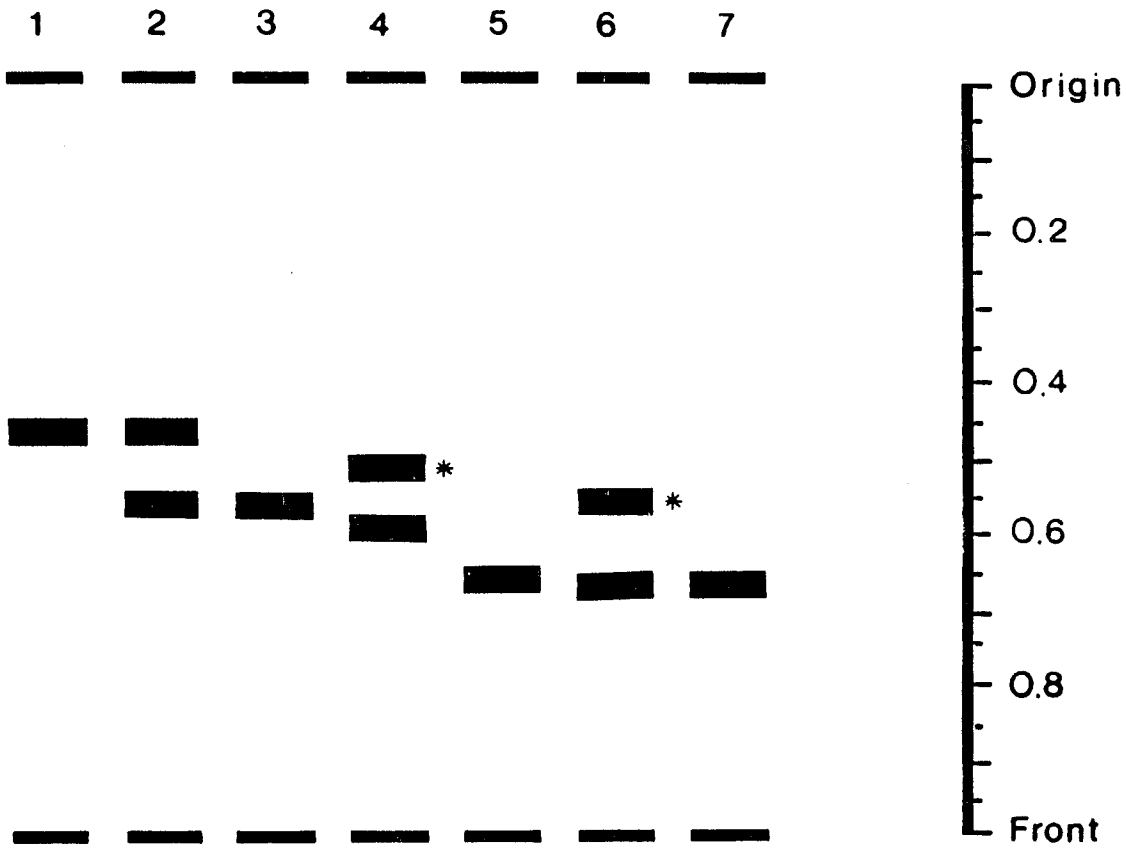


Figure 3-1

Figure 3.2. Ferguson Plot of trehalase from: ● D. teissieri and D. yakuba; ▲ D. erecta (thorax); ○ D. melanogaster; ■ D. simulans and D. mauritiana.

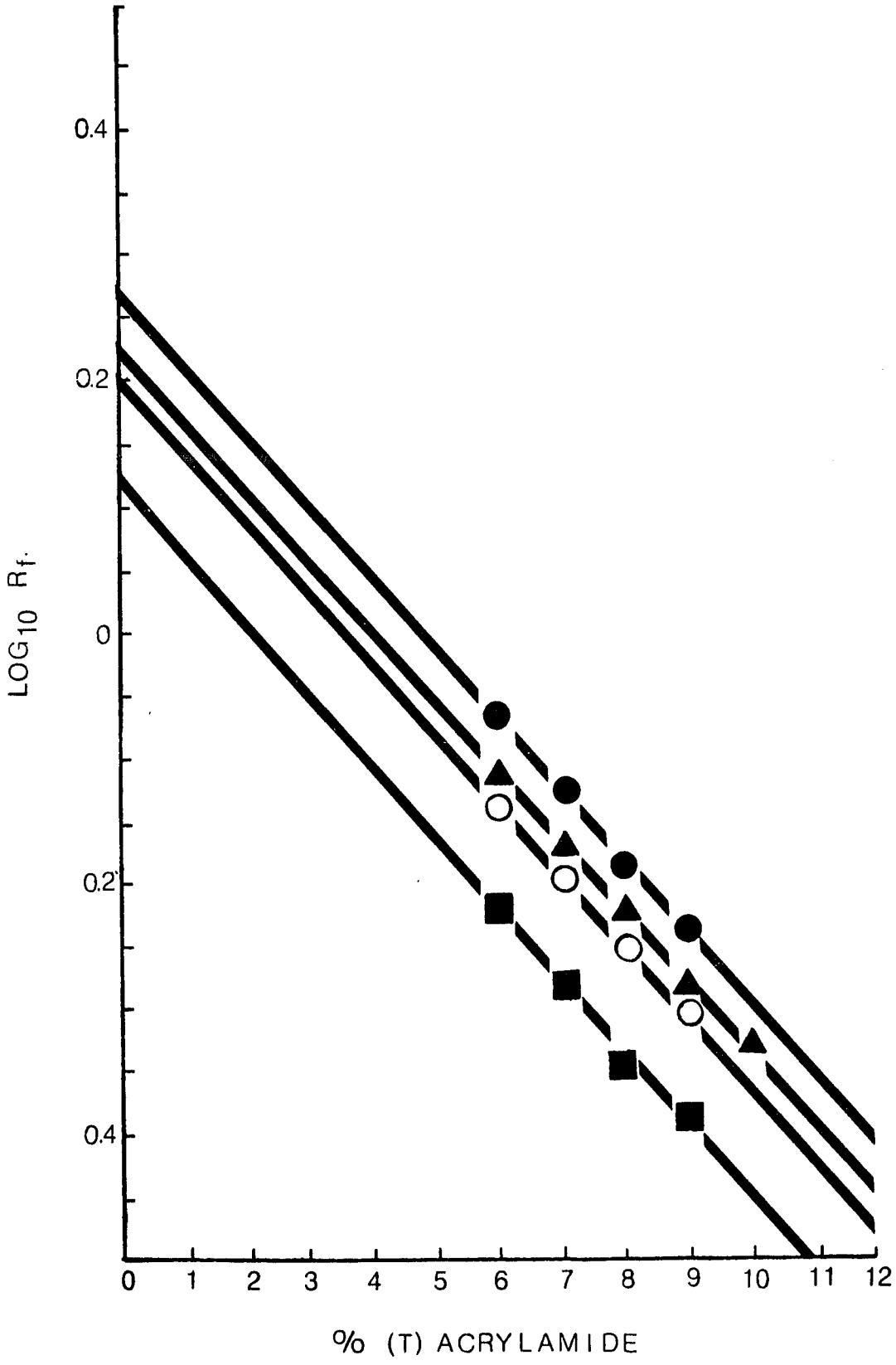


Figure 3-2

Figure 3.3. Ferguson Plot of trehalase from: ▲ D. erecta (thorax); △ D. erecta (abdomen).

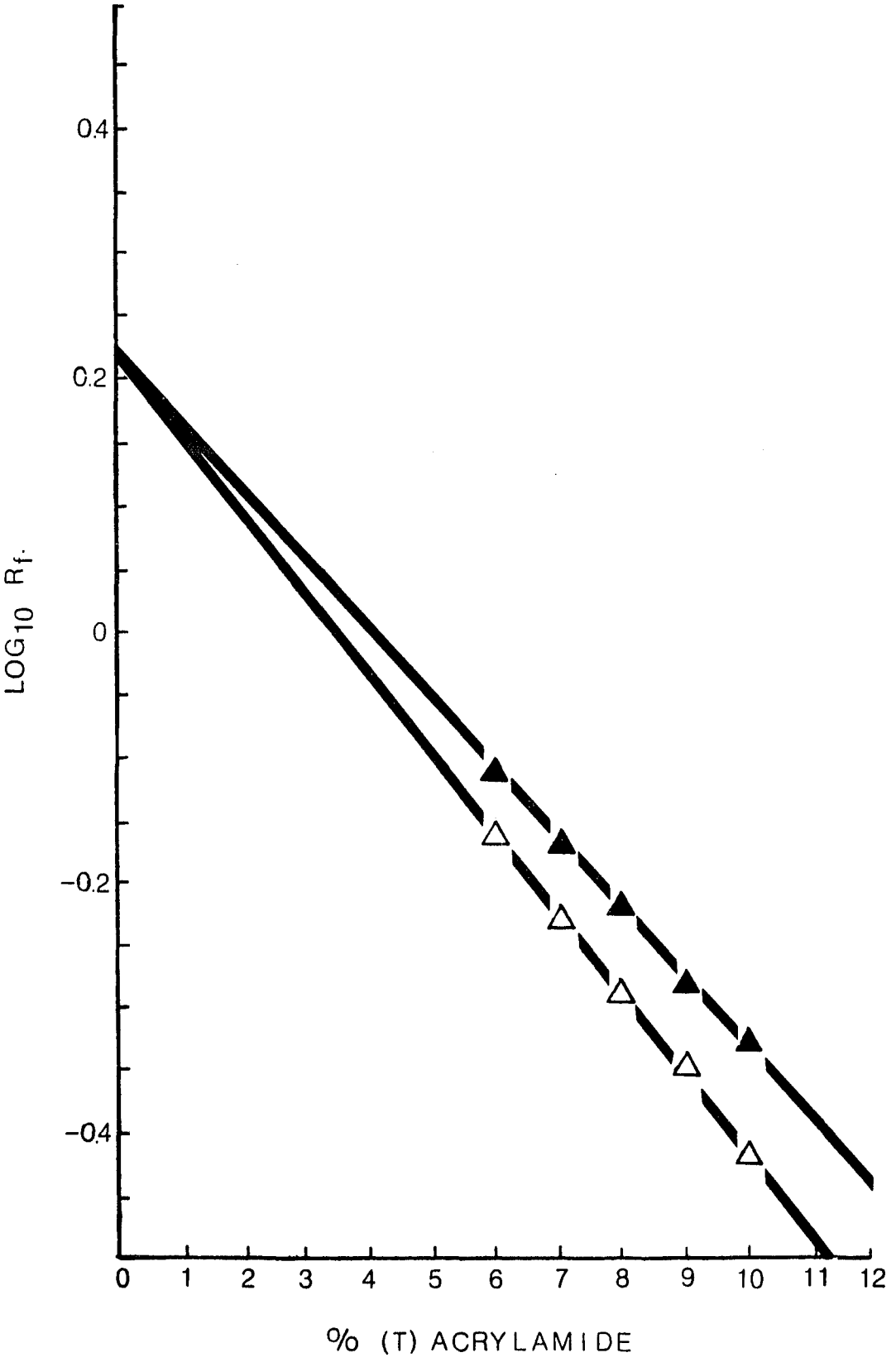


Figure 3-3

Figure 3.4. Ferguson Plot of trehalase from: ○ D. obscura (abdomen); ● D. obscura (thorax) and D. pseudoobscura.

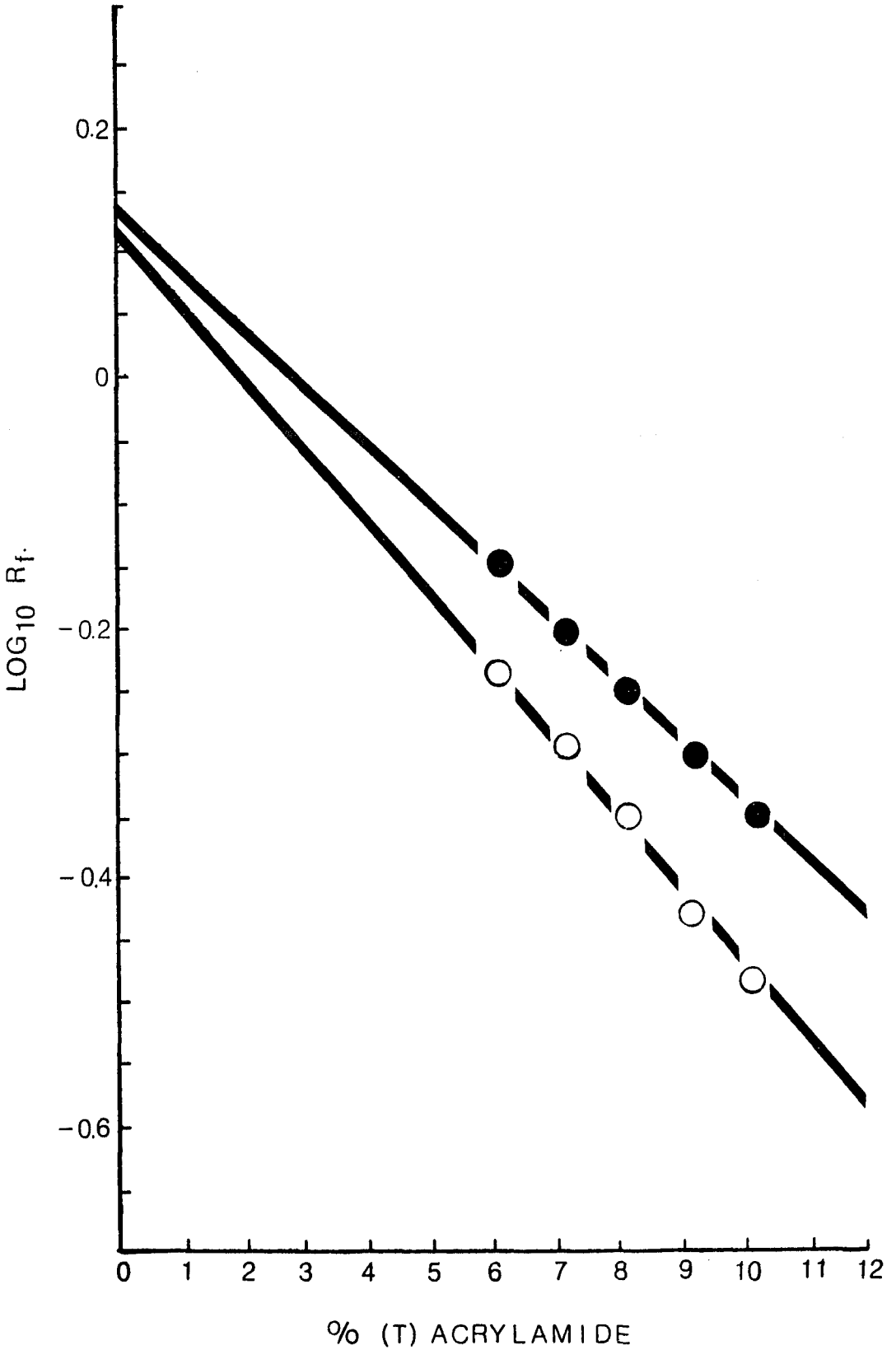


Figure 3-4

co-efficients) measured by the slope of the Ferguson relationship are presented in Table 3-1 and Table 2-14 for D. obscura. Statistically, there is no significant difference among Kr values obtained for trehalases from melanogaster subgroup species. This is true even for the abdominal specific and thoracic trehalase from D. erecta although the Ferguson plots of these forms intersect at 0% T ($F_{0.05[1,6]} = 5.99 > F_s = 1.99$). Statistical analysis of the retardation co-efficients, Kr, of the tissue specific forms from D. obscura indicate that they are charge isomers ($F_{0.05[1,6]} = 5.99 > F_s = 1.60$). The differences in Kr, if real, would correspond to a molecular weight of 83,000 and 68,000 for abdominal and thoracic trehalase from D. erecta and 73,500 and 62,000 for the corresponding forms in D. obscura. The molecular independence of the two forms in D. erecta is further supported by the inability of pancreatic lipase and repeated freeze-thaw treatments to convert the two forms into a single common band.

(iii) Physicochemical parameters

A summary of physicochemical parameters including Km, Vmax, and temperature lability are presented in Table 3-1 for the six sibling species which represent the melanogaster subgroup of Drosophila.

2. Genetic variation

(i) Individual electrophoretic assessment

No variation in electrophoretic mobility was evident in individuals sampled from 31 strains of D. melanogaster, 8 of D. simulans, 2 of D. erecta and D. mauritiana, 1 of D. teissieri and D. yakuba on MBS 4014.10 XIII pH 8.24 when 8% T, 9% T, or 10% T gels were employed.

Table 3-1. Summary of physicochemical parameters for trehalase from melanogaster subgroup species.

Species/Strain	Kr ± Sb	Critical Temp	% Activity	Km^2 ± Sb	Km^3 ± Sem	Max Cat. Act.[4] ΔE/min ± Se.	Protein mg/ml
D. melanogaster							
Oregon R	0.0558±0.0011	50	43.97±1.19[1]	0.715±0.055	0.661±0.099	0.0544±0.0048(0.262)[5]	2.74
Ken Ting	-	50	44.93±2.44	0.690±0.058	0.544±0.069	0.0445±0.0036(0.215)	2.45
Wageningen	-	50	28.78±1.80	0.702±0.066	0.645±0.092	0.0573±0.0051(0.276)	2.02
Kuala Lumour	-	50	29.67±0.25	0.750±0.055	0.693±0.005	0.0498±0.0144(0.240)	2.21
Guam	-	50	23.48±0.80	0.694±0.055	0.622±0.015	0.0550±0.0208(0.265)	2.53
D. simulans							
Cook Islands	0.0571±0.0023	45	60.18±3.43	0.734±0.040	0.627±0.010	0.0586±0.0040(0.283)	2.27
Neuva, Ca.	-	45	39.81±1.19	0.789±0.042	0.731±0.017	0.0720±0.0045(0.374)	2.32
D. mauritiana							
Strain A	0.0571±0.0023	45	39.47±1.71	0.857±0.052	1.054±0.261	0.0514±0.0038(0.248)	2.37
Chaland	-	45	42.28±1.34	1.189±0.059	1.061±0.037	0.0395±0.0022(0.191)	2.75
D. erecta (thorax)							
Strain A	-	50	9.01±0.69	-	-	-	-
Bafout	0.0554±0.0019	50	28.36±1.89	0.674±0.031	0.711±0.011	-	1.09
D. erecta (abdomen)							
Bafout	0.0626±0.0011	45[3]	-	-			
D. teissieri	0.0562±0.0020	50	42.10±1.56	1.031±0.174	0.999±0.111	0.0466±0.0092(0.225)	2.28
D. yakuba	0.0562±0.0020	50	49.24±2.38	1.028±0.073	0.900±0.115	0.0523±0.0093(0.252)	2.46

1) S.E.M. three replicates
2) Woolf Augustinsson Hofstee ± Standard error of regression
3) Lineweaver-Burke ± S.E.M. 2 replicates
4) Change in absorbance/min from W-A-H plots
5) value in ( ) = μMoles trehalose/min/ml

At pH 5.76 (MBS 3017.0 VI) minor differences in electrophoretic mobility could be detected between two groups of five laboratory strains of D. melanogaster. Ken Ting and Kuala Lampur strains were characterized by a band with relative mobility equal to 0.4629 ± 0.0105 whereas Oregon R, Wageningen, and Guam strains had a mean relative mobility of 0.4853 ± 0.0109. The difference is not statistically significant by parametric tests (Student-t, $t_s = 3.58 > t_{0.05} = 1.76$, 14 d.f.). However, the electrophoretic mobility of Ken Ting and Kuala Lumpur strains was consistently less than the three remaining strains. Nevertheless, it is uncertain whether the observed difference reflects the presence of alterations in the trehalase molecule, or is related to minor differences in protein concentration or ionic strength of these samples. In fact, the electrophoretic grouping of these strains does not correspond to that obtained by thermal denaturation analysis (Table 3-1). As the differences in heat stabilities can be shown to segregate according to expected Mendelian ratios, it is concluded that the difference in electrophoretic mobility probably does not reflect genetical variation of trehalase in this case. Although small sample sizes were employed (8-30 individuals), no electrophoretic variation could be detected on MBS 4014.10 among individuals representing the obscura subgroup (i.e. D. pseudoobscura, D. persimilis, D. miranda, D. obscura, D. subobscura), the takahashii subgroup (D. lutea, D. paralutea, D. trilutea, D. takahashii, D. pseudotakahashii, D. prostpennis), the sibling species (D. americana, D. montana, D. virilis), the sibling species D. arizonensis and D. mojavensis, and the following species D. mulleri, D. quadralineata,

D. nitidithorax, D. robusta, D. argentostriata, D. hypocasta, D. pararubida, D. lebanonensis, D. lebanonensis castelli and D. immigrans. The inability to detect a single electrophoretic variant within these species implies that the electrophoretic trehalase monomorphism is widespread in the genus Drosophila.

(ii) Thermal denaturation

Table 3-2 shows the results of a preliminary experiment to determine the influence of temperature on trehalase inactivation as a function of time. Based on these results, a 10 min heat application period was selected for screening populations for genetic variants. Table 3-3 summarizes the results of heat denaturation experiments for mass homogenates of 12 strains of D. melanogaster. As shown in this table these strains can be potentially separated into three groups when samples are incubated at 50°C for 10 min. Six strains retain an average of 44.54 ± 1.36 percent activity, while 4 are characterized by 28.81 ± 1.02 percent activity. The strain from Agana, Guam probably represents a third class with 23.48 percent activity while that from Crimea probably represents a mixture of alleles.

Expected means for heat stability for individuals from crosses between Oregon R (44%) and Wageningen (29%) are presented in Table 3-4. The results of this analysis are shown in Figure 3-5. Although it is not possible to differentiate individual heterozygotes from homozygotes in either backcross the data clearly indicate a shift in the mean of the distribution of individuals assayed which is consistent with an interpretation of a single genetic locus with two co-dominant alleles coding these enzyme forms.

Table 3-2. Residual activity of trehalase (D. melanogaster) as a function of temperature and time.

Time (min)	Temperature				
	35	40	45	50	55
5	108.25±3.99[1]	103.81±0.48	99.37±0.81	68.41±0.45	16.35±0.23
10	106.98±2.73	102.38±2.72	90.48±1.78	43.97±1.19	3.02±0.45
15	104.44±0.22	93.65±1.75	83.49±1.25	29.21±0.81	1.75±0.59
20	100.00±1.78	95.56±1.19	80.16±0.81	21.43±0.67	1.67±0.24

1. S.E.M. three replicates

Table 3-3. Summary of percentage of residual trehalase activity following incubation for 10 min.

Strain	Electrophoretic Mobility Class		Residual Activity at Temperature				
	4014 pH 8.25	3017 pH 5.76	35	40	45	50	55
Oregon R	0.56	0.49	106.98±2.73[1]	102.38±2.72	90.48±1.78	43.97±1.19	3.02±0.45
Ken Ting Taiwan	0.56	0.46	97.28±3.50	88.95±3.12	90.40±1.79	44.93±2.44	7.25±0.22
Samarkand	0.56	-	124.37±5.73	129.05±6.36	103.05±6.36	45.80±1.25	8.36±2.63
Swedish-b-6	0.56	-	114.03±2.35	114.42±2.34	97.30±0.73	42.23±0.99	3.31±0.99
Virginia-1	0.56	-	105.43±2.35	97.97±1.36	79.71±4.03	43.12±2.28	4.89±1.17
Hikone R	0.56	-	100.73±4.19	94.93±2.71	84.78±5.56	46.02±2.96	2.72±1.17
Kuala Lumpur	0.56	0.49	122.90±4.30	107.33±3.20	82.90±2.61	29.67±0.25	2.44±0.25
Wageningen	0.56	0.46	123.09±3.61	118.49±0.31	92.88±2.84	28.78±1.80	3.47±0.08
Mysore, India	0.56	-	110.82±3.74	96.56±5.47	83.68±3.16	27.15±0.24	1.03±0.73
California-2	0.56	-	123.87±3.28	116.46±3.28	89.71±4.29	29.63±0.50	2.06±1.16
Crimea	0.56	-	90.78±6.25	118.37±2.97	88.36±2.74	32.44±2.25	2.66±0.91
Agana Guam	0.56	0.46	101.69±2.66	93.13±5.65	77.04±0.84	23.48±0.80	1.43±0.66

1 Standard error of 3 determinations

Table 3-4. Expected percentage of residual trehalase activity in individuals from crosses:

CROSS PERFORMED	EXPECTED OUTCOME
OREGON R (44%) x WAGENINGEN (29%)	F_1 (37.5%)
F_1 (37.5%) x WAGENINGEN (29%)	1/2 (29%):1/2 (37.5%)
F_1 (37.5%) x OREGON R (44%)	1/2 (37.5%):1/2 (44%)

Figure 3.5. Histogram showing percent activity of trehalase remaining in individual flies following heating. Crosses analyzed include: Oregon R X Wageningen; F_1 X Wageningen; F_1 X Oregon R.

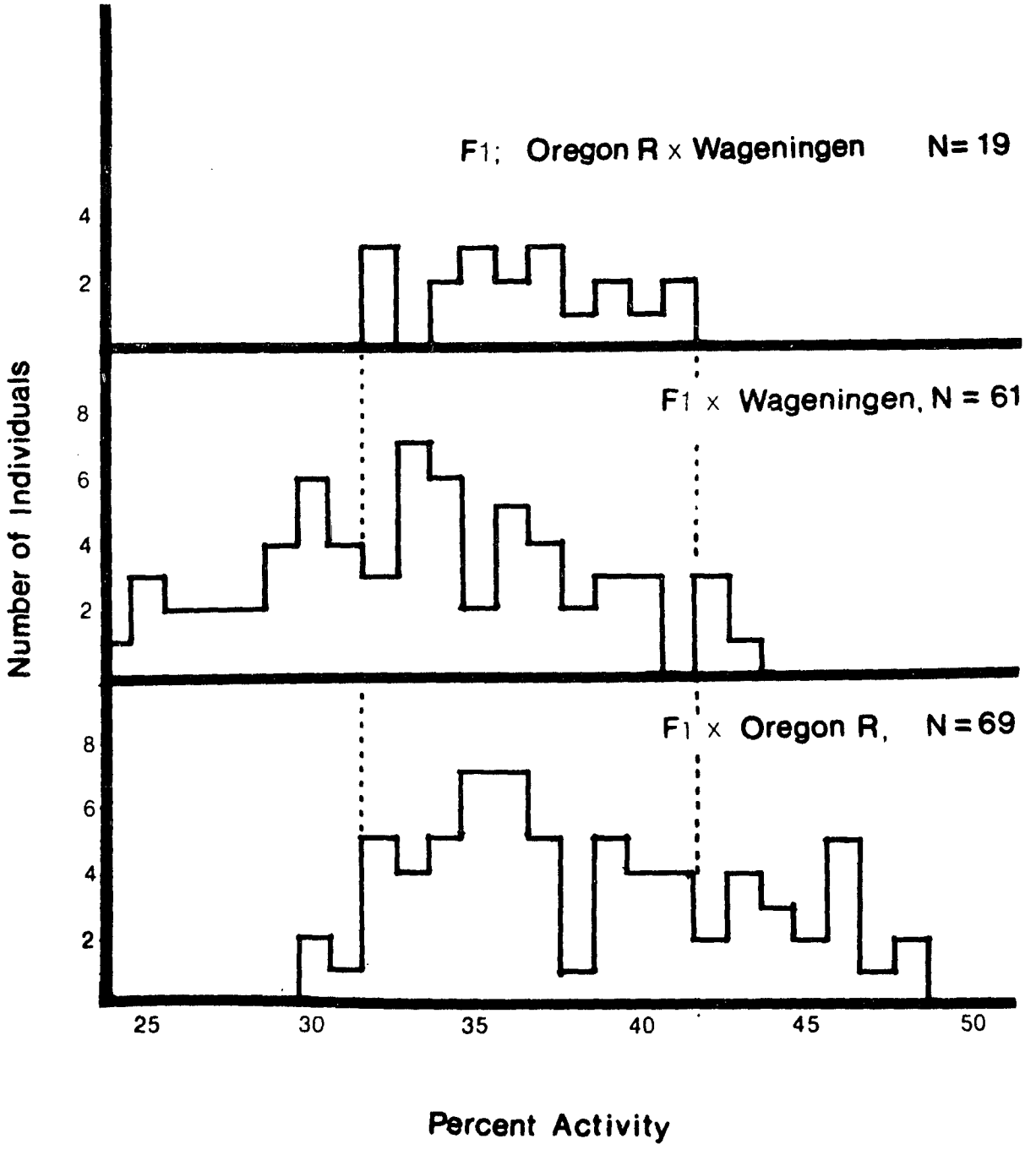

F1; Oregon R x Wageningen N= 19
F1 x Wageningen, N = 61
F1 x Oregon R, N=69
Number of Individuals
4
2
8
6
4
2
8
6
4
2
25
30
35
40
45
50
Percent Activity

Figure 3-5

Table 3-5 indicates the residual activity of trehalase for 4 strains of D. simulans. Although it may be possible to group three of four strains, i.e. Cook Is. Lima Peru, and California-5 into a heat class retaining a mean 57.23 ± 2.63 percent activity as opposed to the 39.81 percent activity for the Neuva sample, the variance among samples perhaps indicates that these strains are not fixed for a particular allele. This contention is supported by the replicate of Cook Is. population sampled at a later time which indicated 45.44 ± 0.90 percent activity. A comparison of residual activity for two strains of D. mauritiana are presented in Table 3-1. These data indicate a similarity between the Chaland and strain A populations and are similar to the Neuva strain.

(iii) Michaelis constants

Estimates of Km obtained from kinetic plots are presented in Table 3-1 for five strains of D. melanogaster representing the three proposed thermal stability classes. The differences observed in Woolf-Augustisson-Hofstee plots are not statistically significant ($p < 0.05$) (comparison of slope -Km of regression V/[S] vs V). Included in Table 3-1 are Km values estimated from Woolf-Augustisson-Hofstee plots for other members of the D. melanogaster subgroup.

(iv) Maximal catalytic activity

Estimates of maximal catalytic activity, Vmax, for strains representing D. melanogaster, D. simulans, D. mauritiana, D. teissieri and D. yakuba are presented in Table 3-1. No data is presented for D. erecta because the sample concentration (wet weight tissue homogenized)

Table 3-5. Summary of residual activity of D. simulans strains following 10 min incubation.

Strain	Residual Activity at Temperature				
	35	40	45	50	55
Cook Islands (1)	96.82±2.95[1]	81.93±4.07	60.18±3.43	10.31±2.47	5.47±2.54
Lima, Peru	89.13±2.31	75.62±3.34	57.71±1.30	10.57±0.36	2.64±0.62
Neuva, California	102.87±2.38	85.56±0.40	39.81±1.19	2.87±0.10	1.06±0.15
California 5	108.08±0.36	92.17±1.29	53.79±0.62	9.85±1.07	1.26±0.36

1 Standard error of three determinations

was much less than that of the remaining species. In addition, Clarke et al. (1979) have indicated that the amount of Adh in D. melanogaster is allometric with body weight, rather than strictly proportional to it. Differences and/or similarities in Vmax reported in this table must therefore be interpreted cautiously as they do not necessarily represent differences or similarities in enzyme structure. Recall that $Vmax = Kp\ [E]_t$ for a single substrate reaction, where Kp is a rate constant and $[E]_t$ is the concentration of active sites. Therefore, any factor which alters the concentration of catalytic sites would change the value of this parameter. Increases in concentration of enzyme due to the differential transcription or translation of the regulatory gene could not be distinguished from changes in enzyme structure which modify the rate constant Kp thus changing enzyme turnover.

IV. DISCUSSION

There are three remarkable features of the trehalase enzyme locus in Drosophila. The first is the presence of species-specific forms which follow the taxonomic relationship proposed for this group by Lemeunier and Ashburner (1976) (Figure 3-6). The second is the lack of any electrophoretically detectable genetic variation within any of these six sibling species but the presence of genetic variants detected by thermal denaturation studies. The third is the pattern of relationship among kinetic constants, electrophoretic mobility and other physicochemical parameters.

Prior to a discussion and interpretation of these data with respect to the genetics of species-specific forms and the amount and significance of the genetic variation at this locus, the properties of the multiphasic buffer systems employed in this investigation will be discussed in terms of their ability to detect genetic variation among proteins.

1. Sensitivity of Electrophoretic Procedure

The properties and theoretical foundation for electrophoresis in MBS are described fully by Jovin (1973a,b,c) and Chrambach et al. (1976), no hence detailed attempt will be made here to explain in these aspects. The electrophoretic resolution characteristic of MBS on polyacrylamide gels is the result of two separate but co-ordinated phases. The first termed "stacking" ensures that all proteins present in the sample are concentrated in a thin starting zone. This is effected by virtue of the electrophoretic mobility of the proteins with

Figure 3.6. Proposed scheme (Lemeunier and Ashburner 1976) showing the phylogenetic relationship between the six sibling species of the melanogaster subgroup.

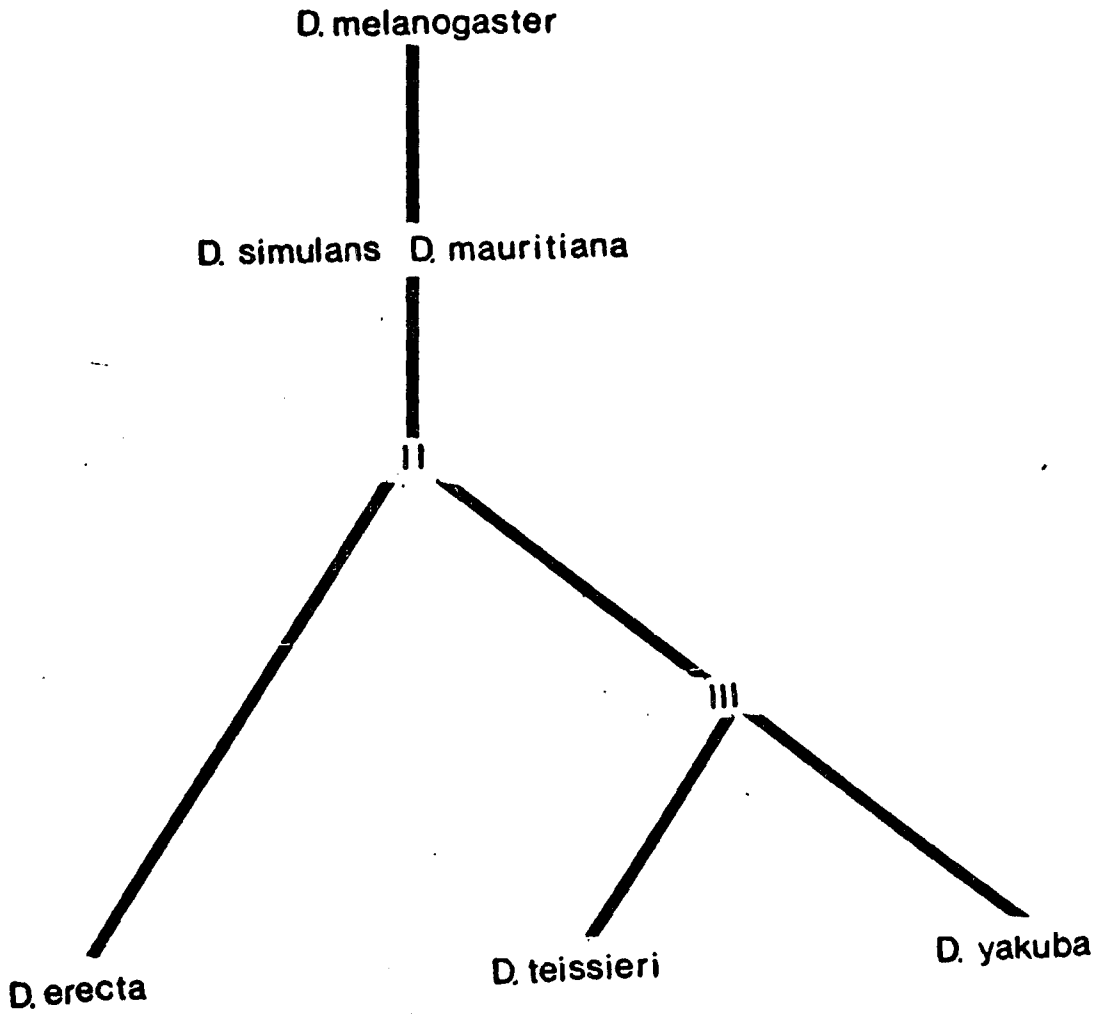

D. melanogaster
D. simulans
D. mauritiana
II
III
D. erecta
D. teissieri
D. yakuba

Figure 3-6

respect to the mobilities of the buffer constituents comprising the "stacking gel". Separation and resolution of these bands is achieved during the second stage, termed "unstacking". This entails removal of proteins from the original thin starting zone (stack) and can be achieved by two means; the effects of molecular sieving and/or by alteration of relative constituent mobilities. Unstacking by molecular sieving results from restrictions on the mobility of the protein imposed by the effects of pore size of the acrylamide gel on the molecule's mean geometrical radius (proportional to Kr). Pore size in acrylamide gels is a complex and not fully understood function of % T (percentage total acrylamide) and % C (portion of crosslinking reagent, e.g. Bisacrylamide or DATD/total acrylamide). Unstacking by relative constituent mobility results when the mobility of the trailing constituent in MBS is greater than that of the protein component in the separation gel. Proteins will remain stacked if their mobility exceeds or equals that of the trailing constituent in the separation phase (phase π) but is less than the mobility of the migrating constituent in the electrophoretic phase which physically preceeds the separation phase (i.e. phase λ).

In the above context, it should be stressed that true "charge isomers" (i.e. proteins which differ only in charge not geometric radius) can be detected only in buffer systems where unstacking is achieved by relative constituent mobilities rather than pore size of the supporting medium. Alterations of polyacrylamide gel concentrations are effective in resolution only if proteins differ in mean geometrical radius (estimated by Kr in Ferguson plots). Therefore, to ensure resolution of the maximum number of potential charge variants, proteins

should be unstacked by relative constituent mobilities in preference to pore size. Allelic variants resulting in alterations in both charge and molecular size can be resolved by either unstacking procedure.

The utilization of MBS over a range of separation pH increases the probability of detecting genetic variability. There are a number of reasons for this. Electrophoresis at low pH effectively ensures the partial ionization of all six charged amino acids. This is not feasible in systems above pH 9.0 (Table 3-6). Perhaps of greater significance, is the ability to detect partial charges at lower pH. A partial charge results from the incomplete ionization of an amino acid due to the interaction of pH and pka. Of the several physical reasons which result in partial charges, the effect of neutral amino acid substitutions on the pka of a charged amino acid are most interesting and are only detectable in carefully designed electrophoretic experiments. For example, neutral substitutions which affect the pka of histidine by $\pm$ 1 unit could only be detected at pH 6.0 where they could result in a 1% difference in electrophoretic mobility if that single residue accounted for 1/40th of the protein charge at this pH (because difference in charge of protein by alteration in pk is 40%). Clearly the optimal strategy to detect all such partial charge effects would be to perform electrophoresis at the mean pka of all six charged residues. Obviously this is not feasible for a number of reasons. Over the range of pH utilized in this study such partial charges could be detected (Table 3-6) to some extent for aspartic, glutamic acid at pH 5.76, histidine at pH 6.0, tyrosine and lysine at pH 8.55. It would

Table 3-6. Percentage ionization of amino acids ± 1 pka as a function of separation pH.

Amino Acid		pH 5.76	6.05	7.00	8.07	8.24	8.44	8.55	8.60	9.00
aspartic	2.9	99.86	99.93	99.99	99.99	99.99	99.99	99.99	99.99	99.99
	3.9*	98.64	99.30	99.92	99.99	99.99	99.99	99.99	99.99	99.99
	4.9	87.87	93.39	99.21	99.93	99.95	99.97	99.98	99.98	99.99
glutamic	3.3	99.63	98.82	99.98	99.99	99.99	99.99	99.99	99.99	99.99
	4.3*	96.65	98.25	99.80	99.98	99.99	99.99	99.99	99.99	99.99
	5.3	74.25	84.90	98.04	99.83	99.89	99.93	99.94	99.95	99.98
hidtidine	5.0	14.81	8.18	0.99	0.09	0.06	0.04	0.03	0.03	0.01
	6.0*	63.47	47.12	9.09	0.84	0.57	0.36	0.28	0.25	0.10
	7.0	94.56	89.91	50.00	7.84	5.44	3.50	2.74	2.45	0.99
tyrosine	9.9	99.99	99.99	99.87	98.54	97.86	96.65	95.72	95.23	88.88
	10.9*	99.99	99.99	99.99	99.85	99.78	99.65	99.56	99.50	98.76
	11.9	99.99	99.99	99.99	99.99	99.98	99.97	99.96	99.95	99.87
lysine	9.8	99.99	99.98	99.84	98.17	97.32	98.82	94.68	94.96	86.31
	10.8*	99.99	99.99	99.98	99.81	99.73	99.57	99.44	99.37	98.43
	11.8	99.99	99.99	99.99	99.98	99.97	99.56	99.94	99.94	99.84
arginine	11.5	99.99	99.99	99.99	99.96	99.95	99.91	99.89	99.87	99.68
	12.5*	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.96
	13.5	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99

1. pka values with asterik taken from Stryer 1975 $\phi = 1/[1 + 10^{\pm(pka-pH)}] \times 100$

be impossible to distinguish partial charge effects on arginine (always totally charged) and due to the similarity of pKa of tyrosine and lysine it would be difficult to distinguish tyrosine-lysine substitutions without the co-inducation of other conformational changes.

The utilization of a pH range has other intersting implications. Preliminary data indicate that trehalase is maximally charged at pH 8.49 (Table 3-7). The apparent decrease in negative charge at higher pH is unexpected (Table 3-6) and perhaps indicates a change in protein conformation which can be induced over a rather narrow pH interval. Such a conformational change could result in the masking or detection of amino acid substitutions, or could alter the mean geometrical radius (Kr) of the protein. This latter consideration indicates the possible existence of experimental errors in procedures which rely only on gel sieving procedures and do not strictly control separation pH.

A comparison of the percentage ionization of the six charged amino acids over the pH interval utilized in this study with the 7.0 - 9.0 pH range utilized in other investigations indicates that the gel sieving and unstacking procedures employed here are at least as powerful as those utilized by other investigators who have detected significant increases in genetic variability. Therefore, the apparent electrophoretic monomorphism of trehalase cannot be ascribed to the inability of the electrophoretic procedure to detect such variation although that it is evident from the preceeding discussion and Table 3-6 that a broader range of pH could detect more partial charge effects and hence a greater number of alleles.

Table 3-7. Maximum charge of trehalase as determined by electrophoresis on non-restrictive polyacrylamide gels

	Mutiphasic buffer system							
	3017		4014					
$\bar{r}_1^{\pi 1}$	-0.125	-0.197	-0.193[3]	-0.214	-0.235	-0.256	-0.298	-0.319
pH	5.76	6.2	7.98	8.07	8.15	8.24	8.44	8.55
$\bar{r}_m^{Na^+}$ [2]	-0.089+0.001	-0.119+0.001	-	-0.206+0.003	-0.208+0.004	-0.217+0.002	-0.221+0.002	-0.206+0.001
% maximum charge	40.27	53.85	-	93.21	94.12	98.19	100	93.21

1. $\bar{r}_1^{\pi}$ relative mobility to sodium of constituent 1 in separation phase π
2. $\bar{r}_m^{Na^+}$ relative mobility to sodium of trehalase; determined by multiplying relative mobility of trehalase x $\bar{r}_1^{\pi}$.
3. trehalase remains stacked in nonrestrictive gels at this pH.

2. Species Differentiation

Three hypotheses may be generated to explain the pattern differentiation of trehalase electromorphs among members of the melanogaster subgroup. The first maintains that the generation of species specific forms results from the fixation of alleles at a single homologous genetic locus. A second, suggests the presence of distinct genetic loci and regulatory elements resulting in the production of species specific isoenzymes. A third, raises the possibility of differential post-transcriptional or post-translational modification of a common enzyme transcript or molecule in these species (c.f. Finnerty et al. 1979, Finnerty and Johnson 1979, Cochrane and Richmond 1979).

The likelihood of the third alternative is reduced by the qualitative observation of approximately equal staining intensity of both electromorphs in D. melanogaster by D. simulans hybrids. This observation necessitates the codominant action of any post-transcriptional or post-translational genetic modifiers, a mechanism which is difficult to envision at the molecular level.

Although, all electorphoretic forms of trehalase from the melanogaster subgroup are related as charge isomers, the conclusion that these forms necessarily represent a series of alleles at a single genetic locus is premature. A detailed consideration of the data presented is also consistent with the second interpreation. Therefore in reality, the species differentiation of trehalase may be the result of the fixation of alleles and the differential expression of isoenzymes

coded by distinct structural loci.

A first line of evidence supporting a non-allelic interpretation for these species specific forms arises from a consideration of the multiple forms of trehalases in D. erecta and D. obscura. The specificity of the tissue distribution of these forms, their presence in larval and adult stages, their relationship as charge isomers, and the inability to interconvert the two forms with lipase incubation, or freeze-thaw cycles supports an isoenzymic interpretation for these two enzyme forms. This isoenzymic interpretation implicates the existence of two separate structural gene loci coding trehalase in thse two species.

A second line of evidence which supports an isoenzymic explanation for the species differences or at least a combination of the allelic and isoenzymic hypotheses involves the genetic and biochemical characterization of the species-specific forms.

The molecular independence of species specific forms draws support from two sources: First, the lack of interspecific overlap in mobility of trehalase forms, coupled with the presence of allelic variation in each electrophoretic form as detected by thermal denaturation. Second, the ability to organize members of the melanogaster subgroup into classes representing their thermal labilities and Michaelis constants. For example, a consideration of critical temperatures used to detect thermal allelic classes can differentiate D. simulans, D. mauritiana and possibly the abdominal form of D. erecta from the remaining melanogaster subgroup species. Similarly, estimates of Michaelis constants, Km, can be used to separate D. teissieri and D. yakuba from the remaining species.

It is also possible to separate D. mauritiana from D. simulans by this criteria. The difference in Km perhaps indicates that not all structural differences in trehalase are revealed by electrophoretic or heat denaturation techniques. However, it should be noted that Km may be expected to be the least reliable indicator of differences in enzyme structure for two reasons. The first is due to the sensitivity of this parameter as a complex function of rate constants to modification by chemical parameters and regulatory molecules, and secondly this parameter could be expected to be the focus of normalizing selection if the enzyme were selectable. In this context, the similarity of this parameter within and between species is perhaps more significant because it indicates that the trehalase enzyme molecule can be modified without significant alterations in catalytic function (e.g. D. simulans, D. melanogaster, D. erecta thorax). Therefore, if different electrophoretic forms were allelic products, one might reasonably expect to find populations of one species group which express the trehalase electromorph of another species group. The inability to detect such overlapping interspecific variation, the presence of genetic variants within species-specific forms, and the characteristic physicochemical properties of species-specific electromorphs, is consistent with the isoenzymic hypothesis.

Based on these data, it is suggested that there are at least two distinct genetic loci coding trehalase in the melanogaster and obscura subgroups and that the expression of species specific trehalase forms likely involves a combination of the differential expression of these loci and the fixation of electromorphs at homologous loci within species

populations.

This genetic hypothesis can be tested directly by the localization of species-specific genes in the Drosophila genome with a suitable molecular probe, similar to those recently employed in quantifying the number of immunoglobulin genes (Siedman et al. 1978) and the organization of the ovalbumin gene (Royal et al. 1979). If the interpretation presented in this study is corroborated by such techniques, then the genetic divergence of these species favors an interpretation which stresses the importance of regulatory genes in speciation.

3. Genetic variability of trehalase locus

Given that the apparent power of the electrophoretic techniques employed in this investigation, to be equal to those utilized by other investigators, the total number of alleles found at the trehalase locus deviates somewhat from the bimodal pattern observed by Coyne and Singh (available data are summarized in Table 3-8). If the distribution of enzymes is bimodal with respect to genetic variability, then one would predict that an electrophoretically monomorphic locus such as trehalase would remain essentially monomorphic when other biochemical procedures are employed. Although, the increase in variability is perhaps not as striking as the XDH or Est-5 data, the electrophoretically homogeneous trehalase locus was shown to contain three alleles, two common and one rare in a sample of only twelve inbred laboratory strains of D. melanogaster when thermal denaturation was investigated. This represents a three-fold increase in the number of alleles which is the typical average increase reported by Coyne for polymorphic loci in

Table 3-8. Increase in the number of alleles at enzyme loci with the application of systematic biochemical procedures.

Data Source[2]	Locus	Previous	Current	Increase
Coyne[1]	Est-5	12	30	3.5
	Adh-6 (Ao)	7	16	2
	Xdh	8	37	4.6
	Odh	7	8	-
	Hk-1	2	2	-
	Mdh	2	2	-
	αGpdh	1	1	-
Singh (1979)	Adh-6 (Ao)	4	16	4
	Pt-7	4	9	2
	Pt-8	4	6	1.5
	Pt-10	3	6	2
	Pt-13	3	10	3.3
	Est-5	13	32	2.5
	Est-6	3	9	3
	Xdh	6	37	6

1. compilation of data presented at annual meeting of Evolution Society at Boulder, Colorado, June 1979.

2. D. pseudoobscura only.

Table 3-8. The data of Singh (1979) in the lower half of this Table indicate a more even distribution in the average increase of the number of alleles then a number of separation criteria are employed but is somewhat less than the six heat sensitive alleles per electrophoretic allele at the Odh locus reported by Sing, Hubby and Trockmorton (1975). It should be point out however, that despite the increase in the number of alleles at the trehalase locus, it still remains relatively monomorphic but as noted previously this may be a consequence of the small number of lines sampled.

The presence of two common and one rare allele at the trehalase locus in D. melanogaster contradicts the general pattern at most enzyme loci where one common and several rare alleles are observed (Li 1978, Ohta 1976). If one accepts that the general pattern is due to the slightly deleterious affects of most rare alleles (Ohta 1976) then following a consideration of the similarity of the physicochemical parameters for the three trehalase alleles (Table 3-1), it is evident that similar forces are not in operation at the trehalase locus.

There is good agreement between the number of alleles found at this locus with that predicted by the relationship between subunit molecular weight and the average number of alleles per locus in Drosophila reported by Koehn and Eanes (1978). From the regression, $Y = 2.20704 \pm 10^{-4} X$ (Figure 1, page 61 of Koehn and Eanes) the predicted number of alleles for an enzyme with subunit MW 70,000 daltons is 3.01. However, it is likely that the number of alleles currently found will increase somewhat with an increase in sample size. Also the data base utilized by Koehn and Eanes may represent techniques which are less sensitive in detecting electrophoretic variation.

The failure of the electrophoretic procedures to resolve the differences in thermal stability of trehalases in D. melanogaster is in part consistent with the expectations of the step-wise mutation model. Although, the amino acid substitutions responsible for the thermal effects are unknown at present, they do not alter electrophoretic mobility and hence cannot be detected as unit or partial charges. However, as described above, the electrophoretic procedures utilized in this investigation are not expected to be able to resolve all possible amino acid substitutions although it is readily apparent that the systems are capable of detecting some non-unit charge effects. This consideration indicates that the conclusion of Coyne et al. (1978) that the appearance of polymorphic and monomorphic classes is "not simply a sampling artifact of the restricted techniques previously used" is perhaps premature until more electrophoretic and physicochemical techniques are performed on all allelic classes which are capable of detecting more types of amino acid substitutions. Coyne's result, for example, may arise because performing electrophoresis at pH 7.0 may have detected more variants in Xdh than Mdh due to the presence of critical histidine residues in Xdh which are not present in the Mdh molecule. Perhaps aspartic acid (pK 3.9) is the critical amino acid residue in Mdh and as seen in Table 3-6, electrophoresis at pH 7.0 would fail to detect any partial charges in this molecule. Nevertheless, although it may be possible to resolve all possible amino acid substitutions by carefuly electrophoretic experiments, it may be impossible for technical reasons. This observation supports the use of theoretical population genetic models which combine the infinite and stepwise mutation model (e.g. Li 1976, see also Fuerst and Ferrel 1979,

Ramshaw, Coyne and Lewontin 1979, Johnson 1974), however care must be exercised when such models are applied to experimental data if the physicochemical procedures employed do not have equal likelihood of detecting genetic variation in all proteins.

4. Maintenance of trehalase alleles

The pattern of genetic variation at the trehalase locus leads to two interrelated questions. The first involves the role of natural selection in the maintenance of the alleles detected by thermal denaturation, the second with the nature of the factors leading to the lack of electromorphs within the genus Drosophila.

(i) Role of natural selection

The concept of conditional neutrality proposed in Chapter 1 represents an attempt to describe the compensatory mechanisms which arise as a consequence of the existence of a "selective unit" which has other components besides the enzyme locus in question. The existence of such components would effectively lead to the shielding of allozymes with altered physicochemical properties. Based on the correlation between hemolymph concentration and duration of flight in Diptera, and the apparent relationship between enzyme function (as measured by kinetic constants) and the efficiency and limits of substrate conversion, it can be argued that the enzyme trehalase is part of a larger selective unit involving the utilization of trehalose, and is in principle selectable. The Michaelis constant, Km, and maximal catalytic activity, Vmax, are expected to be critical parameters in trehalase evolution for these kinetic constants ultimately

define the physiological limits of trehalose utilization.

The similarity of kinetic constants for the three allozymes in D. melanogaster (Table 3-1) indicates that under the assay conditions tested to date the forms are physiologically equivalent and therefy precludes their selective discrimination. Hence they are selectively neutral. Consequently, the frequency of the three alleles would be determined entirely by non-selective forces or linkage relationships to selectable genes. The causal basis for the existence of the rare allele at this locus cannot be ascribed to supposed deleterious properties (c.f. Ohta 1976, Li 1978). The selective neutrality of the two proposed alleles in D. simulans can be established by similar arguments.

The overall similarity in physicochemical parameters determined for closely related species in the melanogaster subgroup of Drosophila, contrasts the widespread differences reported by Danford and Beardmore (1979) for the esterase-6 locus. Morgan (1975) for Adh and Miller et al. (1975) for α Gpdh in D. melanogaster which may under appropriate conditions form the basis for differential selection of these forms. The similarity of Km and Vmax for the three alleles detected by thermal denaturation and the lack of electrophoretic alleles in D. melanogaster found in this study agrees with the prediction of Bernstein et al. (1973) that selection is unable to differentiate heat sensitive alleles, and supports the contention of Finnerty and Johnson (1979) that molecules with similar shape (Kr) would retain similar enzyme activities.

Therfore, it would appear that the similarity in physicochemical properties in alleles detected in D. simulans and D. melanogaster

implicates the action of purifying selection at this enzyme locus in Drosophila. This would explain the apparent lack of electrophoretic alleles and alleles which differ in physicochemical properties at this locus and possibly the electrophoretic monomophism.

Given the apparent selective neutrality of heat sensitive alleles at the trehalase enzyme locus in D. melanogaster and D. simulans it is tempting to accept the generalities proposed by Bernstein et al. (1973) and Finnerty and Johnson (1979). However, there are a number of difficulties associated with these generalities.

First, the selective neutrality of heat sensitive alleles based on their greater frequency when compared to electrophoretic alleles (Bernstein et al. 1973) depends upon the tacit assumption that the mutation rate leading to the production of electrophoretic alleles is identical to the rate of production of heat sensitive alleles.

Secondly, it is not apparent why electrophoretically distinguishable alleles (in contrast to heat labile alleles or alleles with similar Kr) would necessarily have altered physicochemical properties and consequently be removed by the action of purifying selection. The tenability of these generalizations is questioned when one considers the properties of trehalase in the six sibling species which comprise the D. melanogaster subgroup. Although, the genetic relationships between these species-specific forms have not been definetly established, that is, whether they represent allozymes or isozymes (section 2 of this Discussion) it is interesting to note that the electrophoretic forms characterizing D. melanogaster, D. simulans and D. erecta although differing in electrophoretic mobility are identical with respect to Kr and Km. D. melanogaster and

D. simulans are also identical with respect to pH optima (Figure 2-4). These data illustrate that enzymes with different electrophoretic mobility may also be physicochemically and hence physiologically and selectively equivalent. The possibility of selective differentiation of heat sensitive alleles is illustrated by the properties of the enzyme form obtained from D. simulans and D. mauritiana. Although identical in electrophoretic and thermal properties the D. simulans form is readily distinguishable kinetically from D. mauritiana. D. mauritiana Chaland differs from D. mauritiana Strain A when Km values based on Woolf-Augustinsson-Hofstee plots are considered but are identical by estimates from Lineweaver-Burke plots. Because W-A-H plots have been shown to better estimate Km than L-B plots (Dowd and Riggs 1965), the difference in Km may be accurate. If the difference in Km is real, then the similarity in heat denaturation and electrophoretic mobility suggest that it may not be related to any structural modification of the enzyme. The apparent difference in Vmax between enzyme forms of D. simulans Cook Islands and Neuva Ca. and D. mauritiana Strain A and Chaland is probably due to sampling error. The extent of these differencesin D. simulans and D. mauritiana, 0.064 and 0.057 μM trehalose/min respectively falls close to the range, 0.074 μM trehalose/min reported for alleles in strains of D. melanogaster. If differences exist, the apparent structural similarity of the enzyme forms, suggests that the differences are due to differences in enzyme concentration and therefore unrelated to the structural locus. Similarly, the differences in maximal catalytic activity between strains of D. melanogaster and D. simulans may form the basis for selective differentiation of trehalase enzyme forms, if related to the structural

locus.

Unfortunately, these questions cannot be answered at present but must await further physicochemical and genetical characterizations and testing at higher levels of organization (e.g. stability of hemolymph trehalose concentrations, duration of flight) before any unequivocal conclusions can be made. However, it is apparent that these differences could form the basis for differential selection or necessitate some form of physiological compensation.

These data argue against the possibility that only heat sensitive alleles at the trehalase locus are undistinguishable by selection (i.e. similarity in Km between D. melanogaster and D. simulans) and also contrast the apparently clear relationship between shape modification and alteration of thermal stability and enzyme activity for multiple forms of Xdh by Finnerty and Johnson (i.e. similarity of Kr and heat parameters of D. melanogaster and D. teissieri but differences in Km and kinetic parameters). Some of the data (D. mauritiana differences) presented are congruent with those of Singh (1979) and Gosling (1979) which indicate the possible action of selection on enzyme forms contained within electromorphs. The possibility that these enzymes may be selectively discriminated on the basis of parameters not related to enzyme structure increases the relevance of the arguments concerning the existence of regulatory polymorphisms.

Therefore, the overall similarity of kinetic parameters of alleles found in D. melanogaster and D. simulans does not necessarily reflect any generalization concerning the action of selective forces at the molecular level, but may reflect the importance of the relationship

of the enzyme to the unit of selection and selective processes in these species. Nevertheless, if one only considers the similarities of physicochemical parameters of intraspecific alleles the action of purifying selection is clearly implicated at the trehalase locus in D. melanogaster and D. simulans.

(ii) Electrophoretic monomorphism

Knowledge that electrophoretically distinct yet physicochemically identical forms of trehalase exist in closely related species, leads to questions concerning the apparent lack of electrophoretic variants within D. melanogaster. Although this differentiation may reflect some as yet undetermined physicochemical parameter, which differs among enzyme forms, no evidence is evident which would support this hypothesis. A possible explanation for the lack of electrophoretic alleles within species, lies in the considerations of Conrad (1977, 1979). Briefly, it can be argued that the trehalase enzyme molecule in Drosophila has a structure which is not amenable to single amino acid substitutions which alter electrophoretic mobility. It can be argued that such alterations disrupt these functional properties of the enzyme which must be maintained within preset boundaries. Presumably, functional equivalence can be restored by further additional amino acid substitutions, but the number of substitutions required to produce electrophoretically distinct yet functionally identical forms makes the transition improbable.

The difference in charge among electrophoretic forms of trehalase can be calculated from the Y intercept in Ferguson plots (Figure 3-2) or directly from the relative mobilities of trehalases on

non-restrictive gels. Trehalase from D. melanogaster exceeds the negative charge of D. simulans by approximately 11% at pH 8.44 and is 16% less charged than the form from D. teissieri and D. yakuba. However, because there is no apparent relationship between number of amino acid substitutions and electrophoretic charge (see Tables 1 and 2 of Fuerst and Ferrell 1979) it is impossible to estimate the number of amino acid substitutions which are responsible for these differences in electrophoretic mobility. In fact, if one expects molecular shape and function to be preserved largely by electrostatic effects (Perutz 1978) then it is futile to even attempt such an analysis.

A consideration of these factors increases the tenability of the hypothesis that the lack of electrophoretic alleles within a given species is the result of "purifying selection". At the molecular level such selection includes all forces which preserve the structure and function of the enzyme within boundaries set by the physiological system. The alleles which do exist within this species cannot be distinguished electrophoretically, have identical kinetic properties and are expected to be physiologically equivalent, thus effectively neutral under the specified assay conditions. This is perhaps a good demonstration of the efficiency and limits of selection in culling alleles at the trehalase locus in D. melanogaster and D. simulans. A physiological corollary of these arguments is that trehalase is the rate limiting step in energy mobilization and consequently largely determines the fitness of the selective unit(s) of which it is a part.

The significance of the possible difference in Km and Vmax in D. mauritiana is uncertain. As mentioned previously, it has not been established that the difference is related to molecular structure, or

that it is genetical in origin. Should these two criteria be met, the data may be indicative of, among other possibilities, a relaxation of selective pressures or the existence of physiological compensation in this species leading to conditional neutrality.

5. Evolution of species specific forms

The existence of species-specific trehalases within the D. melanogaster subgroup can now be explained within the framework of the preceeding arguments. If these forms are isozymes as suggested in section 2 of this Discussion, then it is evident that there is more than a single genetic locus coding trehalase in the Drosophila genome but that only a single locus is expressed in most species at a given time. Such differential expression leading to the presence of "silent" genetic loci, would permit the accumulation of genetic variants, some of which may code for functionally equivalent trehalase molecules with altered electrophoretic mobility. The generation of electrophoretically distinct alleles would depend upon the duration of time that a locus remained "silent" (i.e. the dynamics of genetic variants at regulatory elements controlling expression) and the number of amino acid substitutions required to preserve function and change mobility. Enzyme forms which differ in kinetic properties, e.g. the difference between D. simulans and D. mauritiana, D. melanogaster and D. teissieri, D. yakuba presumably reflect the different physiological requirements of these species. Therefore, the electrophoretic and kinetic differences observed between species are conceivably due to the interaction of genetic drift and selection on both regulatory and structural genes involved in the biosynthesis of this enzyme.

V. SUMMARY

Trehalases in the Drosophila melanogaster subgroup are hypothesized to be coded by at least two structural gene loci, although in the majority of species only one of these forms is expressed. Species differences in electrophoretic mobility, thermal stability, and/or Km and Vmax may be explained by hypothesis which combine the role of differential expression of these loci and the presence of a fixed electromorphs at an expressed locus.

Within species, there is an apparent lack of electrophoretic genetic variation, yet electrophoretic bands in D. melanogaster and D. simulans can be shown to be heterogeneous by the application of thermal denaturation procedures. The difference in thermal stability of two strains of D. melanogaster has been shown to segregate according to expected Mendelian ratios. The similarity of Michaelis constants, Km, of there enzyme forms in five strains of D. melanogaster and two of D. simulans suggest that they are physiologically equivalent, and hence within the constraints of available information are selectively neutral. No statement can be made concerning the differences in Vmax until the genetic basis for this difference has been determined.

Species specific electromorphs may also be identical in Km or pH optima although differing in electrophoretic mobility, or may differ in both Km and electrophoretic mobility.

Based on data which indicated the electrophoretic monomorphism of trehalase, the presence of alleles detected by thermal denaturation, and the physical properties of species-specific forms, it is suggested

that the evolution of trehalases in the melanogaster subgroup has involved the action of natural selection and genetic drift on both regulatory and structural elements.

VI. GENERAL DISCUSSION

The trehalase enzyme locus in Drosophila was chosen to investigate the role of genetic variants in enzymes in evolutionary processes. Conceptually, this investigation was based on the arguments presented in Chapter 1, concerning the hypothesis of conditional neutrality and the mechanisms leading to the selective differentiation of proteins. Briefly, these arguments invoked the interaction of a number of biochemical and physiological processes in the formation of a selective unit of which a particular enzyme is part. The contribution of that enzyme to the unit of selection and hence to fitness was considered to be variable and dependent in part upon its biochemical properties and relationship to other enzymes in the physiological processes. Conditional neutrality was coined as an expression to define the process of physiological and biochemical compensation for differences in the physicochemical properties of the enzyme in question, which would result in the equivalent fitness of the selective units which contained allelic forms of the enzyme. In an experimental context, the question can be posed as whether differences in the physicochemistry of allelic forms of an enzyme are translated into similar differences at a higher level of phenotypic organization where they could be differentiated by selective processes. With this experimental design one could in theory determine the boundaries within which selection could discriminate allozymic substitutions and conversly the extent of the contribution of an enzyme to the unit of selection. Therefore, it would be possible to assess the relative

importance of natural selection and genetic drift in the dynamics of biochemical polymorphisms, at a functional level.

The biochemical pathway concerned with the metabolism of trehalose was selected as a possible system to investigate these possibilities in Drosophila. Interest in this enzyme pathway arose as a consequence of the knowledge that the dissacharide trehalose is the major reserve substance in dipterans and has been shown to be a critical factor in a number of physiological processes, but most notably duration of flight. The enzyme trehalase, involved in the hydrolysis of trehalose, was chosen for study because of the simplicity of the catabolic portion of this pathway (one step) and its obvious relevance in the production of glucose as an energy source. However, it should be noted that this enzyme is not necessarily solely responsible for the relationship between trehalose and flight duration. As described in the literature review, the de novo synthesis of trehalose from glycogen reserves may also play an important role.

Although the work presented here is not complete with respect to the determination of the functional significance of allozymes a number of interesting possibilities have been ascertained. The similarity in Km of allelic enzyme forms detected by thermal denaturation in populations of Drosophila melanogaster and D. simulans is indicative of the likelihood that the alleles code functionally equivalent products and are therefore selectively equivalent or neutral. The kinetic constants, Km and Vmax are expected to be critical parameters in reactions which utilize reserve substances, in that at substrate concentrations much less than Km at a given Vmax, the efficiency of catalytic conversion decreases markedly. Therefore, enzymes with lower

Km but identical Vmax would mobilize a greater proportion of a reserve substance at physiologically useful concentrations. A more complete discussion of this point is presented in Chapter 1.

The present investigation of the trehalase locus in Drosophila has established that:

1) The trehalase enzyme locus is atypical in that no electrophoretically detectable genetic variation was evident in any of the 32 Drosophila species investigated. However, there were marked differences in the electrophoretic mobility of enzyme forms between species, some of which represented groups of sibling species. The presence of multiple forms of trehalase was established in D. quadralineata, D. erecta and D. obscura. The forms in the latter two species were investigated in some detail and were shown to be tissue specific.

2) The presence of trehalase isoenzymes, and sharp discontinuities in the physicochemical properties of species-specific forms are congruent with a hypothesis which implicates the presence of two structural gene loci coding trehalase in Drosophila and maintains that the presence of species-specific forms results from the differential expression of these loci and the fixation of a single electrophoretic form within a given species.

3) It was suggested that the pattern of species-specific differences observed in the melanogaster subgroup of Drosophila may have been the result of the joint effects of genetic drift and selection (within physiological constraints) on regulatory and structural gene loci coding trehalase. This hypothesis cannot be directly tested without the

development of suitable techniques currently employed in molecular genetics.

Future work on the trehalase enzyme locus will involve:

1) The development and utilization of molecular probes to locate, quantify, and determine the relationship between structural gene loci coding trehalase, and if possible the associated regulatory elements.

2) A hypothesis has been proposed which predicts the presence of a number of alleles at a "silent" genetic locus with properties and frequencies different from the expressed locus. The biochemical properties of such alleles at a "silent" locus could be determined if suitable isolation and translational procedures could be developed.

3) The further characterization of alleles present within species such as D. melanogaster or D. mauritiana should permit further statements concerning the selectability of these enzyme forms, and possibly in the determination of the unit of selection. An indication of the possibility of physicochemical differences among the allozymes already found could be determined by determining the effects of allelic substitution on duration of flight, or trehalose utilization in these species.

In any event, the trehalase enzyme locus in Drosophila should provide considerable information concerning the evolutionary dynamics of gene-enzyme systems.

APPENDIX I

1. Statistical Tests

Because of the nature of its formulation, it can be argued that the acceptance or rejection of the neutral gene hypothesis is in reality a statistical problem. In effect, the opposing theories make rather different predictions concerning the overall distribution of allelic frequencies and heterozygosities within and between populations. Because either theory can be modified to account for slight alterations in the observed data sets, the statistical procedure must test the congruence of theory with all or a large number of independent predictions. Obviously, the observation of the selective maintenance of a particular allozyme does not invalidate the hypothesis as such a finding is expected under the neutral gene hypothesis. Conversly, data obtained from a functional approach indicating the physicochemical identity of allelic products cannot form the basis for a rejection of the selectionist hypothesis. These considerations, however, do not invalidate the experimental approach for two reasons. It can be argued that the statistical formulation of the neutralist-selectionist controversy is transcended by the obvious relevance of the outcome to biochemical evolution, and secondly, the experimental approach can provide an indication of the mechanisms leading to neutrality or selection and thereby indicate necessary theoretical modifications in either hypothesis, and assess the likelihood of the results of the statistical test.

A number of statistical tests and testing procedures have been proposed to resolve the neutralist-selectionist controvery. Some, but

not all of these will be reviewed here. This approach is currently the most widely employed as a means of determining the validity of the neutral gene or selectionist theories.

A somewhat qualitative comparison between the predictions of the neutralist and selectionist theories has been performed by Ayala et al. (1972), Ayala et al. (1974) and Ayala and Gilpin (1974) for five Drosophila species of the willistoni subgroup. They report that their data are at variance with certain interpretations of the neutral gene theory which they have derived. Thus, they report that the effective number of alleles predicted by the neutral gene hypothesis with the infinite allele and stepwise model greatly exceeds that actually found in willistoni subgroup. In addition, the distribution of single locus heterozygosities deviates from that which they predict for the neutral gene hypothesis (i.e. normally distributed about the mean). However, at this point it should be noted that the theoretical distribution of single locus heterozygosity is not known (see Stewart 1976, Fuerst, Chakraboraty and Nei 1977), therefore one cannot reasonably accept Ayala's interpretation, although it may be correct. Fuerst et al. (1977) did not observe any statistically significant departure of the distribution of single locus heterozygosity for the willistoni data from their estimated distribution although this may be due in part, to the lack of the power in the statistic employed. Ayala et al. (1974) report that the correlations in allelic frequencies found among widely separated populations and species are at variance with those expected under the neutral gene hypothesis. Similarly, Zouros (1974) has reported that the degree of genetic similarity in the genetic constitution of the mulleri subgroup of Drosophila measured by heterozygosity per locus or

in terms of overall genetic similarity correlated better with the similarity in ecological niche of these species rather than their degree of reproductive isolation. Bulmer (1971) interpreted the correlations in allelic frequencies in D. pseudoobscura as evidence against selective neutrality. Borowsky (1977) has suggested a statistical analysis which describes the degree to which two related species parallel one another in their patterns of geographic variation of allozyme frequencies. He maintains that such parallel patterns observed in the reanalyzed data of Ayala et al. (1974) and McKechnie et al. (1976) are unambiguous proof of selection. The neutral gene theory could explain the observed correlation if gene flow was sufficient to prevent population differentiation but this, as pointed out by Ayala, would exacerbate the apparent departure of the effective number of alleles from that predicted by the neutral theory. Alternatively, it can be argued that the observed correlation may be the result of insufficient time to achieve mutation-drift equilibrium.

Finally, Ayala et al. (1974) and Ayala and Gilpin (1974) concluded that the distribution of genetic identity among loci assayed from species of the willistoni subgroup differed from that predicted by the neutral gene theory. However as discussed by Chakraborty et al. (1978) the apparent deviations reported are not warranted.

Perhaps, the most detailed statistical procedures employed and extensive analyses performed to date are those of Nei, Chakraborty and Fuerst (1976), Fuerst, Chakraborty and Nei (1977) and Chakraborty, Fuerst and Nei (1978). They have examined available gene frequency and heterozygosity data from 95 vertebrate and 34 invertebrate species

at a minimum of 20 gene loci. The following parameters were considered; mean and variance of heterozygosity within species, distribution of single locus heterozygosity within species, variance of heterozygosity among species, average heterozygosity and proportion of polymorphic loci, genetic identity and correlation of heterozygosity between populations and species and the mean and variance of genetic distance. Based on these statistical analyses they conclude that the majority of available electrophoretic data are consistent with the neutral or mutation-drift hypothesis. Significant departures include Ayala's data on interspecific variance of heterozygosity and apparent departures of this data and the Hawaiian picture wing Drosophila when the distribution of heterozygosity within species and the pattern of genetic differentiation between species are considered.

Statistical analyzes of the distribution patterns of heterozygosity led Yamazaki and Maruyama (1972, 1974) to conclude that enzyme polymorphisms are selectively neutral, but blood group polymorphisms are not. However, the procedures employed are controversial (see Ewens and Feldman, Yamazaki and Maruyama 1974).

Ohta (1975) has concluded that most genetic variation is selectively neutral. This conclusion was based on the apparent overall goodness of fit of the expected equilibrium distribution of allelic frequencies derived under the assumption of stepwise and infinite allele mutations with the observed data for D. willistoni and human protein polymorphism. However, there was a large excess of rare alleles under either mutational model. A similar excess of rare alleles has been noted by Chakraborty et al. (see Li 1968, p. 377) in 1/3 of 140 species tested in their analysis.

Despite the overall apparent goodness of fit of the electrophoretic data obtained under "standard" conditions it is not possible to state unequivocally that polymorphisms are not maintained by other hypotheses which involve selection and result in qualitatively similar predictions with respect to the observed parameters. It is possible, however, to exclude some of these as discussed by Fuerst et al. (1979). The excess of rare alleles is problematic to both strict neutral and balance (selectionist) hypotheses.

Lewontin and Krakauer (1973) have derived a statistical test which can indicate under certain circumstances (c.f. Lewontin and Kraukauer 1975, Nei and Maruyama 1975, Roberston 1975a,b) indicate whether the variance in heterozygosity between populations is consistent with that expected if selection or drift played a predominant role. Tsakas and Krimbas (1975) have applied this statistic to genetic variation in populations of Dacus oleaand suggest a method which may permit an estimate of the minimum number of selected loci. McKechnie et al. (1976) have applied the Lewontin-Krakauer test to electrophoretic data obtained for populations of Euphydryas butterflies and interpreted the magnitude and distribution of the observed heterozygosity to be inconsistent with the neutral gene hypothesis. Brittanacher, Sims and Ayala (1978) have applied the test to data obtained from an electorphoretic survey of butterflies of the genus Speyeria. They reported a significant heterogeneity of F values and concluded that either selection is maintaining the observed polymorphisms or that the Lewontin-Kraukauer test is sensitive to deviations in population structure. Such sensitivity is known to play an important factor (c.f. Lewontin and Krakauer 1975). Perhaps of greater significance is the

observation that the Lewontin-Krakauer test should "not be used if the distribution of gene frequencies is extremely skewed", as, for example, a J shaped distribution or a U shaped with one tail much higher than the other. As the majority of electrophoretic data fits a skewed U shape the test may not be applicable to the majority of studies.

Ewens (1972) has developed a statistical test (see also Ewens and Gillespie 1974, Kirby 1975) which measures departures from neutrality as deviations from the eveness of allelic frequencies predicted by a neutral, infinite allele model. Coyne and Felton (1977) have applied this test on their data and indicate departures from neutrality for Xdh, Adh-6 and Odh, but part of the deviations observed may result from pooling data sets. Weir, Brown and Marshall (1976) have applied Ewens tests to the data of Ayala and Tracey (1974) and indicate that most of the polymorphisms are in accordance with the neutral mutation theory of Kimura and Ohta (1971). They are critical of Ewens test for its lack of power, especially when the number of alleles are low unless the sample size is large ($n > 400$) and for its assumption of the infinite allele model and independence among mutations.

Weir et al. (1976) suggest a test which is based on the assumption of a step model of electrophoretic mutation and a comparison of the observed number of alleles in each step with respect to their expected frequency under the neutral hypothesis. The results of this analysis indicate that 4 out of 27 loci reported by Ayala et al. (1974) consistently depart from the expected neutral distribution, and an additional 4 sporadically depart depending upon the population observed. The remainder agree with the neutral distribution. However, the validity

of this approach depends on the strict applicability of the charge state model which they employ. As discussed earlier, the charge state model may not be sufficiently accurate to predict the electrophoretic mobility of proteins even when the amino acid substitutions are known.

Watterson (1977, 1978a) has advanced the "homozygosity test of neutrality" as a good statistic for assessing the operation of selection. The F statistic generated detects departures in the frequency of homozygotes from neutrality in the direction of heterosis. The model assumes that all heterozygotes are equally fit and all homozygotes are equally unfit and thus the test may be unrealistic. Watterson (1978b) has applied this statistic to the data of Coyne (1976) and Singh et al. (1976) and concludes that strict neutrality does not appear to be the case, but that if further alleles were to be distinguished in the samples, neutrality could become a possible explanation. However, in contrast to this conclusion and Ayala's consideration of the Ne, it appears that a remarkable deviation of the actual data noted by Li (1978) is that there is an excess of rare alleles than what is predicted by the neutral gene theory. Watterson and Anderson (1978) have compared the analysis of Odh and Adh-6 data utilizing Ewens statistic by Coyne and Felton (1978) and found no significant alteration in the interpretation. Coyne and Felton (1978) remark that although the two tests may yield different results with some future data sets, the application to available data does not indicate a substantive difference between them.

Schaffer, Yardley and Anderson (1977) have modified a method suggested by Fisher and Ford which should be sensitive to the

action of directional selection at a genetic locus. An analysis of the gene frequency at the α-amylase locus in Drosophila pseudoobscura was consistent with the neutral hypothesis (Yardley, Anderson and Schaffer 1977).

In summary, there are a number of reservations concerning the applicability of several statistical tests although the following comments are not specifically addressed to any one test described above. First, is the question if similarities of allele frequencies in closely related species necessarily indicate the action of selection upon allozyme loci, or if these loci serve merely as markers indicating the action of selection at the chromosomal level. This objection is somewhat alleviated by the general inability to detect linkage disequilibrium among allozyme loci, but such disequilibrium may be masked by the presence of hidden alleles (c.f. review of linkage disequilibrium). The second reservation concerns the independence of the tests employed in any one study. For example, if the effective number of alleles observed differs from that expected by either theory, then similar differences may be expected for heterozygosity because Ne is estimated from:

$$Ne = 1 / (1-\bar{H})$$ where $\bar{H}$ is the mean heterozygosity at a locus. Finally, it is not known if the increase in number of alleles typical of the data of Coyne (1976), Singh et al. (1976) will continue its bimodal pattern. Such bimodality would jeopardize numerous conclusions of the statistical tests currently employed, and necessitate the employment of neutral models with variable mutation rates, as are being formulated by Nei et al. (1976) (see also Zouros 1979).

APPENDIX II

I. Experimental Approach

The experimental approaches which will be described in this section represents an attempt to resolve the neutralist-selectionist controversy by demonstrating the existence or absence of selective forces at the enzymatic and or physiological level of organization. The approaches to be discussed include: 1) single gene investigations 2) enzyme-environmental correlations, 3) correlates of enzyme variation, 4) existence of co-adapted gene complexes.

1. Single gene investigations

Studies concerned with the dynamics of single gene loci in laboratory populations have revealed that heterotic and frequency dependent selection may be operating. Richmond and Powell (1970) found an excess of heterozygotes for D. paulistorum tetrazolium oxidase as did Wills and Nichols (1971) for octanol dehydrogenase in D. pseudoobscura when octanol was introduced into the culture medium. Wills and Nichols (1972) suggest that such single gene heterosis is likely but masked by the effects of genetic background. Yamazaki (1972) has offered an alternate explanation for the Wills and Nichols experiment which may indicate a flaw in experiments designed to detect single gene effects by inbreeding. In a series of experiments, Kojima and co-workers (Kojima and Yarborough 1967, Kojima and Tobari 1969, and Huang et al. 1971) have presented evidence consistent with the action of frequency dependent selection at the esterase-6 locus in

D. melanogaster. Fontidevila et al. (1975) have indicated the possibility of selective factors determining the frequency of alleles at the esterase-5, Odh and Mdh loci in D. pseudoobscura. Nassar (1979) demonstrated frequency dependent changes in gene frequency and the Lap (leucine aminopeptidase locus) in D. melanogaster. Powell (1973) reported independent changes in gene frequency at Lap-5, and Est-5 loci in cage populations of D. pseudoobscura. Joseph and Singh (1979) report directional selection at the Est-5 locus in five independent populations of rose aphids. The loss of the fast allele in asexual populations is probably compensated by its gain during sexual generations. Laurie-Ahlberg and Merrel (1979) found consistent differences in aldehyde oxidase (Ao) gene frequencies between strains selected for DDT resistance and control strains but the allozyme locus was found to be in strong linkage disequilibrium with inversions. In contrast to these studies, Yamazaki (1971) found no significant trend in sex-linked esterase-5 alleles over a 2-3 year period in replicated laboratory populations of D. pseudoobscura. Powell (1973) could find no changes at the Est-7 locus despite changes at Est-5 and Lap in D. willistoni. Similarly, Yardely, Anderson and Schaeffer (1977) could not detect the occurence of balancingor frequency dependent selection at the amylase locus in D. pseudoobscura. The changes in gene frequency observed in the small populations utilized was consistent with that predicted by the neutral hypothesis.

Although often interpreted as supportive of either the selectionist or neutralist theories data concerning the change in allelic frequency at a single allozyme locus are generally argued to be confounded by the

genetic background. Therefore, in experiments which have observed singificant changes in gene frequency, it is not clear if selection operated directly in these loci or was manifested indirectly through the genetic background on a group of coadapted genes which functionally do not include the allozyme locus in question. Conversly, a similar argument can be formualted for experiments which do not show changes. In this case, the effects of selection at a single locus is claimed to be masked by the genetic background. The importance of genetic background on the outcome of such experiments has been demonstrated by the report of Jones and Yamazaki (1974). Changes in gene frequency at the esterase-5 locus was dependent upon the number of chromosomal lines which were employed to start the population cages. This result is interpreted as demonstrative of the effects of linkage disequilibrium on allozyme loci in such selection experiments. A further danger in the interpretation of single gene selection experiments arises following a consideration of the preliminary report by Anderson, Sakeda and Turner (1979) on changes in gene frequency at the α-amylase-1 locus in D. pseudoobscura with respect to three associated inversions CU, TL and EP. Although 1,000 lines were used to start the cage, and the observation of a balanced polymorphism for TL and EP inversions and loss of CU, the conclusion that the frequency changes at the amylase locus were independent of the inversion and deviate from that predicted by the neutral theory is premature because the study makes a tacit assumption that TL and CP inversions are genetically identical. Obviously this is not necessarily the case, and may result in the differential association of amylase genes within TL inversion sequences leading to errors in the predicted neutral gene

frequencies.

In general, the difficulty with this approach which attempts to demonstrate the action of selection is the lack of physicochemical evidence which would support the conclusions of the study. For example, studies which have demonstrated changes in gene frequencies in experimental populations in response to a particular selective agent would be more convincing in their conclusions if differences were observed in the physicochemical properties of the alleles which were relevant to the selective agent. Conversly, in selection experiments which do not detect changes in gene frequency, physicochemical data could establish if the alleles were functionally monomorphic as a consequence of their identical properties or due to physiological compensation for their differences. If the alleles differed and no compensation were evident, then it is evident that selection cannot penetrate the genome to change their frequency.

A number of studies have investigated the physicochemical properties of allozymes coded by alleles at single gene loci. Differences have been reported by Watt (1977) for alleles at the phosphoglucose isomerase locus in Colias butterflies, by Danforth and Beardmore (1979) in esterase-6 in D. melanogaster, by Berger (1974) for allelic forms of esterase-5 in D. pseudoobscura and by Yardley (1979) for amylase allozymes. However, it should be noted that such differences do not indicate that the enzymes are selected or selectively maintained. It merely indicates that the allozymes are selectable. In contrast no differences could be detected in biochemical properties of soluble malate dehyrogenase (Hay and Armstrong 1976) or xanthine dehydrogenase allozymes (Yen and Glassman 1967). The interpretation that these

alleles are selectively neutral is correct but must be qualified by stating the parameters measured. The existence of functionally identical alleles does not indicate that selection is inoperative at the enzyme level. The lack of dissimilar allelic products at such loci may be a consequence of the action of purifying selection. In this context, the presence of dissimilar allozymes may indicate the relaxation of selection at the particular locus. Clearly, any investigation which attempts to establish the operation of selection at an enzyme locus must combine the functional and experimental approach. This has been accomplished in three gene-enzyme systems; alcohol dehydrogenase (Adh), α glycerophosphate dehydrogenase (α Gpdh) and to a lesser extent in amylase (amy). These studies are reviewed below.

(i) Alcohol dehydrogenase

The potential action of selection at the Adh enzyme locus in D. melanogaster has been intensively investigated by numerous laboratories. Only a small sample of available studies will be described here as a means of illustrating the approach employed.

Day, Hillier and Clarke (1974) have investigated the properties of two alleles Adh^F and Adh^S from populations of D. melanogaster. They could detect no differences in Michaelis constants $K_{ethanol}$, K_{NAD}, $K'_{ethanol}$, K'_{NAD} or quantity of enzyme. However, there was a marked difference in the relative catalytic efficiency of the two enzymes, which was presumed to be caused by the structural difference. Adh^F was found to have greater catalytic activity and therefore was expected to confer greater alcohol resistance. This hypothesis was

examined in an experimental situation and a preliminary report has been published (Daly and Clarke 1979). The results of selection experiment employing 2% ethanol was found to be in agreement with the *in vitro* prediction. Thus the Adh^F alleles conferred increased viability relative to Adh^S when ethanol was present. These data are interpreted as an indication of the ability of selection to discriminate alleles directly at the Adh locus rather than through closely linked alleles.

Morgan (1975) has demonstrated differential selection with respect to two homozygous Adh genotypes by subjecting flies to vapor of 1-penten-3-ol. The genotype characterized by higher V_{max} was as predicted to be at a selective disadvantage as the metabolite of the dehydrogenation (ethyl vinyl ketone) is a lethal product.

A detailed investigation of the Adh polymorphism in *D. melanogaster* has been reported by Oakeshott (1967a,b, 1977). Oakeshott has demonstrated that differences in physicochemical properties are responsive to selective factors. Of note was the observation that the direction of selection varied not only with the fitness component investigated but also with the type of alcohol employed. In addition, it was maintained that the response of Adh to selection is not affected significantly by loci other than Adh.

McDonald and Ayala (1978) have reported the apparent selective advantage of *D. melanogaster* strains with high enzyme activity and maintain that this difference is a consequence of difference in "regulatory genes". As discussed previously, the conclusion may be premature.

Cavener and Clegg (1978) have indicated that selection imposed by ethanol at the Adh locus, results in the co-evolution of at least one other locus (α Gpdh) as a consequence of increased acetate and NADH levels.

Van Delden, Boerema and Kamping (1978) have demonstrated the increase in Adh^F in large population cages on ethanol media. The egg to adult survivorship of Adh^{SS} genotypes was significantly less than Adh^{SF} of Adh^{FF} genotypes. In addition they report that on normal food the survivorship of Adh^{SF} is greatest. However, it is unlikely that this latter effect is related to the Adh locus. Consequently it cannot be used as a valid mechanism to explain the maintenance of the Adh polymorphism at the single gene level.

Despite the overall congruity of the Adh data with a selectionist interpretation, there are some difficulties. The nature of the physiological toxification process leading to the detoxification of alcohols in Drosophila is not simple in that the process requires the activity of subsequent enzymes to metabolize the aldehydes produced by the reaction (c.f. David and Bocquet 1976). Van Delden et al. (1978) have reported that the same increase in Adh^F frequency in response to ethanol can be generated by methanol. Because methanol is not a substrate for Adh, this result indicates that the primary selective action of alcohol may not involve the Adh enzyme locus. Complexities in the interpretation of population data may arise if a recessive lethal (1(2)stm) which is closely associated with Adh^F is in high frequency in the population (Leibenguth and Steinmetz 1976). In addition, the kinetic interpretation of Adh activity is often simplified

by investigators. In fact, there is good evidence which indicates that the reaction is an ordered bibi reaction and involves a conformational change induced by NAD binding (Knopp and Jacobson 1972). Clarke et al. (1979) have indicated that enzyme activity is dependent upon the media. This finding accentuates the importance of relating electrophoretic differences to structural differences with the required alterations in physicochemical properties necessary for the selection regime employed.

Finally there may be some indication that the effect is limited to D. melanogaster. In D. pseudoobscura for example the Adh is encoded by either separate loci, which are characterized by overlapping substrate affinities. Genetic variation at one locus leading to a chemically distinguishable allozymes may not be selected due to possible compensatory mechanisms (c.f. Chapter 1).

(ii) α-glycerophosphate dehydrogenase (α-Gpdh)

The αGpdh enzyme locus in Drosophila is generally characterized by low heterozygosity (Powell 1975, Koehn and Eanes 1978). The conservative nature of amino acid substitutions and slow rate of evolution in Drosophilidae of this homodimeric enzyme has been ascertained by Collier and MacIntyre (1978) in an investigation of the reassociation ability of subunits derived from twenty species.

In Drosophila melanogaster variants have characterized by Alahiotis, Miller and Berger (1977). A lattitudinal cline in gene frequency could be explained by a selection regime involving environmental temperature. Thus, the slow αGpdh allele exhibited

positive thermal modulation of Km (a mechanism which ensures reaction rate constancy) whereas the fast allele was independent of temperature. The interpretation of these data were supported by the observation that αGpdh form present in tropical species of Drosophila, D. willistoni and D. equinoxialis exhibited negative thermal modulation and exhibited the activity pattern of the α Gpdh^F allele in D. melanogaster. Temperate species, D. virilis and D. americana were fixed for an allele exhibiting positive thermal modulation similar to that of the αGpdh slow allele in D. melanogaster. Therefore it seems reasonable to conclude that the cline is the result of selective forces operating on the enzyme locus.

Bewley and Lucchesi (1977) have reported the properties of α-Gpdh alleles in D. melanogaster. Surprisingly a null allele was detected which was viable but flightless. O'Brien and Shimade (1974) conducted a comparative survey of the metabolic enzymes in wild type and α-Gpdh null alleles but found no apparent difference. Bewley and Lucchesi have suggested that the nonlethality of the null mutant indicates the lack of general plerotropic effects and the existence of a compensatory mechanims for the function of the α glycerophosphate shunt (cycle).

(iii) α-amylase

The α-amylase gene locus, although well characterized genetically and biochemically (Doane et al. 1975) has not as yet been investigated thoroughly in an evolutionary context. De Jong and Scharloo (1976) have surmized the action of selection on the amylase locus as has Hickey (1977). However, as pointed out by Yardley (1978)

the amylase allele $Amy^{4,6}$ investigated in both studies is the result of a gene duplication. Therefore, it seems inappropriate to compare the properties of individuals containing $Amy^{4.6}$ to a single allele Amy^{1}. Yardley et al. (1977) could not detect the operation of selection at this locus in experimental populations of D. melanogaster. Nevertheless the locus contains many interesting structural, and potentially regulatory mutants which should yield considerable information concerning the operation of selection at the molecular level.

2. Enzyme environemntal correlations

The theoretical considerations of a number of workers have established the approximate conditions whereby polymorphism will be more likely in variable rather than constant environments (c.f. Levins 1968, Gillespie and Langely 1974, 1976, Taylor 1976 and review by Soule (1976) for examples. Based on these conclusions, one might predict that a positive correlation should exist between enzyme variability and environmental heterogeneity, if the allozymes were segregating independently of other genetic elements and were adaptive.

Several authors (Powell 1971, Levinton 1973, Bryant 1974, McDonald and Ayala 1974, Band 1975, Gooch and Hedrick 1979) have demonstrated a positive correlation between heterozygosity and temporal and spatial environmental variability in natural or laboratory populations. Conversely, Sabbath (1974) found no correlation between niche breadth and genetic variability in eleven sympatric populations of Drosophila. Similarly, although the degree of chromosomal polymorphism correlates well with an index of environmental diversity in marginal or

central populations, the allozymic data do not in general follow a similar pattern (Lewontin 1974, Zouros et al. 1974, Marinkovic, Ayala and Andjelkovic 1978). These data are often interpreted as indicative of the potential differential action of selection on chromosomal and genic polymorphisms. The lack of correlation is not expected if level of heterozygosity of allozymes were adaptive.

An adaptive significance for the levels of heterozygosity has been suggested (Soule 1979). Thus several investigators have attempted to detect a positive correlation between levels of heterozygosity and components of fitness. For example, Soule (1979) observed a negative correlation between heterozygosity and phenotypic asymetry and under the assumption that asymetry reflect developmental instability suggests a correlation with fitness. Singh (1979) has reported that mean weight of american oyster is greater in individuals heterozygous at any one of the seven polymorphic loci investigated and that there was an association of individual weight once the number of loci polymorphic. Stalker (1976) indicated that homozygosity at acid and alkaline phosphatase in females of D. euronotus correlated with developmental time and "being sperm free." Although these correlations between levels of heterozygosity as measured by enzyme polymorphisms and "fitness" are interesting it is not celar that it represents a clear indication of adaptiveness of protein polymorphism. The same relationship can be observed for isogenic chromosomal segments in various heterozygote combinations (c.f. Zali and Allard 1976). Therefore the allozymes surveyed may in fact merely serve as chromosomal markers and are not adaptive per se.

An association of a gene frequency cline and an environmental gradient is often interpreted as evidence indicating the action of selective rather random forces, however, the association may be spurious or related to local differentiation and gene flow (c.f. Ward and Neel 1976). Nevertheless, the existence of numerous environmental and genic clines is well established as demonstrated by Mitton and Koehn (1975), Koehn (1969), Johnson et al. (1969), Band (1975). In some cases (Koehn 1969 and Alahiotis et al. 1977) kinetic analysis of the proteins support the view that these correlations are adaptive. A possible mechanistic basis for niche-allozyme associations has been provided by Rockwood-Sluss et al. (1973). Their data indicate the assocation of several enzymes with variation in the concentration of certain chemicals in the host plant of Drosophila pachea. Richardson, Smouse and Richardson (1977) have observed a similar association between the taxonomic diversity of the larval substrates and the electrophoretic diversity of the Drosophila populations using those substrates and proposed a number of plausible physiological mechanisms. Johnson and Powell (1974) have shown that the Adh genotype in D. melanogaster which predominates in areas with higher average temperature most frequently survives heat shock under laboratory conditions. Significant allozyme-environmental correlations were observed for Est-2 and Adh-1 gene frequency and heterozygosity and Pgm gene frequency in a survey of D. buzzatii populations by Mulley et al. (1979). Tsuno (1975) has observed differences in allozymes frequencies of two esterase loci depending upon sample location (brewery vs. lumberyard). Although no functional basis for the majority of such correlations has been established, it may be possible that such associations are caused by

the action of natural selection. Selection at the chromosomal level, or on closely linked loci cannot be discounted until the functional basis is established.

3. Correlates of enzyme variation

It is becoming well established that the levels of heterozygosity observed for a given enzyme or group of enzymes may be related to one or more physiological or structural parameters. The interpretation of the significance of these data with respect to the resolution of selectionist-neutralist controversy is itself often controversial and certainly it is unlikely that any one correlation can explain all the data.

Prakash (1977) following an investigation of 43 gene loci in D. persimilis reported that loci coding hydrolytic and non-specific enzymes were more variable than loci coding enzymes in the glycolytic pathway, krebs cycle and those with a specific function. This observation is consistent with the hypothesis advanced by Gillespie and Kojima (1968) and Kojima et al. (1970) that levels of enzyme polymorphism reflect the degree of environmental variation in substrates (i.e. the substrate-specificity hypothesis). Thus enzymes involved in energy production (Group I) are expected to be genetically less variable than those which metabolize substrates originating from the external environment (Group II) as the latter group of substrates would be expected to be more variable. The selectionist interpretation of this correlation would maintain that the higher degree of heterozygosity found in Group II enzymes is a consequence of the diversity in substrates

or synonomously, higher environmental heterogeneity. It could however, be argued with equal vigor that Group II enzymes could contain a higher proportion of selectively neutral alleles. This situation could arise if the substrate specificity of several enzymes within this category overlapped as is the case with nonspecific esterases and alcohol dehydrogenases (c.f. Bargiello et al. 1977, Singh 1976). Singh (1975, 1976) has investigated the substrate specificity hypothesis by determining the substrate specificity of eight alcohol dehydrogenase loci in D. pseudoobscura. It was concluded that the substrate specificity for enzymes is not an important factor in the determination of greater amounts of genetic variation observed in enzymes which utilize external substrates than those involved in energy production since no evidence for substrate specific differences among genetic variants of enzyme loci in Group II or Adh could be found. Band (1975) could find no significant difference in levels of polymorphism among glucose and non-glucose metabolizing enzymes in D. melanogaster. These results suggest that the correlations described by Gillespie and Kojima should not be hastily accepted until the enzymes in question are more fully characterized at a functional level.

Johnson (1974) has proposed that the degree of heterozygosity at an enzyme locus correlates with the regulatory role of the enzyme. Regulatory enzymes are expected to be of greater evolutionary consequence than non-regulatory enzymes if for no other reason than that their effect would be expected to involve more than a single gene locus. Zouros (1975) has reviewed the available electrophoretic data from 61 species of Drosophila and indicated the following order in the levels

of polymorphism; enzymes with multiple substrates > enzymes with single substrates, also enzymes with multiple substrates > regulatory enzymes > non-regulatory enzymes. But unlike the classical interpretation suggested by Johnson or Gillespie and Kojima, Zouros (1976a, 1979) implies that the differences in the amount of electorphoretic variation of these enzyme classes may be explained by differences in the rate of "electrophoretic mutation" among these classes and therefore does not necessarily reflect functional differences.

Zouros (1976b) has also demonstrated that heterozygosities of multimeric enzymes (i.e. enzymes composed of more than one subunit) are lower than heterozygosities of monomeric enzymes. He suggests that this finding contradicts the expectation of heterozygote superiority simultaneously for both multimeric and monomeric enzymes. Therefore, if one accepts that the level of heterozygosity at multimers is adaptive in nature, than the higher variation at monomeric enzymes implies that a higher proportion of alleles at these enzymes are neutral or maintained by some other form of natural selection. Bargiello et al. (1977) have offered a different interpretation for the nature of selective forces acting upon multiple enzyme forms. They suggest that individuals with multiple forms of an enzyme, that is isoenzymes rather than allozymes may be adapted to a wider range of environmental conditions, and that mutations which disrupt the functional range of such an enzyme system are deleterious. If the functional range of the enzyme system is not disrupted by the presence of such alleles then they are defined as being "conditionally neutral". This concept of conditional neutrality or compensation for differences in

gene expression resulting from substitution is somewhat similar to the considerations of Latter (1975), Forkmann and Seyfert (1977) and Collier (1979).

Koehn and Eanes (1977, 1978) have observed a positive correlation between subunit molecular weight and number of observed alleles for Drosophila and humans, a relationship between subunit size and heterozygosity was evident only in Drosophila populations. Harris, Hopkinson and Edwards (1977) detected no difference in molecular weight between monomorphic and polymorphic loci in humans with a total sample of 87 gene loci. Li (1979) suggests that this may be the result of low average heterozygosity of the human population which effectively masks the relationship. Both sets of data were analyzed statistically, and found to be critical of the mutation-drift hypothesis. In contrast to this conclusion, Nei Fuerst and Chakraborty (1978) have examined the relationship between subunit molecular weight and heterozygosity in six different groups of organisms, including 9 species of primates, 32 of rodents, 56 of reptiles, 12 of salamanders, 64 of teleost fishes and 29 of Drosophila. Their results are not inconsistent with the mutation drift hypothesis, and support Koehn and Eanes contention that subunit molecular weight is an important factor for determining the level of protein polymorphism. Therefore it would appear that mutation rate and hence genetic variability is dependent on the number of amino acids in a protein. Recent advances in the determination of the structure of the eukaryotic gene (i.e. the existence of intervening sequences) implies that a more complex relationship between gene size, protein size and mutation rate may exist.

In addition to the correlation between subunit size and genetic variation, Koehn and Eanes (1978) have reanalyzed Ayala's willistoni data by comparing estimates of genetic identity as a function of molecular size of proteins. Although the results of their analysis are too extensive to report here, there were obvious correlations between the patterns of protein divergence and the subunit molecular weight. These results indicate that it may be of interest to reexamine the statistical tests performed by Nei, Fuerst and Chakraborty in terms of subunit size and associated differences in mutation rate.

For the most part, the aspect of molecular structure and protein variation has been excellently reviewed by Koehn and Eanes (1978) and their considerations will not be reiterated here. There are however, two recent papers concerning molecular structure which have a direct bearing on the prediction of the amount of protein variation. The first is the observation of Miyata, Miyuzawa and Yasunga (1979) that the frequency of amino acid substitutions relative to the frequency expected by chance decreases linearly with an increase in the physicochemical differences between the amino acid pairs involved in a substitution. Although Miyata et al. indicate some exceptions, these data support the contention that natural selection acts in such a way to select proteins which retain their existing confirmation. Proteins which deviate substantially would be removed by purifying selection. A similar inference can be drawn from the considerations of Conrad (1977, 1979) on molecular adaptability. Conrad's theory predicts that proteins which are amenable to change would be structurally perturbed to a lesser extent than those which have not evolved such

intrinsic mechanisms. Thus the differences in "electrophoretic mutation rate" and number of alleles in protein groups may be related to the structural stability of the protein rather than simply due to their relative size. A synthesis of the considerations of Koehn and Eanes and Conrad would lead to the prediction that larger proteins would have proportionally greater stability to perturbation introduced by amino acid substitutions because their functional density (i.e. proportion of sites involved in specific functions which are sensitive to substitution, Zuckerland 1976) is less than small proteins. Therefore one might predict that higher molecular weight proteins would have a greater number of variants than predicted on the basis of size alone. It is of interest to note that the analysis of Koehn and Eanes (1978) indicates that small enzymes possess more alleles than the infinite allele model would predict from their heterozygosities. The larger subunit enzymes have approximately the number of alleles predicted for their average heterozygosity. These considerations may be interpreted in a number of ways and consequently it is not immediately obvious if they support the neutralist or selectionist theories.

4. Co-adapted gene complexes

The presence or absence of co-adaptation between allozyme loci has been one of the major areas investigated as a test of the neutral gene hypothesis (see reviews by Hedrick, Jain and Holden 1978, Mukai et al. 1971, Baker 1975, Zouros 1976a). Co-adaptation of gene loci has been detected by calculating the amount of linkage disequilibrium present between loci, or by investigating the segregation

of allozymes within chromosomal inversions such as the standard or arrowhead configuration found in D. pseudoobscura. The interpretation of these analyses can be complicated because the presence of linkage disequilibrium can be indicative of several factors. These include selection, epistasis or non-additive interaction between loci, non-random mating, gene flow, linkage, genetic drift as well as the interaction of one or more of these factors (see Hedrick et al. 1978, Lewontin 1974 for a complete review). The presence of stable amounts of linkage disequilibria between allozyme loci in large randomly mating populations has been interpreted as evidence that epistasis is present or that selection is operating on the loci in question especially when the allozymes are structurally or functionally related. The absence of linkage disequilibrium between enzyme loci does not imply that selection is inoperative, since for example, permanent disequilibrium will not result if selection acts in an additive manner on the loci investigated (Mukai et al. 1979). Gillespie and Langely (1976) have investigated the theoretical behavior of multilocus systems in random environments. As expected, for the additive model used, linkage disequilibrium was found to be zero. However, it was found that when the presence of a negative correlation in fitness of loci across environments was introduced into the model, the equilibrium state at the two loci was highly interdependent. Feldman et al. (1975) have shown that stable disequilibrium between two gene loci can be destroyed by the presence of new mutations. The incidence of new mutations at polymorphic loci may be greater due to intragenic recombination (Morgan and Strobeck 1979, Strobeck and Morgan 1978, Ohno et al. 1969).

To date, a number of studies are available which have investigated the association of enzyme loci to each other or other polymorphic systems (inversions). These have been extensively reviewed by Mukai et al. 1971 and Hedrick et al. 1978. Therefore, only certain cases will be described here.

Significant disequilibrium has been reported between Mdh-1 and amylase (Mukai et al. 1971) as well as between acetylaldehyde oxidase and xanthine dehydrogenase in D. subobscura (Zouros and Krimbas 1973) and D. mojavensis (Zouros and Johnson 1976). The basis for this interaction was believed to reside in the apparent structural and physiological relationship between these loci and was therefore interpreted to be selectively maintained. The data should be reanalyzed in light of the indication that the correlation in activity changes at these loci under the influence of mal and lxd loci are most likely due to post-translational modification. Cavener and Clegg (1979) have demonstrated the functional relationship between Adh and α Gpdh, in response to ethanol as mediated by acetate and NADH affects. Despite the clear functional relationship only a small amount of linkage disequilibrium could be detected among alleles at these loci. Baker (1975) has investigated the pattern of linkage disequilibrium among alleles at four tightly linked esterase loci in D. montana (1 centimorgan apart) He concludes that the polymorphisms are maintained by the action of selection, but as the functional nature of these esterases is unknown, the nature of the selective forces maintaining the apparent co-adaptation between these alleles is equally obscure. In contrast Tsuno (1975) could not detect linkage disequilbria between two esterase loci (15 centimorgans apart) although alleles at both loci responded to changes

in environment (i.e. brewery vs. lumberyard). Similarly Zouros and Johnson (1976) could not detect linkage disequilibrium between two linked esterase loci. The lack of linkage disequilibrium observed between allozyme loci is indicated by the data of Langely, Tobari and Kojima (1974) and Zouros et al. (1974). These investigators could not detect linkage disequilibrium between 21 allozyme loci on the second and third chromosomes of Japanese population of D. melanogaster (Langely et al. 1974) or could detect significant non-transient disequilibria among 2 of a sample of 18 loci in D. subobscura. Zouros, Golding and Mackay (1977) suggest that the inability to detect stable disequilibria between allozymes may be a result of pooling alleles. Such pooling may arise as a consequence of hidden alleles within electromorphs. Weir and Cockerham (1978) have critized the formulation devised by Zouros et al. to reach this conclusion but report that their modifications do not alter the general conclusions. The lack of linkage disequilibrium between allozyme loci may indicate that allozymes are selectively neutral or interact in an additive manner.

Linkage disequilibria between allozymes and polymorphic inversions have been reported by Prakash and Lewontin (1968) between certain alleles and an inversion for the α-amylase locus and a larval protein locus. Kojima et al. (1970) have reported disequilibria for three loci Adh, Odh and Est C and three inversions In 2LB, In 3AG and In 3RG, respectively in D. melanogaster (Katsunuma population). Associations between alleles and inversions have also been reported by Prakash and Levitan (1974), Langely et al. (1974), Loukas and Krimbas (1975) report linkage disequilibrium among genes in Est-9 complex locus, and between this complex locus and inversions but no association could be

detected between hexokinase and these genes. Lack of correlation between heterokaryosis and heterozygosis has been reviewed by Zouros (1976a). He maintains that the lack of correlation between cytological and electrophoretic variation in several Drosophila species, indicates that allozymes are in general not members of the co-adaptive complexes characterizing inversions. A similar conclusion was reported by Watanabe and Watanabe (1977) in an experimental investigation of the association between Adh and inversion In (2L)B in Japanese populations of D. melanogaster. That inversions are comprised of co-adapted genes has been demonstrated by Watanabe and Yamazakai (1976). Thus, it would appear that the selective forces maintaining inversion polymorphisms are not involved in the maintenance of allozymes.

As a consequence of these conclusions, Zouros (1976a) concludes that "the amount of electrophoretic variation is determined by properties intrinsic to the molecule and is independent of factors such as background genotype and differences in the environment". The same argument may apply for allozymes closely associated with inversions. In this case it is still not established that the allozymes are responsible for the altered fitness of the inversion or merely associated with it by historical accident and close linkage.

Templeton et al. (1976) have reported the presence of strong epistatic forces between electrophoretic markers on the same chromosome arm in experiments designed to define the unit of selection in parthenogenetic strains of Drosophila mercatorum. They warn that the apparent selective neutrality of allozyme markers "may arise as an artifact in a co-adapted genetic complex if the genetic markers used do not identify the unit of selection". This problem of the unit of

selection and allozyme polymorphisms has been considered by Annest and Templeton (1978) DeBenedictis (1978) and is the subject of Chapter 1 in this thesis.

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