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A KINETIC AND THERMODYNAMIC COMPARISON OF BEEF HEART, BEEF MUSCLE AND FLOUNDER MUSCLE LACTATE DEHYDROGENASES

by Uwe Borgmann

A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements for the Ph.D. degree in Biology

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ABSTRACT

The dehydrogenation of lactate to pyruvate, catalysed by lactate dehydrogenase, proceeds by an ordered ternary-complex mechanism. A study has been made of the steady-state and pre-steady-state kinetics of the reaction in both directions, at various substrate and coenzyme concentrations, and over a range of temperatures (0 to 50°C), for beef heart, beef muscle and flounder muscle lactate dehydrogenase. The steady state data, combined with the pre-steady-state data in either direction, are consistent with a four-step model, and all eight rate constants can be evaluated. Comparison of the pre-steady-state data in the two directions indicates that a more complex model, containing five steps, is necessary to explain all of the results. Ten rate constants can therefore, in theory, be evaluated, and this has been done for beef heart lactate dehydrogenase. The pre-steady-state in the forward direction (lactate to pyruvate) could not be measured for the two muscle enzymes and hence, the four-step model is used to compare the three homologous enzymes. The relationship between the four and five-step models is discussed. The concentration of active enzyme can also be calculated from the kinetic results and is consistent with the value obtained from the protein concentration and the molecular weight.

The thermodynamic parameters, free energy ($\Delta G^{\ddagger}$), enthalpy ($\Delta H^{\ddagger}$), and entropy ($\Delta S^{\ddagger}$) of activation, were calculated for each of the eight rate constants. Enthalpy and entropy profiles reveal that the relative heights of the activation barriers are very similar in all three enzymes, differences in kinetic behaviour resulting mainly from differences in the stable binary and ternary enzyme-substrate complexes. These complexes are, in general, at lower enthalpy and entropy levels for the beef enzymes than for the flounder enzyme. A high degree of compensation is found between the enthalpies and entropies of activation, resulting in relatively small differences between the free energies (and rates) for homologous steps of different enzymes. The results are discussed in terms of solvation and weak bonding effects, but it is not possible to decide precisely what causes the compensation between enthalpy and entropy.

Adaptation to environmental temperature is examined, based on the above observations and additional experiments on thermal denaturation. Low temperature adaptation is observed in the ectothermic enzyme (flounder) as indicated by a reduced enthalpy of activation for k_{cat}, increased catalytic efficiency, and decrease in the temperature at which maximum velocity is observed at low substrate concentrations. This is a result of the altered bonding in the enzyme-substrate complexes. Adaptation to higher temperatures in the endothermic enzymes (beef heart and muscle) is suggested by a decreased sensitivity to heat denaturation,

especially in the presence of substrates. A direct correlation is found between the degree of bonding in the enzyme-substrate complexes and the decrease in rate of heat denaturation caused by addition of substrates.

Résumé

La déhydrogénation du lactate au pyruvate, catalyzée par le lactate déhydrogenase, procède par le mécanisme "ordered ternary-complex". Les kinétiques "steady-state" et "pre-steady-state" de la réaction furent étudiées dans les deux sens, à des concentrations variées de substrat et de coenzyme, et sous une gamme de températures (0 à 50°C), pour les enzymes du coeur de boeuf, du muscle de boeuf et du muscle de flet. L'information "steady-state", combinée avec l'information "pre-steady-state" en chaque direction, est compatible avec le modèle à quatre échelons, et toutes les huit constantes de vitesse peuvent être évaluées. La comparaison des données "pre-steady-state" dans chaque sens indique qu'un modèle plus complexe, avec cinq échelons, est nécessaire pour expliquer tous les résultats. Il est alors possible d'évaluer dix constantes de vitesse (en théorie). Ceci fut accompli pour le lactate déhydrogenase du coeur de boeuf. Le "pre-steady-state" dans la direction de lactate à pyruvate ne put pas être mesuré pour les deux enzymes des muscles, aussi le modèle à quatre échelons fut-il utilisé pour la comparaison des trois enzymes homologues. La relation entre les modèles à quatre échelons et à cinq échelons est discutée. La concentration d'enzyme actif peut être calculée d'après les résultats kinetiques, et sa valeur est compatible avec celle dérivée de la concentration de protéine et du poids moléculaire.

Les paramètres thermodynamiques, énergie libre ($\Delta G^{\neq}$), enthalpie ($\Delta H^{\neq}$), et entropie d'activation ($\Delta S^{\neq}$) furent calculées pour chacune des huit constantes de vitesse. Les courbes d'enthalpie et d'entropie révèlent une grande similitude des barrières d'activation chez les trois enzymes. Les différences dans le comportement kinétique résultent des différences entre les complexes stables binaire et ternaire. Ces complexes des enzymes de boeuf ont une enthalpie et une entropie généralement plus basses que les complexes d'enzyme de flet. Une compensation élevée existe entre l'enthalpie et l'entropie d'activation, résultant en des différences relativement faibles entre les énergies libres (et les vitesses) pour les échelons homologues d'enzymes différents. Les résultats sont discutés par rapport aux effets de la solvation et des liaisons faibles mais il n'est pas possible d'expliquer définitivement la cause de la compensation entre enthalpie et entropie.

L'adaptation à la température ambiente est examinée, sur la base des observations ci-dessus et d'autres essais de dénaturation thermale. On observe une adaptation aux basses températures dans l'enzyme ectothermique (flet) indiquée par une diminution de l'enthalpie d'activation pour k_{cat}, par une efficacité catalytique accrue, et par un abaissement de la température correspondant à la vitesse maximale aux faibles concentrations de substrat. Ceci est dû à des liaisons altérées dans les complexes d'enzyme-substrat.

Une adaptation aux hautes températures dans les enzymes endothermiques (les enzymes de boeuf) est suggérée par une sensitivité réduite à la dénaturation thermale particulièrement en présence de substrat. Il existe une corrélation directe entre le degré de liaison dans les complexes d'enzyme-substrat et la réduction de la vitesse de dénaturation thermale est provoquée par l'addition de substrats.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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I

INTRODUCTION

A. Temperature Adaptation in Enzymes

Adaptation to environmental temperature, and temperature fluctuations, may be accomplished in one of several ways. Mammals and birds regulate the amount of heat produced and dissipated to the environment and hence maintain a constant body temperature. Most mammals, for example, have a body temperature between 37 and 39°C, and most birds between 39 and 42°C (see Prosser, 1973). Fish may also maintain a relatively constant body temperature. The brain and eye temperature of the bluefin tuna varies by only 5 or 6°C in an environment where temperature fluctuates between 7 and 23°C (Linthicum and Carey, 1972). The body or core temperature of certain tunas may be 10°C higher than the surrounding sea water (Carey et al., 1971). Such organisms, whose major source of body heat is their own metabolic activity, are termed endotherms; conversely, those which obtain their body heat principally from the environment are called ectotherms (Hochachka and Somero, 1973). Some ectotherms may also regulate their body temperatures, although behaviourally. Snakes and lizards, for example, absorb much radiant heat by basking in the sun and can gain heat by conduction from rocks and sand (Prosser, 1973). In fact, most lizards gain considerable independence of macroclimate conditions by means of behavioural regulation (Licht, 1967).

Many ectotherms, however, must tolerate considerable fluctuations in their body temperature, which is essentially

equal to that of the environment. Hence, the body temperature of most fish, with the exception of some sharks and tunas, lies within a degree or so of the environmental temperature (Carey et al., 1971). The enzymes of such species, therefore, must be able to function under a wide variety of temperatures and, if adaptation to temperature occurs, it must do so at the molecular level. In fact, many temperate zone fishes that experience habitat (body) temperature variations of as much as 20 to 30°C, provide good examples of metabolic compensations to temperature (Somero, 1972). Adaptations will now be examined in endothermic and ectothermic enzymes, with reference to both the absolute temperature under which the enzymes must function, and the temperature fluctuations the enzymes must tolerate.

It has been suggested that the enzymes from cold-adapted ectothermic animals might show adaptation to lower temperatures by having a greater catalytic efficiency (i.e. a greater reaction rate and lower free energy of activation than the homologous endothermic enzymes (Somero et al., 1968; Somero and Hochachka, 1971; Hochachka and Somero, 1973; Low et al., 1973)). The activation energy (E_a), calculated from the maximum velocity (V_{max}), was one of the first and most commonly used indices of enzymatic efficiency (Somero et al., 1968; Hochachka and Somero, 1973). Since the free energy of activation (and also the reaction rate) depends on the entropy of activation as well as E_a, activation energies present only circumstantial evidence of catalytic efficiency. Nevertheless, many studies

have been done in which only the activation energy was measured, and correlations between E_a and body temperatures have been observed in some enzymes (Hochachka and Somero, 1971; 1973). Regardless of catalytic efficiency, one advantage of a low E_a in ectothermic enzymes is the decreased sensitivity of catalysis to temperature fluctuations, a problem which is much less serious in endothermic organisms, due to their relatively constant body temperature (Vroman and Brown, 1963).

Lower activation energies for ectothermic enzymes, compared to the homologous endothermic enzymes, have been observed for adenosine deaminase (Harbison and Fisher, 1973a), AMP-aminohydrolase (Zydowo and Purzycka-preis, 1972), myofibrillar ATPase (Bendall, 1969) and glyceraldehyde-3-phosphate dehydrogenase (Greene and Feeney, 1970). Similarly, E_a for trout citrate synthase (Hochachka and Lewis, 1970) is lower than for the pig enzyme (Kosicki and Srere, 1961), and extremely low E_a values have suggested adaptation in lactate dehydrogenase from Arctic fish (Behrisch, 1972) and succinic dehydrogenase from Antarctic fish (Somero et al., 1968). Moreover, correlations have been observed between E_a values and habitat temperature among ectotherms themselves. This has been noted for pyruvate kinase from fish and crustacea (Somero, 1969a) and pyruvate kinase from fish (Somero and Hochachka, 1968). Trout lactate dehydrogenase from warm acclimated fish has a higher E_a than from cold acclimated fish (Hochachka and Lewis, 1971). In addition to the above

examples, there have been a few cases for which it has been shown that E_a is not only lower in the ectotherm, but the free energy of activation is also lower (catalytic rate per mole of enzyme is higher). Some examples are glyceraldehyde-3-phosphate dehydrogenase (Cowey, 1967), glycogen phosphorylase-b (Assaf and Graves, 1969) and lactate dehydrogenase (Low et al., 1973). Similarly, E_a, and at low temperatures the free energy of activation, are lower in fish from cool waters as compared to those from equatorial regions (Johnston et al., 1973).

Some observations contradicting the above have also been noted. Rat pyruvate kinase has a lower activation energy than does trout pyruvate kinase (Somero and Hochachka, 1968). No relationship is observed between the E_a for lactate dehydrogenase and environmental temperature among crustacea and fish (Hochachka and Somero, 1968; Somero, 1969a). Similarly, no relationship is seen for acetycholinesterases among various species of fish (Baldwin, 1971). The E_a for warm and cold acclimated trout acetylcholinesterase (Baldwin and Hochachka, 1970) and isocitrate dehydrogenase (Moon and Hochachka, 1971) is very similar.

In the exceptions noted above, acetylcholinesterase has a very low activation energy and strong selection for lowering the E_a still further may therefore not exist. Similarly, although no correlation between environmental temperature and E_a was observed for fish and crustacean lactate dehydrogenases, a correlation was observed among the same animals for pyruvate kinase, perhaps because of its larger E_a, and hence greater

selective pressure, as compared to lactate dehydrogenase (Somero, 1969a). It has been suggested that E_a plays no role in immediate temperature compensation, but is involved in long-term (evolutionary) adaptation (Somero, 1969a). If this is the case, one might expect differences in E_a between ectotherms and endotherms, but perhaps little difference within each group. In all except one of the examples quoted above, in which ectotherms were compared with endotherms, the ectothermic enzyme did have the lower E_a.

Since substrate concentrations in vivo seldom reach saturating levels (Somero, 1972; Hochachka and Somero, 1973), the temperature dependence of V_{max} may be a highly misleading index of what is occurring in the cell (Somero, 1972). The other important kinetic parameter which determines the rate of an enzyme reaction is the "apparent enzyme-substrate affinity" which is often equated with the reciprocal of the Michaelis constant (K_m) (Somero and Hochachka, 1971). Since K_m is the substrate concentration required to reach half maximal saturation, lower K_m values (or higher ES affinities) will increase the reaction rate at subsaturating concentrations of substrates. In fact, most modulators of enzymes exert their influence by changing the ES affinity rather than V_{max} (Atkinson, 1968; Hochachka and Somero, 1971; Somero, 1972). It is now recognized that the regulation of the ES affinity is a common type of metabolic control (Atkinson, 1966).

For many enzymes, the K_m decreases as the temperature decreases, resulting in an increase in ES affinity which is

similar to the action of positive modulators and has therefore been called "positive thermal modulation" (Hochachka and Somero, 1973). The decrease in kinetic energy at low temperatures may therefore be partially or totally counteracted by the apparent enhanced affinity (Hazel and Prosser, 1974). The K_m-temperature plot is usually "U" or "J" shaped, with the minimum in K_m occurring near the lowest temperature normally experienced by the enzyme in nature; consequently, decreases in K_m with temperature are observed only within the physiological temperature range of the organism (Somero and Hochachka, 1971; Hochachka and Somero, 1973). Positive thermal modulation has been observed among fish lactate dehydrogenases (LDH) (Somero and Hochachka, 1969; Behrisch, 1972) and isocitrate dehydrogenases (Moon and Hochachka, 1971), turtle LDH (Beall and Privitera, 1973), frog LDH (Levy and Salthe, 1971), platypus and echidna LDH (Baldwin and Aleksiuk, 1973) and LDH, pyruvate kinase and glucose-6-phosphate dehydrogenase from aquatic poikilotherms (Somero, 1969a).

In many cases, ectotherms have been observed to produce several enzyme variants, depending on the acclimation temperature. Each variant has a minimum in K_m at or below the acclimation temperature, such that positive thermal modulation is always observed near the acclimation temperature. Hence, the minimum in K_m is observed at a higher temperature in enzymes from warm acclimated animals than from cold acclimated ones. Some examples are trout acetylcholinesterase (Baldwin

and Hochachka, 1970), isocitrate dehydrogenase (Moon and Hochachka, 1971; 1972) and fish lactate dehydrogenase (Hochachka and Lewis, 1971; Wernick and Künnemann, 1973). King crab pyruvate kinase exists in two variants of a single protein species, which are interconverted by temperature changes, each variant having low K_m values at the temperature at which it is the dominant form (Somero, 1969b).

Comparison between different species indicates a similar pattern. The minimum in K_m is observed near the lower temperature range on the organism for fish pyruvate kinase (Somero and Hockachka, 1968) and lactate dehydrogenase (Hochachka and Somero, 1968). Similarly, for LDH from the reptile Thamnophis sirtalis, the tropical and temperate genotypes each have minimum K_m values correlated to their environmental temperatures (Aleksiuk, 1971).

Positive thermal modulation, and the occurrence of minimum K_m values near the lower temperature range of the organisms have been well documented for ectotherms, as demonstrated by the above examples. Some reports indicate that these phenomena are less prevalent in endothermic enzymes. For example, K_m has a greater temperature dependence (i.e., greater positive thermal modulation) for lobster glycogen phosphorylase than for the rabbit enzyme (Assaf and Graves, 1969). The K_m for pyruvate kinase from seal flipper, a relatively ectothermic tissue, had a much higher temperature dependence than from the endothermic rat (Somero and Johansen, 1970). Although the minimum in K_m for pyruvate kinase was

observed at or near the lower temperature extreme of the habitat (body) temperature for various ectotherms, the minimum in K_m for the rat was observed at a temperature much lower than that of the body (Somero, 1969a). Acetylcholinesterase from mullet, ladyfish and goatfish displays U-shaped K_m-temperature curves; however K_m from tuna, which demonstrates some degree of endothermy, is temperature independent from 25 to 40°C (Baldwin, 1971). Similar results were reported for warm and cold acclimated fish: warm adapted animals have enzyme variants with less positive thermal modulation than cold adapted fish. This is observed for goldfish choline acetyltransferase (Hebb et al., 1972) and trout liver citrate synthase (Hochachka and Lewis, 1970). This suggests that positive thermal modulation is less pronounced in endothermic and warm adapted ectothermic organisms.

Exceptions to the above have, however, been noted. The K_m for rabbit and fish glyceraldehyde-3-phosphate dehydrogenase demonstrated similar temperature dependences in one study (Greene and Feeney, 1970) and the cod and lobster demonstrated smaller temperature dependences than rabbit in another (Cowey, 1967). The K_m for 6-phosphogluconate dehydrogenase decreased markedly with decreases in temperature in the enzyme from endothermic seal tissue (obtained from deep within the animal), whereas the enzyme from the heterothermic flipper tissue showed a lesser temperature dependence (Behrisch and Percy, 1974). There was a large difference in temperature dependence of V_{max} between hen and carp AMP-aminohydrolase,

but a lesser difference in the velocity at low substrate concentrations (Zydowo and Purzycka-Preis, 1972), indicating greater positive thermal modulation in the hen. At present, therefore, insufficient evidence is available to conclude that enzymes from ectothermic organisms have a special temperature-compensation mechanism as far as the relationship of K_m to temperature is concerned (Hazel and Prosser, 1974).

Some studies have been done on the first-order rate constant with respect to substrate (i.e., the rate at very low substrate concentrations). When related to the Michaelis-Menten equation,

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

this constant is given by V_{max}/K_m. At very low substrate concentrations, S is very much smaller than K_m and hence, the rate is given by $(V_{max}/K_m)[S]$. The temperature dependence of the first order rate constant, V_{max}/K_m can also be used as an index of adaptation in ectothermic and endothermic enzymes. For example, the E_a for chicken adenosine deaminase V_{max}/K_m values is about 12 kcal.mole^{-1}, whereas for the homologous scallop enzyme, E_a is about 0 (Harbison and Fisher, 1973a). Similarly, V_{max}/K_m has a larger temperature dependence in the hen than in the carp (Zydowo and Purzycka-Preis, 1972). Hence, E_a for V_{max}/K_m from ectotherms appears to be lower than from endotherms. In the latter example, the hen has a higher temperature dependence for both V_{max} and V_{max}/K_m.

However, the difference in temperature dependence between hen and carp is larger for V_{max} than for V_{max}/K_m (Zydowo and Purzycka-Preis, 1972). Hence, a correlation is observed between the temperature dependence of both V_{max} and V_{max}/K_m and the origin of the enzyme (endothermic or ectothermic), although "positive thermal modulation" is larger in the endotherm. Perhaps more consistent results could be obtained if the temperature dependence of V_{max}/K_m between endotherms and ectotherms is compared, rather than the temperature dependence of K_m. In addition, V_{max}/K_m may be much easier to interpret, relative to an enzyme model, than K_m (Harbison and Fisher, 1973b; 1974). Unfortunately, the temperature dependence of V_{max}/K_m has been examined much less frequently than the temperature dependence of K_m in studies on adaptation.

To this point, no mention has been made of the mechanisms by which thermal adaptation may occur. It has been mentioned above that enzymes from animals adapted to low temperatures may have a greater catalytic efficiency compared with enzymes from animals adapted to higher temperatures. It has been suggested that this difference in catalytic efficiency may be related to a decreased rigidity in the structure of ectothermic enzymes (Hochachka and Somero, 1973; Low and Somero, 1974. A comparison of ectothermic and endothermic lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and glycogen phosphorylase-b has indicated a high degree of compensation between the entropy and enthalpy of activation (i.e. enzymes with a high activation enthalpy also have a high

activation entropy) and this has led to the suggestion that more weak bonds are formed during activation in enzymes from ectotherms (Low and Somero, 1974). It has been suggested that increased rigidity in the endothermic enzymes may make it more difficult to form or break such bonds in these enzymes, with the result that catalytic rates are lower (Low and Somero, 1974). However, a high activation enthalpy for endothermic enzymes indicates that either fewer weak bonds are formed or more weak bonds are broken during activation. The actual number, or relative number (between enzymes), of bonds present in either the enzyme-substrate complexes or the activated complexes cannot be estimated from these studies.

Although decreased rigidity may increase catalytic rates, endothermic enzymes may benefit from increased rigidity if this reduces the rate of heat denaturation, since endothermic enzymes must normally function at relatively higher temperatures (Hochachka and Somero, 1973; Low and Somero, 1974). If rigidity affects both denaturation rates and catalytic rates (i.e. by increasing E_a), a negative correlation would be expected between activation energy and the denaturation rate. This has been observed for fish myofibrillar ATPase. Cold adapted fish have lower E_a values and greater denaturation rates than equatorial fish (Johnston et al., 1973; 1975).

To summarize, the general trends observed in thermal adaptation in enzymes are:

1) E_a (calculated from the temperature dependence of V_{max}) is usually lower in ectotherms than in endotherms and this may result in greater catalytic rates. E_a may play no role in immediate or short-term temperature compensation and differences might therefore not be observed between various ectotherms adapted to different temperatures (Somero, 1969a).

2) The K_m-temperature curve demonstrates adaptation in enzymes obtained from ectotherms (Hochachka and Somero, 1968; Somero, 1969a) but, at present, no definite correlation can be made between the degree of positive thermal modulation and endothermy or ectothermy (Hazel and Prosser, 1974).

3) V_{max}/K_m may be an important index of adaptation when comparing ectothermic and endothermic enzymes (Harbison and Fisher, 1973a).

4) These observations, especially the first point, might be explained by an increased rigidity in the enzyme molecule which may also be related to reduced sensitivity to heat denaturation (Low and Somero, 1974).

In the present investigation, the kinetics of beef heart, beef muscle and flounder muscle lactate dehydrogenase will be examined in detail in an attempt to answer the following questions. Can adaptation be observed in V_{max}, K_m or V_{max}/K_m? Is the catalytic efficiency greater in the ectothermic enzyme? What is the actual difference in the degree of bonding between enzyme and substrate in the homologous enzymes, how does this contribute to structural rigidity (as

measured by entropy changes) and how is this related to adaptation in V_{max}, V_{max}/K_m or K_m? What is the relationship between denaturation rates and the degree of bonding between enzyme and substrate? In order to answer these questions, data from the stopped-flow apparatus will be used to supplement information obtained from traditional steady-state studies.

B. The Role of Lactate Dehydrogenase

Lactate dehydrogenase (LDH)(EC 1.1.1.27) is an enzyme which lends itself readily to laboratory study. One of the products (or reactants, depending on the direction of the reaction) is NADH (nicotinamide adenine dinucleotide: reduced form), the concentration of which can be measured spectrophotometrically at 340 nm. The reaction therefore, need not be coupled with other reactions. This is especially useful in stopped-flow work. LDH is also relatively easy to purify and, if obtained commercially, is relatively inexpensive. Furthermore, it is very abundant in many tissues, such as flounder muscle, from which it was purified for this study.

LDH is an important enzyme in the metabolism of glucose through anaerobic fermentation. In most aerobic organisms, this has become a preparatory step for further oxidation of fermentation products by oxygen (Lehninger, 1970). During fermentation, each molecule of glucose is converted, by a series of enzymic reactions, to two molecules of pyruvate ($CH_3COCOOH$). At the same time, two molecules of ATP are produced and two molecules of NAD^+ are reduced to NADH. During glycolytic fermentation, the two molecules of NADH and pyruvate are then converted into NAD^+ and lactate ($CH_3CHOHCOOH$) by LDH. Hence, NAD^+ is conserved and the end products of glycolysis are lactate and ATP. This process requires no molecular oxygen (Lehninger, 1970). If oxygen

is available, pyruvate can be oxidized by removal of hydrogen atoms (or the equivalent electrons) to produce carbon dioxide. The electrons (or hydrogen) are transferred to NAD^+ to form NADH and H^+. This NADH, along with that formed during glycolysis up to the stage of pyruvate, then releases its electrons to the respiratory chain which donates the electrons to molecular oxygen forming water and ATP (Lehninger, 1970). In this case, NAD^+ is regenerated by the respiratory chain instead of by LDH.

During maximal activity of muscles, aerobic oxidation of glucose is supplemented by anaerobic glycolysis with a resultant increase in lactate. Hence LDH in muscles is important for the conversion of pyruvate to lactate and regeneration of NAD^+. The excess lactate diffuses into the blood and is later either further oxidized by the liver and heart, or converted into glucose or glycogen by the liver (Lehninger, 1970). In order to further oxidize the lactate, or convert it to glycogen by reversal of glycolysis, lactate must be reconverted into pyruvate. LDH is therefore, important in heart and liver for the conversion of lactate into pyruvate. The LDH reaction can therefore occur in either direction, depending on the tissue and metabolic requirements of the organism.

Electrophoresis has shown the existence of several isozymes of LDH (Markert and Moller, 1959; Fondy et al., 1964). There are two major isozymes, each consisting of four subunits of the same type, the heart isozyme (H_4) and the muscle isozyme

(M_4), so named because of the tissue from which they were first isolated and their differences in function (Kaplan, 1964). The remaining isozymes result from random aggregation of the two subunits (H_3M, H_2M_2 and HM_3). The H and M subunits appear to be under the control of different genes (Cahn et al., 1962; Kaplan, 1964; 1965). H_4LDH and M_4LDH are quite different kinetically (Cahn et al., 1962; Pesce et al., 1964; Kaplan, 1965). The K_m for pyruvate and the turnover numbers are lower for H_4LDH than for M_4LDH, the properties of H_2M_2LDH being intermediate between those of H_4 and M_4LDH (Kaplan, 1964)... Further, for chicken and beef LDH, the rate of heat denaturation is much lower for H_4LDH than for M_4LDH (Fondy et al., 1964).

The kinetics of the heart and muscle LDHs are related to their physiological role in their respective tissues (Kaplan and Goodfriend, 1964). The M type LDH is predominant in tissues such as muscle which is geared for sudden spurts of activity, and catalyses primarily the conversion of pyruvate into lactate. The H type LDH is present in aerobic tissues which perform sustained activity and converts lactate into pyruvate (see above; Kaplan and Goodfriend, 1964; Kaplan et al., 1968; Cahn et al., 1962). In birds, for example, the breast muscle of sustained flyers contains substantial amounts of the H type, whereas breast muscles of birds performing only short sudden flights contain primarily the M type LDH (Wilson et al., 1963). Under certain conditions, such as low oxygen tensions, the M type LDH increases in abundance

relative to the H type, as is seen in rat heart tissue (Penney, 1974). Conversely, under conditions of very high oxygen tensions, the production of the M subunit is suppressed and only the H type subunit is synthesized, even in skeletal muscle (Dawson et al., 1964).

The two types of LDH are seen in most animals (Kaplan, 1964). In fish, for example Trematomus, tuna and lungfish, the two major forms are present together with their hybrid forms, resulting in five possible isozymes (Hochachka and Somero, 1968). An exception to this simple rule is the trout, which possesses five different subunits (A, B, C, D, and E) resulting in 14 possible isozymes (Hochachka, 1966; Hochachka and Somero, 1968). The subunits which can interact in varying proportions to produce isozymes are A and B, B and C, and D and E. Fish isozyme patterns, like those of mammals and birds, are affected by oxygen availability. For example, during acclimation to higher temperatures, when oxygen availability is reduced (due to the low solubility at high temperatures and increased utilization), the M type LDH is observed to increase relative to the H type (Bolaffi and Booke, 1974). In many flatfish (which commonly live in environments with relatively low oxygen tensions) the M type LDH predominates, even in the heart tissue (Cahn et al., 1962; Kaplan, 1964; Cowey et al., 1969; Gesser and Poupa, 1973). Adaptation of LDH to temperature, as demonstrated by positive thermal modulation or the production of isozymes with different temperature characteristics, has been discussed in the previous section.

The differences in function of muscle and heart LDH result largely from differences in substrate inhibition and K_m values. At high pyruvate concentrations (above 2 mM), H_4LDH is inhibited by substrate, preventing conversion of large amounts of pyruvate into lactate. M_4LDH, however, still proceeds at near maximal velocities and can therefore continue to produce lactate (Cahn et al., 1962; Kaplan, 1961; Kaplan and Goodfriend, 1964; Kaplan et al., 1968). Inhibition is believed to occur by formation of an abortive ternary complex (LDH:NAD^+:pyruvate) (Fromm, 1961; Lee et al., 1965; Lee et al., 1966; Kaplan et al., 1968), and higher concentrations of pyruvate are required to produce this complex in the M_4 isozyme (Kaplan et al., 1968). Further, the higher K_m for M_4LDH (Pesce, 1964) prevents saturation of this isozyme until much higher concentrations of pyruvate are reached and the rate of catalysis of pyruvate therefore continues to increase with pyruvate concentration. In the present study, only substrate concentrations below inhibition were used and concentrations of inhibitory complexes were therefore never high enough to play any significant role in the kinetics.

C. Mechanism of Action of LDH

Lactate dehydrogenase has been studied extensively, and several reviews on this enzyme have been published (Kaplan, 1964; 1965; Laidler and Bunting, 1973). The enzyme has a molecular weight of 130,000 to 154,000 and consists of four subunits (Fromm, 1963; Kaplan, 1964; 1965), each of which can bind one substrate and one coenzyme molecule (Fromm, 1963). Since the transient observed in pre-steady-state experiments can be described by a single exponential, it has been suggested that the four sites of the molecule are kinetically independent and identical (Bennett and Gutfreund, 1973).

Several types of experimental studies, including equilibrium isotope exchange (Silverstein and Boyer, 1964) and product inhibition (Zewe and Fromm, 1962), indicate that the mechanism is ordered and passes through a ternary complex. A simplified model of the reaction, and the one used in the following sections, can be represented as follows:

$$E \underset{k_{-1}}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2 B}{\rightleftharpoons}} EAB(\text{or } EXY) \underset{k_{-3}X}{\overset{k_3}{\rightleftharpoons}} EY \underset{k_{-4}Y}{\overset{k_4}{\rightleftharpoons}} E$$

where E is the free enzyme, A is NAD^+, B is lactate, X is pyruvate and Y is NADH. In addition to the above active complexes, at least two abortive ternary complexes can also be formed, EAX and EBY (Zewe and Fromm, 1962; Fromm, 1963). EAX is formed by adding pyruvate to the EA complex and EBY by adding

lactate to the EY complex. These two complexes are believed to be responsible for substrate inhibition in the reverse and forward directions respectively (Fromm, 1961; Zewe and Fromm, 1962; Lee et al., 1965; Lee et al., 1966; Kaplan et al., 1968). Recent transient-kinetic studies have added to the number of steps used in some models. For example, a second EY complex has been included, resulting in an additional isomerization step between two forms of EY (e.g. Criddle et al., 1968). Gutfreund and coworkers routinely use a model for LDH containing at least 11 steps (Bennett and Gutfreund, 1973; Holbrook and Gutfreund, 1973; Stinson and Gutfreund, 1971).

A great deal of pre-steady-state work has been done on LDH, the objective usually being to elucidate further details of the reaction mechanism. The pre-steady-state kinetics of pig heart LDH in the lactate to pyruvate direction indicate an initial burst in the reaction rate, followed by a slower steady-state rate (Holbrook and Gutfreund, 1973; Whitaker et al., 1974). The steady-state is reached in about 20 milliseconds. The rate-limiting step in the steady-state has been attributed to either the release of NADH or the isomerization of two EY complexes (Criddle et al., 1968; for a review, see Laidler and Bunting, 1973). In our model this is equivalent to step 4 (i.e., k_4). The initial burst corresponds to a step previous to the EY complex. Hence an initial high rate of production of EY is observed, followed by a slower steady-state rate once most of the enzyme is in the form of the EY complex. In the pig muscle LDH, no initial transient can be

followed on the stopped-flow apparatus; if oxamate is added to the reaction mixture, a transient is observed, similar to that found in the heart enzyme (Bennett and Gutfreund, 1973). The oxamate is believed to bind to the EY complex, forming a ternary complex which prevents the release of NADH, thereby slowing down the steady state and reproducing a situation similar to that found in the pig heart. This difference between pig heart and pig muscle will also be observed between beef heart and beef muscle, as discussed later.

The equilibrium for the lactate dehydrogenase reaction lies heavily in favour of lactate and NAD^+ (Lehninger, 1970: ΔG^o =-6 kcal.). Hence it is possible to do experiments in the pyruvate to lactate direction in which the NADH concentration is below that of the enzyme, resulting in only a single turnover of the enzyme. Almost all of the NADH is converted into NAD^+. At high pyruvate concentrations, the transient for the single turnover is equivalent to the catalytic rate constant for the pig muscle enzyme (Stinson and Gutfreund, 1971). Since the release of NAD^+ cannot be rate limiting (i.e. the enzyme is not regenerated since catalysis is completed after a single turnover), the rate limiting step in both the single turnover experiment and the steady-state must be step -2 in our model. These same phenomena will be observed in all the LDHs discussed later.

All of the above studies have been conducted at room temperature. Südi (1974 a and b) studied the transient kinetics of

pig heart LDH at 6.3°C. In a fairly comprehensive study, in which both absorption and fluorescence in both directions were monitored, Sŭdi obtained similar results to those described above, but was also able to observe a second exponential in the transient in the pyruvate to lactate direction using fluorescence. Six rate constants were calculated, corresponding to k_2, k_{-2}, k_3 and k_{-3} in the above model and two rate constants for the isomerization of EAB and EXY. The slowest step observed in the forward direction corresponded to the release of pyruvate (k_3) and the slowest step in the reverse direction was calculated to be interconversion of the two ternary complexes.

It is important to point out that, although a considerable amount of work has been done on LDH, none of the pre-steady-state experiments have been carried out at more than one temperature. Activation enthalpies are therefore, not reported in any of the above studies. If adaptation to temperature is to be examined in homologous enzymes, it is necessary to obtain activation enthalpies for the rate constants in the above model. The object of the present study was to obtain as many of the rate constants as possible, and their temperature dependences, in homologous enzymes obtained from both ectothermic and endothermic animals.

II

THEORETICAL

The model for the reaction mechanism has been presented in part C of the introduction. As mentioned previously, additional steps can be included in the model. However, the steady-state data combined with pre-steady-state data in either the forward or reverse direction are interpretable in terms of the above scheme, and all eight rate constants can be calculated. Inclusion of additional steps would make interpretation of the results very cumbersome, and all rate constants could not be calculated from the experimental data. For one of the enzymes (beef heart LDH) information will be presented which suggests additional steps, and this will be considered in the discussion.

The analysis of the steady-state data will be discussed first, followed by the pre-steady-state kinetics in the forward (lactate to pyruvate) direction and a comparison of pre-steady-state and steady-state kinetics. Finally, the pre-steady-state in the reverse direction will be examined, and the results compared with the steady-state. Pre-steady-state kinetics in the two directions can be examined independently of one another since it is necessary to have pre-steady-state data in only one direction in order to calculate all eight rate constants; the above model can therefore be tested and, if necessary, improved.

Steady-State Kinetics

The steady-state equation for the forward direction for an ordered ternary complex mechanism given above can be written

as (Laidler and Bunting, 1973)

$$\vec{v} = \frac{\vec{V}_{max}[A][B]}{K_{AB} + K_{mA}[B] + K_{mB}[A] + [A][B]} \qquad (1)$$

Here K_{mA} is the Michaelis constant with respect to A (NAD^+), K_{mB} is the Michaelis constant with respect to B (lactate), and K_{AB} is an additional constant which can be obtained from kinetic measurements with subsaturation concentrations of both A and B (see Materials and Methods). The explicit forms of the four constants, in terms of the individual rate constants, are:

$$\vec{V}_{max} = \frac{k_3 k_4 [E]_o}{(k_3 + k_4)} \qquad (2)$$

$$K_{mA} = \frac{k_3 k_4}{k_1 (k_3 + k_4)} \qquad (3)$$

$$K_{mB} = \frac{k_4 (k_{-2} + k_3)}{k_2 (k_3 + k_4)} \qquad (4)$$

$$K_{AB} = \frac{k_{-1} k_4 (k_{-2} + k_3)}{k_1 k_2 (k_3 + k_4)} \qquad (5)$$

The following relationships are useful in obtaining some of the rate constants:

$$\frac{\vec{V}_{max}}{K_{mA}} = k_1 [E]_o \qquad (6)$$

$$\frac{K_{mB}}{K_{AB}} = \frac{k_1}{k_{-1}} \qquad (7)$$

If $[E]_o$, the total enzyme concentration, is known, k_1 and k_1/k_{-1}, and hence k_{-1}, can be determined from steady-state kinetics alone. Similarly, if the pyruvate → lactate reaction is studied the steady-state results give k_{-4} and k_4/k_{-4}, and hence k_4.

For the reaction in the pyruvate → lactate direction the steady-state equation is:

$$\overleftarrow{v} = \frac{\overleftarrow{V}_{max}[X][Y]}{K_{XY} + K_{mX}[Y] + K_{mY}[X] + [X][Y]} \qquad (8)$$

where, for example

$$\overleftarrow{V}_{max} = \frac{k_{-1}\, k_{-2}\, [E]_o}{k_{-1} + k_{-2}} \qquad (9)$$

$$K_{mX} = \frac{k_{-1}\,(k_{-2} + k_3)}{k_{-3}\,(k_{-1} + k_{-2})} \qquad (10)$$

Combining equations 2, 4, 9 and 10 gives

$$\frac{\overleftarrow{V}_{max}/K_{mB}}{\overleftarrow{V}_{max}/K_{mX}} = \frac{k_2 k_3}{k_{-2} k_{-3}} \qquad (11)$$

The Michaelis constants for B and X (the second substrates to be added on) are mathematically cumbersone and are of little value in providing rate constants unless further information is available. Some simplification is obtained if the Michaelis constant is divided by the maximum velocity; for example

$$\frac{K_{mB}}{\overleftarrow{V}_{max}} = \frac{k_{-2} + k_3}{k_2 k_3 [E]_o} = \frac{k_{-2}}{k_2 k_3 [E]_o} + \frac{1}{k_2 [E]_o} \qquad (12)$$

$$\frac{K_{mX}}{\vec{V}_{max}} = \frac{k_{-2} + k_3}{k_{-2}k_{-3}[E]_o} = \frac{1}{k_{-3}[E]_o} + \frac{k_3}{k_{-2}k_{-3}[E]_o} = C + D \qquad (13)$$

The symbol C will be used to represent either $1/k_{-3}[E]_o$ or $k_3/k_{-2}k_{-3}[E]_o$, and D to represent the remaining term. The terms to which C and D actually relate are deduced on the basis of experiment, as discussed later (see page 32). The value of C + D can be obtained either from $K_{mX}/\vec{V}_{max}$ or $K_{mB}/\vec{V}_{max}$, multiplied by $k_2k_3/k_{-2}k_{-3}$.

The experimental values of C + D can then be plotted as an Arrhenius plot. Both C and D should be linear on such a plot, and a curve equal to the summation of two lines on the Arrhenius plot can be fitted to the data. These two lines can be labelled C and D, but it is not known which is which. The radio C/D is equal to

$$\frac{C}{D} = \frac{k_{-2}}{k_3} \quad \underline{or} \quad \frac{k_3}{k_{-2}} \qquad (14)$$

From steady-state data alone it is not possible to decide between the two possibilities; a procedure for deciding is considered later (see page 32).

If however, it can be shown that k_{-2} is much smaller than k_{-1} (see Results, part A; Stopped-flow measurements in the reverse direction), the K_m for pyruvate can be simplified without the necessity of dividing through by $\vec{V}_{max}$. K_m reduces to

$$K_{mX} = \frac{k_{-2}}{k_{-3}} + \frac{k_3}{k_{-3}} \qquad (15)$$

K_{mX} can now be plotted on an Arrhenius plot in the same way that C + D was plotted. Again, the data should fit on a curve

obtained by summing two straight lines on such a plot. The equation for V_{max}, of course, also simplifies.

$$\overset{+}{V}_{max} = k_{-2}[E]_o \qquad (16)$$

To summarize, steady state measurements can lead to:

(1) k_1 and k_{-1} from studies in the lactate → pyruvate direction.

(2) k_4 and k_{-4} from studies in the pyruvate → lactate direction.

(3) $1/k_{-3}[E]_o$ and $k_3/k_{-2}k_{-3}[E]_o$, but one cannot determine which is which; and,

(4) if it can be shown that $k_{-2} << k_{-1}$, it is also possible to obtain k_{-2} and $\frac{k_{-2}}{k_{-3}} + \frac{k_3}{k_{-3}}$.

Pre-Steady-State Kinetics in the Forward Direction

The pre-steady-state equations for an ordered ternary-complex mechanism have been worked out by Hijazi and Laidler (1973). There are three kinetic intermediates and a triphasic exponential rise to the steady-state would be expected (Maguire et al., 1974). In the present work, only a single exponential was observed (see Figures 2 and 6); this indicates that two of the steps in the above mechanism are too fast to be detected on the stopped-flow apparatus (Maguire et al., 1974). If the slow step observed in the pre-steady-state is k_3 ($k_3 << k_2B$), an equilibrium can be assumed between E, EA and EAB:

$$EAB = \frac{k_2[B]\ [EA]}{k_{-2}} \qquad (17)$$

and

$$EA = \frac{k_1[A]\ [E]}{k_{-1}} \qquad (18)$$

The total enzyme concentration E_o is thus

$$[E]_o = [E] + [EA] + [EAB] + [EY] = [E](a) + [EY] \qquad (19)$$

where

$$a = 1 + \frac{k_1[A]}{k_{-1}} + \frac{k_1 k_2[A]\ [B]}{k_{-1}\ k_{-2}} \qquad (20)$$

The rate of change of EY with time is

$$\frac{d[EY]}{dt} = k_3\,[EAB] - k_4\,[EY] \qquad (21)$$

whence

$$\frac{d[EY]}{dt} = \frac{k_1 k_2 k_3 [E]_o[A][B]}{k_{-1}\ k_{-2}(a)} - \left[\frac{k_1 k_2 k_3\ [A][B]}{k_{-1}\ k_{-2}(a)} + k_4\right][EY] \qquad (22)$$

Integration and substitution for (a) leads to

$$EY = \text{const.}\ (1-e^{-\lambda t}) \qquad (23)$$

where

$$\lambda = \frac{k_1 k_2 k_3\ [A][B]}{k_{-1}k_{-2} + k_1 k_{-2}[A] + k_1 k_2[A][B]} + k_4 \qquad (24)$$

Since the rate of product (Y) formation is given by $d[Y]/dt = k_4[EY]$, substitution and integration leads to

$$Y = v_{ss}t + C\,e^{-\lambda t} + C' \qquad (25)$$

where v_{ss} is the steady-state velocity and C and C' are constants. With saturating concentrations of A, λ simplifies to

$$\lambda = \frac{k_3\,[B]}{\frac{k_{-2}}{k_2} + [B]} + k_4 \qquad (26)$$

A plot of Y against time yields $v_{ss} + C'$, and λ can then be obtained by plotting log $(Y - C' - v_{ss})$ against time (Hijazi and Laidler, 1973).

The concentration of X as a function of time is expressed in a similar manner as for Y; the value of λ is the same, but the values of the two constants are in general different. The formation of Y must show an initial lag, which is caused by the time required for the concentrations of the intermediates to reach steady-state levels. However, if (and only if) k_4 is rate limiting in the steady-state the concentration X might show an initially high rate of production ("initial burst") (see Laidler and Bunting, 1973).

In our experiments the reaction was monitored by observing the absorption at 340 nm; NADH absorbs at this wavelength whether or not it is bound to the enzyme. With no NADH or pyruvate present at the beginning of the measurements, the sum of the concentrations of NADH and E:NADH is thus equal to the concentration of pyruvate formed. The technique thus follows the production of the first product X.

In the experiments to be described, an initial burst was observed in the production of X (Figure 2), and this indicates that reaction 4 is rate limiting in the steady-state (i.e. the rate limiting step must occur after formation of X). The constant λ is thus approximately

$$\lambda = \frac{k_3 [B]}{\frac{k_{-2}}{k_2} + [B]} = \frac{\vec{\lambda}_{max} [B]}{K_{\lambda B} + [B]} \qquad (27)$$

Variation of B thus gives the constants k_3 and k_{-2}/k_2. The latter ratio, $K_{\lambda B}$ is the concentration of B at half-maximal pre-steady-state velocities, and is analogous to the Michaelis constant in steady-state studies.

The hyperbolic relationship between λ and B (equation 27) is observed if, as stated above, k_3 is the slow step in the pre-steady-state. Alternatively, if k_2 is the slow step, it can be shown that λ would vary linearly with B. Finally, if k_1 were the slow step, λ would be independent of B. The experimentally observed hyperbolic relationship (Figure 8) indicates that, according to the model used, k_3 must be the slow step.

To summarize, the pre-steady-state studies provide evidence that reaction 4 is the slow step in the steady-state, and reaction 3 in the pre-steady-state. The measurements also allow values of k_3 and k_{-2}/k_2 to be determined. The remaining kinetic constants are obtained from steady-state studies, in conjunction with the stopped-flow measurements.

Combined Pre-Steady-State (Forward) and Steady-State Data

Equation 27, from the pre-steady-state, gives k_3. Multiplication of k_3 by C/D and by D/C (equation 14) gives two values, one of which is equal to k_{-2}. Substitution back into the original equations for K_m/V_{max} [eq. (12) and (13)] for both of the possibilities gives two alternative estimates for $k_{-3}[E]_o$ $k_3/k_{-2}[E]_o$ and $k_2[E]_o$ Division of k_{-2} by $k_2[E]_o$ then gives two estimates for $k_{-2}/k_2[E]_o$. However, k_{-2}/k_2 was determined from the pre-steady-state data, and one can compare its temperature dependence with the two estimates for

$k_{-2}/k_2[E]_o$. In this way, it is possible to determine which estimate of k_{-2}/k_2 is correct, i.e., which terms C and D are equal to.

Further substitution then gives all the constants k_2, k_{-2}, k_3 and k_{-3}, and gives the value of $[E]_o$.

Pre-Steady-State Kinetics in the Reverse Direction

As previously mentioned, the equilibrium of the overall reaction lies heavily in favour of lactate + NAD^+, and it is possible to follow the reaction in the direction pyruvate + lactate even when very low concentrations (eg. 5 μM) of NADH are used. The binding of NADH to beef heart lactate dehydrogenase is sufficiently strong that, in a solution containing for example 10 μM NADH and a slightly higher enzyme concentration, essentially all of the NADH is bound to the enzyme and the binding is too fast to be observed in the stopped-flow apparatus. With the concentration of NADH below that of the enzyme, the free enzyme is not regenerated; only a single turnover of the enzyme is observed (Stinson and Gutfreund, 1971), and the release of NAD^+ (k_{-1}) would not be rate limiting. It is thus possible to obtain the rate constant associated with the release of lactate from the ternary complex, and the binding constant of pyruvate to the enzyme. Together, the pre-steady-state and steady-state results therefore permit the calculation of all of the rate constants in the four-step model.

The scheme with which the pre-steady-state results are concerned is

$$E + Y \underset{k_4}{\overset{k_{-4}[Y]}{\rightleftharpoons}} EY \underset{k_3}{\overset{k_{-3}[X]}{\rightleftharpoons}} EAB \xrightarrow{k_{-2}} EA + B$$

This scheme suggests a multi-phasic approach to the equilibrium, similar to the tri-phasic exponential expected for the forward direction. Again, however, only a single exponential is observed (Figures 3 and 7). The relationship between λ and $[X]$ depends on which step is rate limiting, and is analogous to the pre-steady-state in the forward direction. If the rate limiting step for the pre-steady-state in the reverse direction is k_{-2}, equilibrium exists up to EAB and hence,

$$[EY] = \frac{k_{-4}[E][Y]}{k_4} \qquad (28)$$

$$[EAB] = \frac{k_{-3}\, k_{-4}[E][Y][X]}{k_3\, k_4} \qquad (29)$$

and the total enzyme concentration is

$$[E]_o = [E] + [EY] + [EAB] + [EA] = [E]f + [EA] \qquad (30)$$

where

$$f = 1 + \frac{k_{-4}[Y]}{k_4} + \frac{k_{-3}\, k_{-4}[X][Y]}{k_3\, k_4} \qquad (31)$$

The rate of production of EA is

$$\frac{d[EA]}{dt} = k_{-2}[EAB] \qquad (32)$$

and combining this with (29), (30) and (31) gives

$$\frac{d[EA]}{dt} = \frac{k_{-2}\, k_{-3}\, k_{-4}[X][Y]\left([E]_o - [EA]\right)}{k_3\, k_4\left[1 + \frac{k_{-4}[Y]}{k_4} + \frac{k_{-3}\, k_{-4}[X][Y]}{k_3\, k_4}\right]} \qquad (33)$$

If the concentration of X (pyruvate) is much larger than that of the enzyme, only a small proportion of the pyruvate is consumed so that its concentration is approximately constant. However, the concentration of Y (NADH) is lower than that of the enzyme and [Y] is therefore not a constant. Equation 33 thus cannot be integrated. This equation suggests a hyperbolic relationship between d[EA]/dt and [Y]. At sufficiently high concentrations of Y, equation 33 becomes

$$\left(\frac{d[EA]}{dt}\right)_{[Y]\to\infty} = \frac{k_{-2}\{[E]_o - [EA]\}[X]}{\frac{k_3}{k_{-3}} + [X]} \qquad (34)$$

This assumes, therefore, that the pre-steady-state is unaffected by changes in the concentration of Y (NADH). Experimentally, no detectable difference was observed in the pre-steady-state rate when 5 or 10 μM NADH was used. Hence with 10 μM NADH, use of equation 34 appears justified. Since the concentration of X is constant, this can be integrated to give

$$[EA] = [B] = \text{const}\ (1 - e^{-\lambda t}) \qquad (35)$$

where

$$\lambda = \frac{k_{-2}[X]}{\frac{k_3}{k_{-3}} + [X]} \qquad (36)$$

This is of the form of the Michaelis equation and is conveniently written as

$$\lambda = \frac{\overleftarrow{\lambda}_{max}[X]}{K_{\lambda X} + [X]} \qquad (37)$$

The parameter λ can be obtained from a plot of log (1 - [B] / const.) against time (eq. 35), and by varying [X] the quantities $\overleftarrow{\lambda}_{max}$ and $K_{\lambda X}$ can be obtained, as was previously demonstrated for the forward direction. Experimentally, the relationship between λ and [X] is hyperbolic (Table 11), like the relationship between λ and [B] (Figure 8). Hence, using the same argument as in the forward direction, the slowest step in the pre-steady-state must be k_{-2}. If k_{-3} were the slow step, the relationship between λ and [X] would be linear.

To summarize, single turnover experiments in the stopped-flow apparatus indicate that k_{-2} is much smaller than k_{-3} [X], and permit the determination of k_{-2} and k_3/k_{-3} (equation 36).

Combined Pre-Steady-State (Reverse) and Steady-State Data

For the reverse direction, the maximal steady-state and pre-steady-state rates prove to be identical (see Figures 16 and 22). Hence, k_{-2} must be rate limiting in the steady-state as well as the pre-steady-state ($k_{-2} \ll k_{-1}$; equations 9 and 16). K_{mX} therefore reduces to equation 15. Subtraction of k_3/k_{-3} (equation 37) from K_{mX} then gives k_{-2}/k_3. Since k_{-2} is known, k_3 can be obtained, and k_{-3} can be obtained by multiplying (k_{-3}/k_3) by k_3. Finally, k_2 can be obtained by dividing equation 11 by $k_3/k_{-2}k_{-3}$. All eight rate constants are thus obtained. The intermediate four rate constants, k_2, k_{-2}, k_3, and k_{-3}, are obtained twice, once using pre-steady-state data in the forward direction

and a second time using pre-steady-state kinetics in the reverse direction.

Calculation of $\Delta G^{\neq}$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$

From their temperature dependence the enthalpies and entropies of activation can be calculated, use being made of the relationships (Glasstone, Laidler and Eyring, 1941; Laidler, 1965, 1969):

$$k = \frac{\underline{k}T}{h} e^{-\Delta G^{\neq}/RT} \quad (38)$$

$$= \frac{\underline{k}T}{h} e^{\Delta S^{\neq}/R} e^{-\Delta H^{\neq}/RT} \quad (39)$$

where $\Delta H^{\neq}$ is related to the experimental activation energy E_{act} by

$$\Delta H^{\neq} = E_{act} + RT, \quad (40)$$

$\underline{k}$ is the Boltzmann constant and h Planck's constant.

III

MATERIALS AND METHODS

A. Enzyme Preparation

i. Enzyme Isolation

Beef heart and beef muscle LDH, lactate, pyruvate, NAD^+ and NADH were obtained from the Sigma Chemical Company, St. Louis, Mo. Flounder muscle LDH was extracted from flounder obtained in ocean trawls near St. Andrews, New Brunswick, according to the following procedure. The fish were frozen immediately and kept frozen until enzyme was required. The fish were then thawed, filleted and homogenized in a blender in an equal volume of the same buffer used in the kinetic studies (phosphate, 0.05M, pH 7.4). The slurry was centrifuged at 30,000 g for 30 minutes. Further purification of the enzyme was dependent on the type of analysis performed.

ii. Affinity Chromatography

For steady-state work, the centrifuged homogenate was purified on an affinity chromatography column of AH sepharose 4B (Pharmacia Fine Chemicals) to which oxamate was attached (O'Carra and Barry, 1972; Spielmann et al., 1973). The sepharose was permitted to swell in excess 0.5M NaCl overnight, was washed several times in the same solution and then in distilled water. For every 2.5 grams of sepharose (which makes approximately 10 ml of gel) 0.5 g or more of potassium oxalate was added, followed by 0.75 g or more of 1-ethyl-3 (3-dimethyl-aminopropyl) carbodiimide, in a final volume of about 20 ml. This mixture was stirred gently and permitted to react overnight. Additional oxalate and carbodiimide was added each successive day or until the sulfonate test showed

a yellow colour (0.2 - 0.5 ml gel slurry in 1 ml saturated sodium borate plus 3 drops of 3% sodium 2,4,6-trinitrobenzene sulfonate, permitted to react for 30 minutes). Unreacted AH sepharose is orange in colour with the sulfonate test. When the reaction was complete, the gel was washed with phosphate buffer and packed in a small column (0.9 X 10 cm).

Oxamate is a powerful inhibitor of LDH and binds to the enzyme whenever NADH is present, which permits one step purification using the oxamate-sepharose column (O'Carra and Barry, 1972; Spielmann, et al., 1973). The crude homogenate is put onto the column and flushed-through using phosphate buffer containing 0.1 mM NADH. This results in the formation of a LDH-oxamate-sepharose complex. Next the column is washed with buffer containing 0.1 mM NADH, and the bound LDH is subsequently removed by flushing with straight buffer. The resulting purification of the enzyme is shown in Table I. Fractions 1 to 5 were eluted with 0.1 mM NADH, and 6 to 9 with 0.01 mM NADH. Once the NADH is omitted (fractions 10 to 12), the LDH is eluted from the column with an approximately 40-fold purification and a specific activity of 1080 units/mg. The turnover number at 20°C for pyruvate reduction (Figure 22) was calculated to be 660 sec^{-1}. This value is obtained from stopped-flow studies independent of enzyme concentration. Assuming a monomer molecular weight of 35,000 (see Introduction, part B), this leads to a specific activity of 1,130 units/mg. Hence, the LDH from fraction 11 in Table I appears to be quite pure.

TABLE I

Affinity Column Purification

Fraction #	Volume (ml)	Activity (Units/ml)	Total Activity (Units)	Protein (mg/ml)	Specific Activity (Units/mg)
crude	1.5	200	300	7.2	27.8
1	10	0	0	0.91	0
2	10	0	0	0.25	0
3	10	0	0	0.004	0
4	10	0	0	0	-
5	10	0	0	0	-
6	10	0	0	0	-
7	10	0	0	0	-
8	10	0	0	0	-
9	10	0	0	0	-
10	10	1.3	13	0.001	1300
11	10	27	270	0.025	1080
12	10	0	0	0	-

1 unit = 1 μM NADH oxidized per minute

enzyme activity recovered = 283/300 = 94%

protein recovered = (1.19 x 10) / (7.2 x 1.5) = 100%

purification = 1080/27.8 = 39 fold

Specific activity obtained in another purification attempt was 1,120 units/mg. (The calculated specific activity for fraction 10 in Table I is probably less acurate because of the small amount of protein present). This preparation was found to run as a single band on disc gel electrophoresis.

iii. Ammonium Sulfate Preparation

For stopped-flow work the crude homogenate was partially purified by ammonium sulfate fractionation, which permitted much larger quantities of LDH to be collected. Approximately 70% of the activity is recovered within the 40 to 50% saturation fraction. Purification, as indicated by specific activity, was approximately 5-fold. LDH activity per unit absorbance at 340 nm however, was increased over 15-fold by this procedure. Stopped-flow work cannot be performed using the crude homogenate because total absorption at 340 nm (the wavelength used in all kinetic studies) is too high. The ammonium sulfate purified LDH therefore, was used for all stopped-flow work. Since the steady-state kinetics of column purified and ammonium sulfate purified LDH are identical, it is assumed that pre-steady-state kinetics will not be affected by these procedures.

iv. Protein Determination

Protein concentration was determined by the method of Groves, Davis and Sells (1968). Since nucleic acids have an absorption minimum at approximately 230 nm, it is possible to choose, for each buffer system used, a wavelength above

230 nm at which nucleic acids have the same absorbance as at 224 nm. For the phosphate buffer system used in this study, the wavelengths 224 and 239 were found to have the same absorbance. The absorption of protein at 224 less that at 239 is then proportional to the protein concentration, and is independent of the nucleic acid concentration. The average difference in absorption for the two wavelengths using 10 μg/ml beef heart LDH, α-chymotrypsin, β-galactosidase, alkaline phosphatase or bovine serum albumin was found to be 0.09. The standard deviation was calculated to be 20%, which is similar to the standard deviation calculated using the standard Lowry method (19%). Groves, Davis and Sells have found that this method is less sensitive to variation in primary protein structure (i.e., variations in amino acid composition) than the Lowry method.

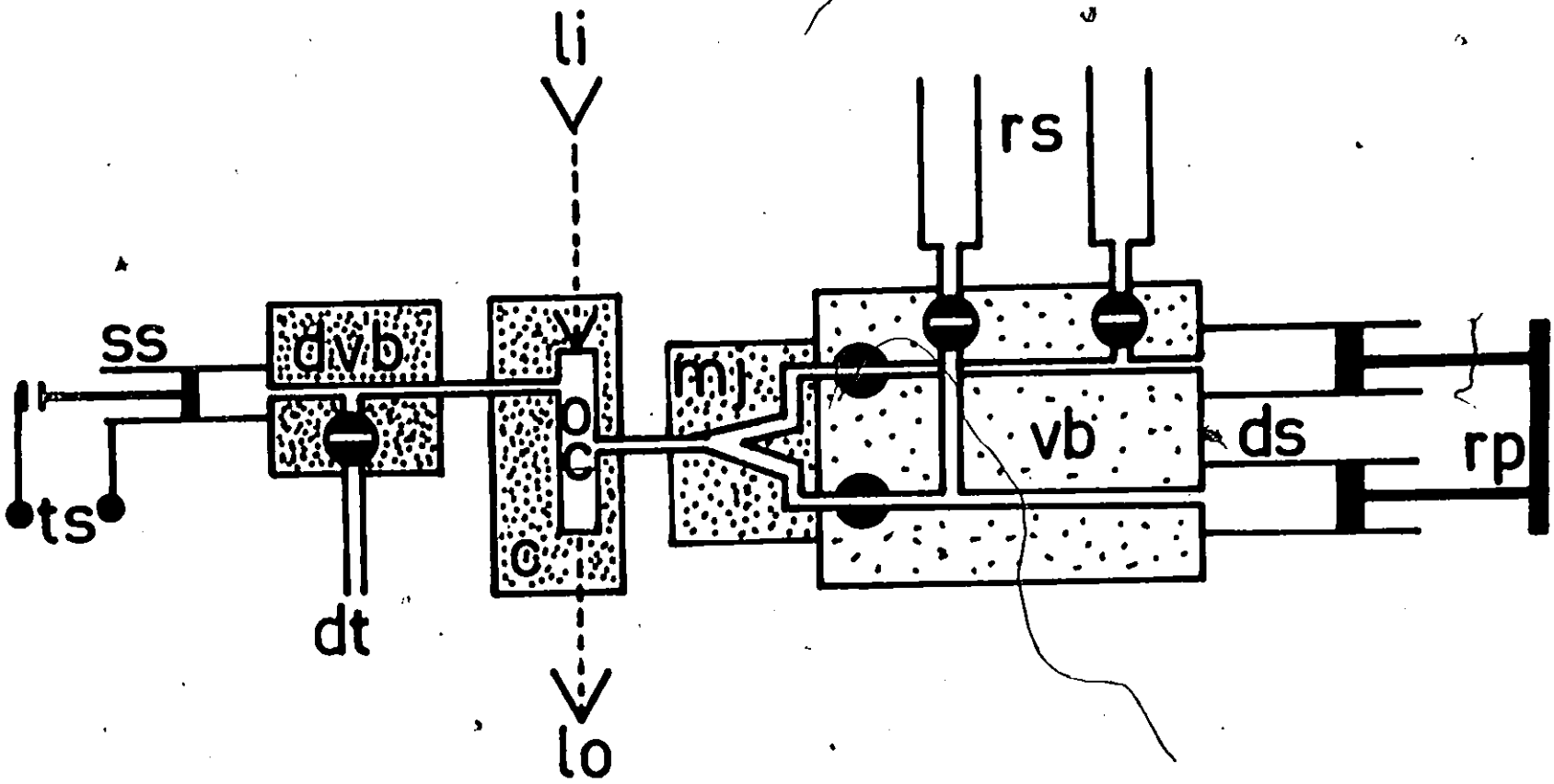
B. Enzyme Analysis

Enzyme concentrations of 0.25 mg/l were used for the steady-state work, 200 mg/l were used to measure the pre-steady-state (beef heart) and 500 mg/l were used for the single turnover experiments. 10 μM NADH was used for the single turnover experiments, and all experimental work was carried out in 0.05M phosphate buffer, pH 7.4.

A Unicam SP-1800 spectrophotometer was used for the steady-state work. The pre-steady-state was followed using a Durrum-Gibson stopped-flow apparatus, shown schematically in Figure 1. The enzyme and substrate solutions are stored in the two reservoir syringes on top of the stopped-flow

Figure 1

A schematic representation of the stopped-flow apparatus. Legend: C - cuvette, dt - drain tube, dvb - drain valve block, ds - drive syringes, li - light input, lo - light output, mj - mixing jet, oc - observation chamber, rp - ram plunger, rs - reservoir syringes, ss - stop syringe, ts - trigger switch, vb - valve block.


li
rs
ss
dvb
m
oc
c
vb
ds
rp
ts
dt
lo

apparatus. The drive syringes are filled from these reservoirs. During an experiment, the ram plunger (operated by compressed nitrogen) injects the solutions into the mixing jet and then through to the observation chamber. Simultaneously, the solution from the previous experiment is ejected and forces the stop syringe out, when then triggers the oscilloscope. It takes 3 to 5 milliseconds to inject and mix the reactants and start recording. Most of the stopped-flow observations were made in the first 50 to 100 ms of the reaction. The signal from the photomultipier tube is displayed on the storage oscilloscope, to which a polaroid camera is attached to photograph the observed trace. An example of a pre-steady-state experiment in the forward direction is shown in Figure 2, and a single turnover experiment in the reverse direction is shown in Figure 3. Transmittance is measured at 340 nm.

The steady-state measurements were made over the temperature range from 5 to 50°C. A Lauda model-NB-D constant temperature refrigerated bath was coupled to the spectrophotometer cell housing and the temperature was checked with a Yellow Springs model-43 telethermometer. Pre-steady-state measurements (for beef heart LDH only) were made from 0 to 15°C, the pre-steady-state being too fast to follow at higher temperatures. Single turnover experiments were made up to 50°C. The temperature for the stopped-flow apparatus was controlled by means of a Haake model-KT-33 low temperature circulator.

Figure 2

Pre-steady-state in the lactate to pyruvate direction. The straight line represents the steady-state and the initial exponential approach to the steady-state is the pre-steady-state. Vertical scale: 95-100% transmittance; horizontal scale: 0-100 milliseconds. Lactate: 7.5 mM; NAD^+ : 2 mM; T : $4^{\circ}C$.

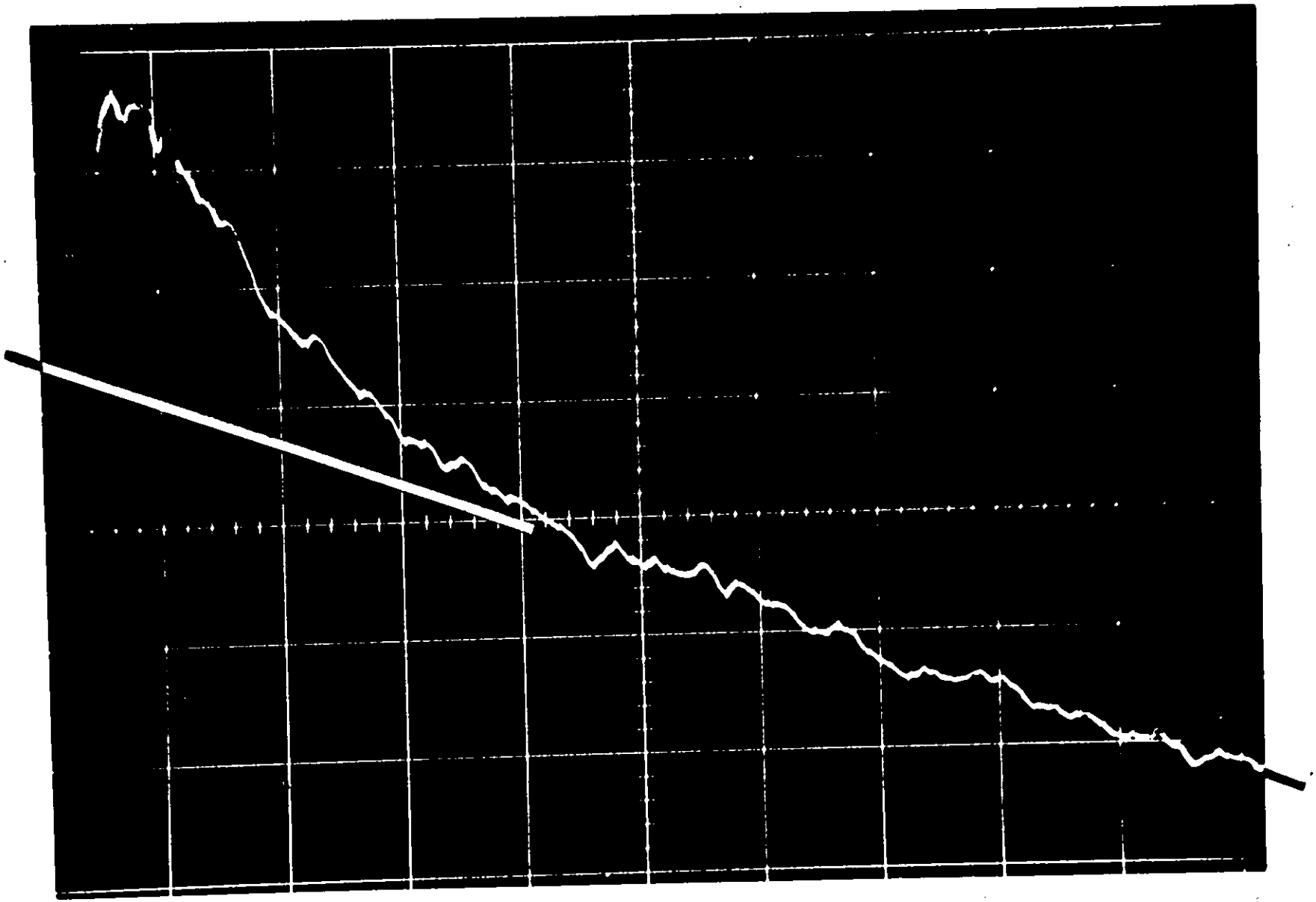
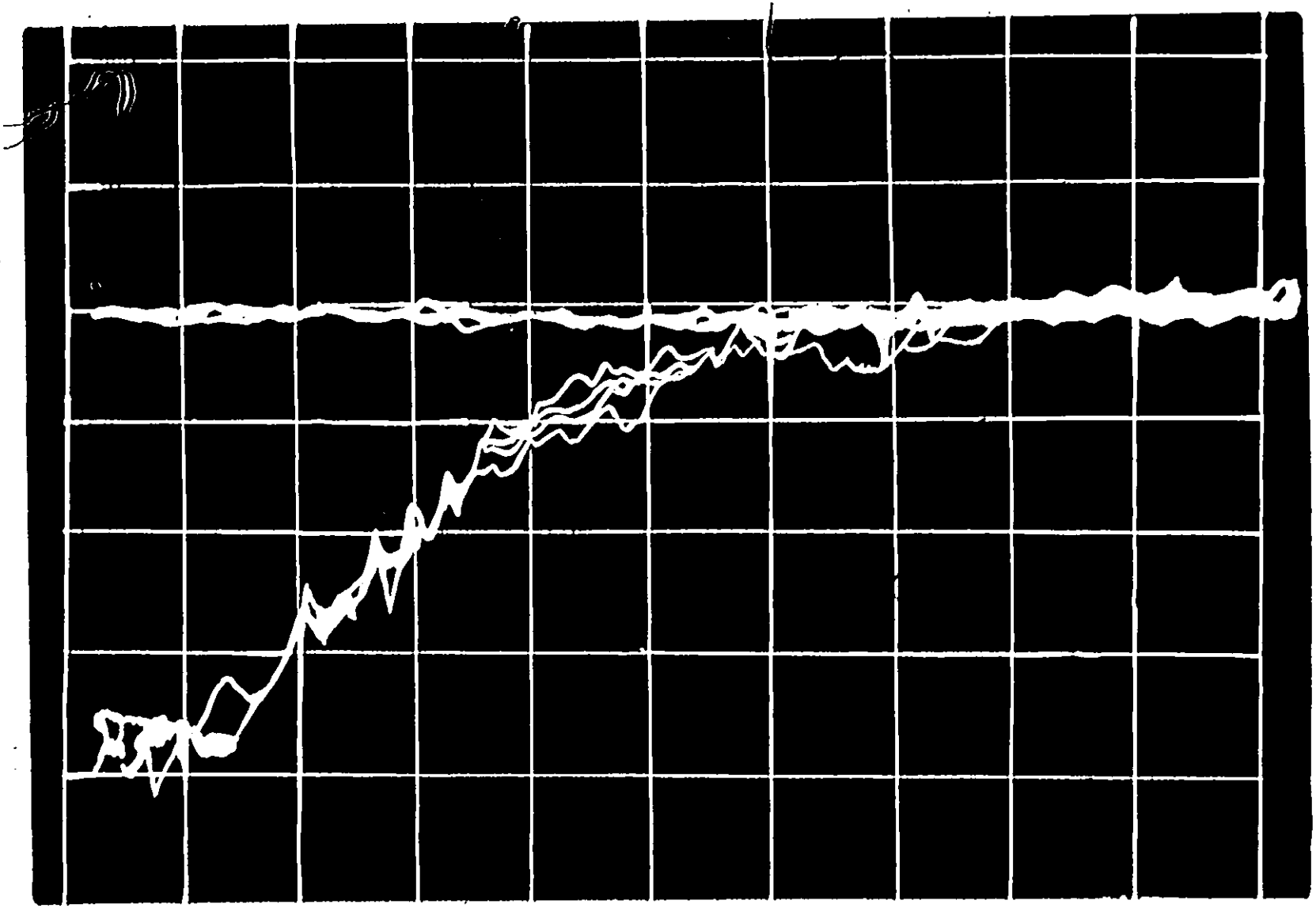


Figure 3

Pre-steady state in the pyruvate to lactate direction: a single turnover experiment. The horizontal line represents the equilibrium and the curve is the exponential approach to the equilibrium (pre-steady-state). Four traces are shown superimposed on one another. Vertical scale: 90-100% transmittance; horizontal scale: 0-50 milliseconds. Pyruvate: 1 mM; NADH : 10 μm; T : 9°C.



C. Analysis of Results

The kinetic results were analysed by means of Wilkinson plots ((substrate)/rate versus (substrate)). Under saturating concentrations of A, equation 1 becomes equivalent to the Michaelis-Menten equation,

$$v = \frac{V_{max}[B]}{K_{mB} + [B]} \qquad (41)$$

Rearrangement gives

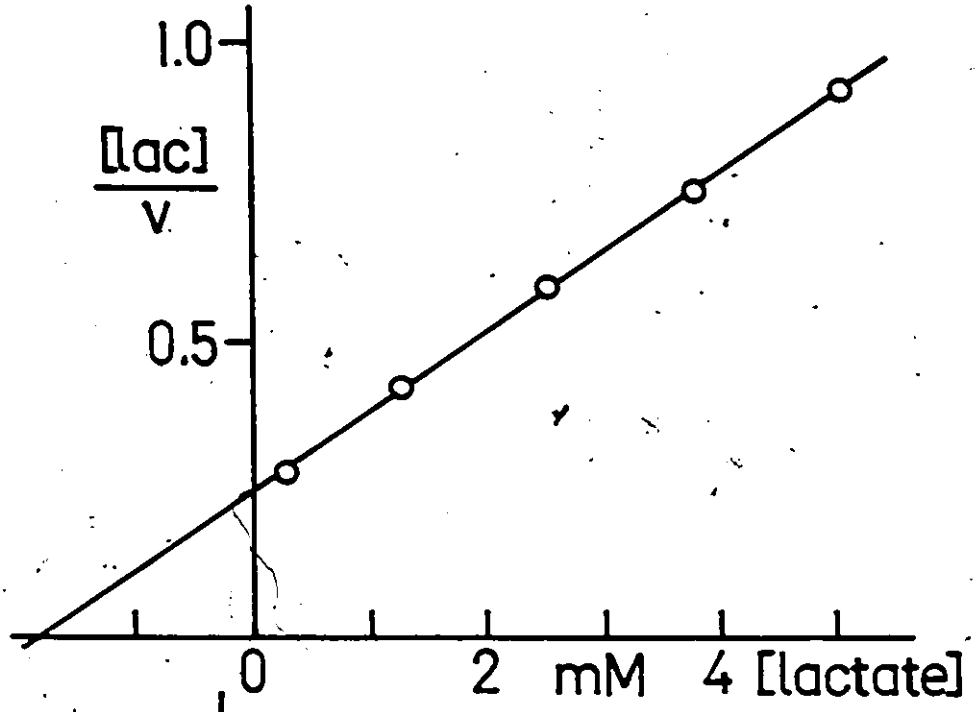
$$\frac{B}{v} = \frac{K_{mB}}{V_{max}} + \frac{[B]}{V_{max}} \qquad (42)$$

By setting $[B]/v$ equal to zero and solving for $[B]$, the "X" intercept is obtained, which is equal to K_{mB}, and V_{max} is equal to the inverse of the slope. There are several advantages of this type of plot as compared to the more common Lineweaver-Burk plot: K_{mB} can be read off directly from a Wilkinson plot (the inverse of K_{mB} is obtained from the Lineweaver-Burk plot), the only parameter which needs to be calculated is $[B]/v$ (as compared to two parameters, $1/v$ and $1/[B]$, for the Lineweaver-Burk plot); and a direct, rate versus substrate, plot can be superimposed on a Wilkinson plot (see for example, Figure 8). Some examples of Wilkinson plots for each of the four substrates are shown in Figures 4 and 5.

Wilkinson plots permit calculation of K_{mA}, K_{mB}, K_{mX} and K_{mY}. Further calculations are required to obtain K_{AB} and K_{XY}. The small extent of reaction (ie., small total absorb-

Figure 4

Wilkinson plots of rate as a function of lactate and pyruvate concentrations. Coenzyme concentration: 2 mM NAD^+ (top figure), 0.1 mM NADH (bottom). Temperature: 45°C (top), 15°C (bottom). Units for velocity are Absorbance per 1000 seconds. Enzyme: beef heart LDH.


1.0
[lac]
v
0.5
0
2 mM 4 [lactate]

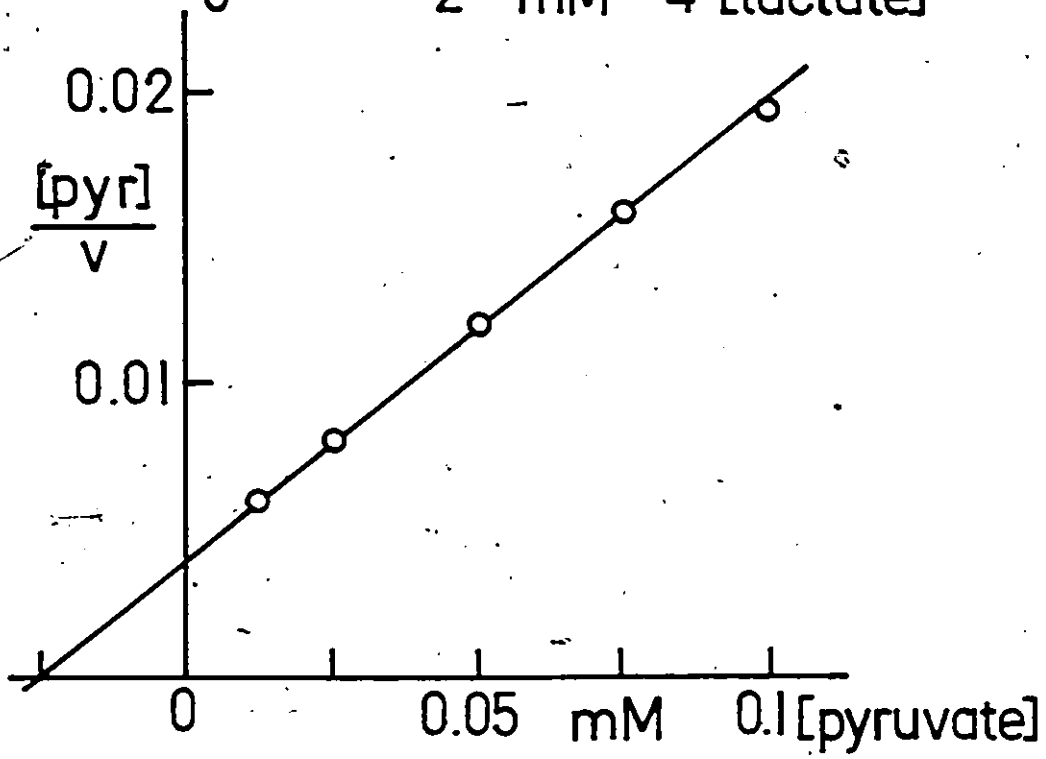
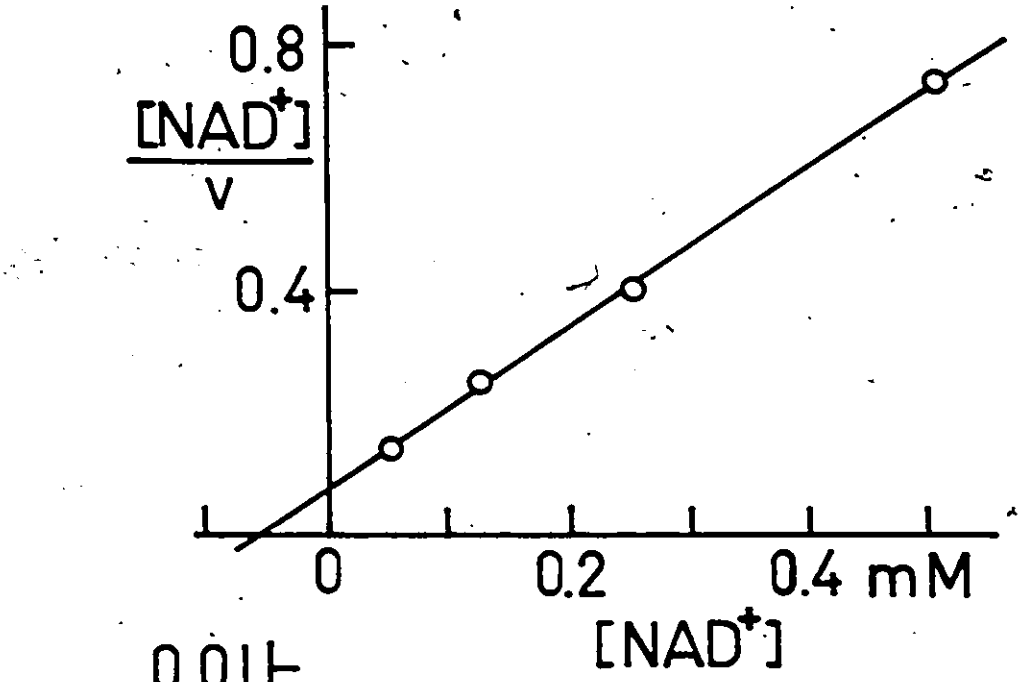
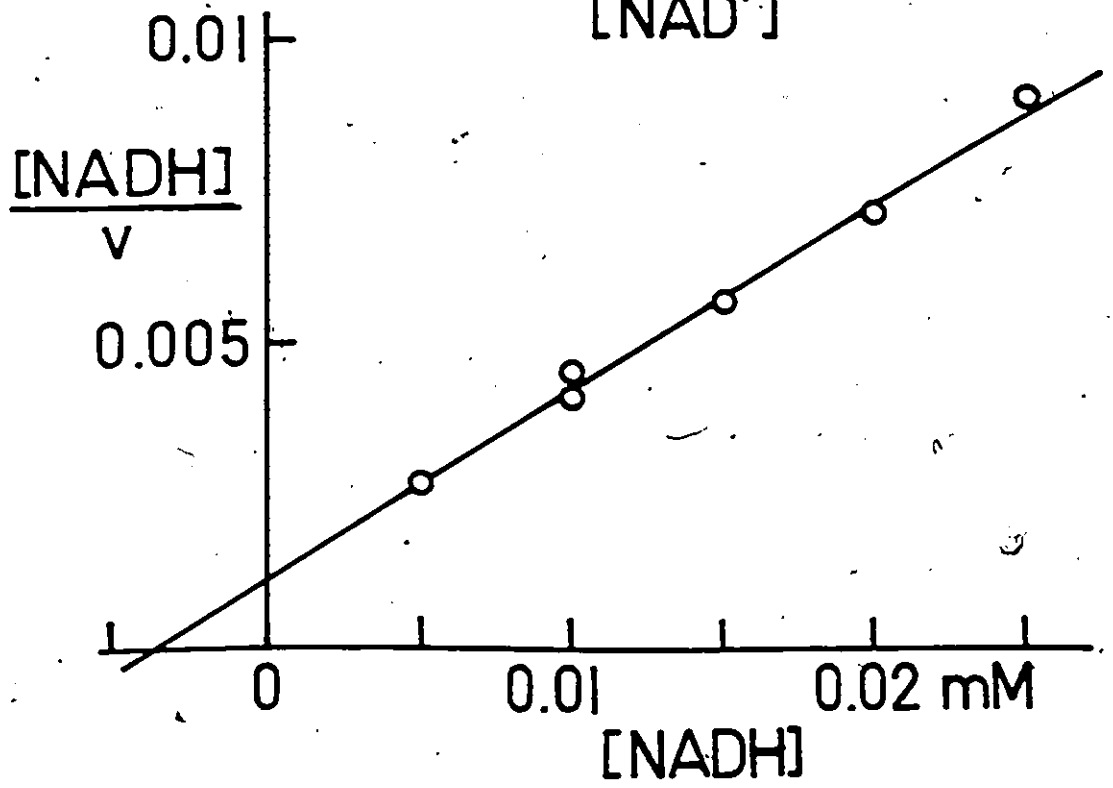

0.02
[pyr]
v
0.01
0
0.05 mM 0.1 [pyruvate]

Figure 5

Wilkinson plots of rate as a function of NAD^+ and NADH concentrations. Temperature: 25°C. Units for velocity are Absorbance per 1000 seconds. Enzyme: beef heart LDH.


0.8
[NAD+]
v
0.4
0
0.2
0.4 mM
[NAD+]


0.01
[NADH]
v
0.005
0
0.01
0.02 mM
[NADH]

ance change) in the forward direction under subsaturating conditions of both substrates made determination of K_{AB} difficult, however, K_{XY} could be determined. The steady-state equation for the reverse direction (equation 8) can be rearranged to give

$$\frac{[X][Y]}{v} = \frac{K_{XY}}{V_{max}} + \frac{K_{mX}[Y]}{V_{max}} + \frac{K_{mY}[X]}{V_{max}} + \frac{[X][Y]}{V_{max}} \qquad (43)$$

A plot of $[X][Y]/v$ against X (ie., a Wilkinson plot with altered vertical scale) at one concentration of Y has slope (J) and intercept (I) which give

$$J = \frac{K_{mY}}{V_{max}} + \frac{[Y]}{V_{max}} \qquad (44)$$

and

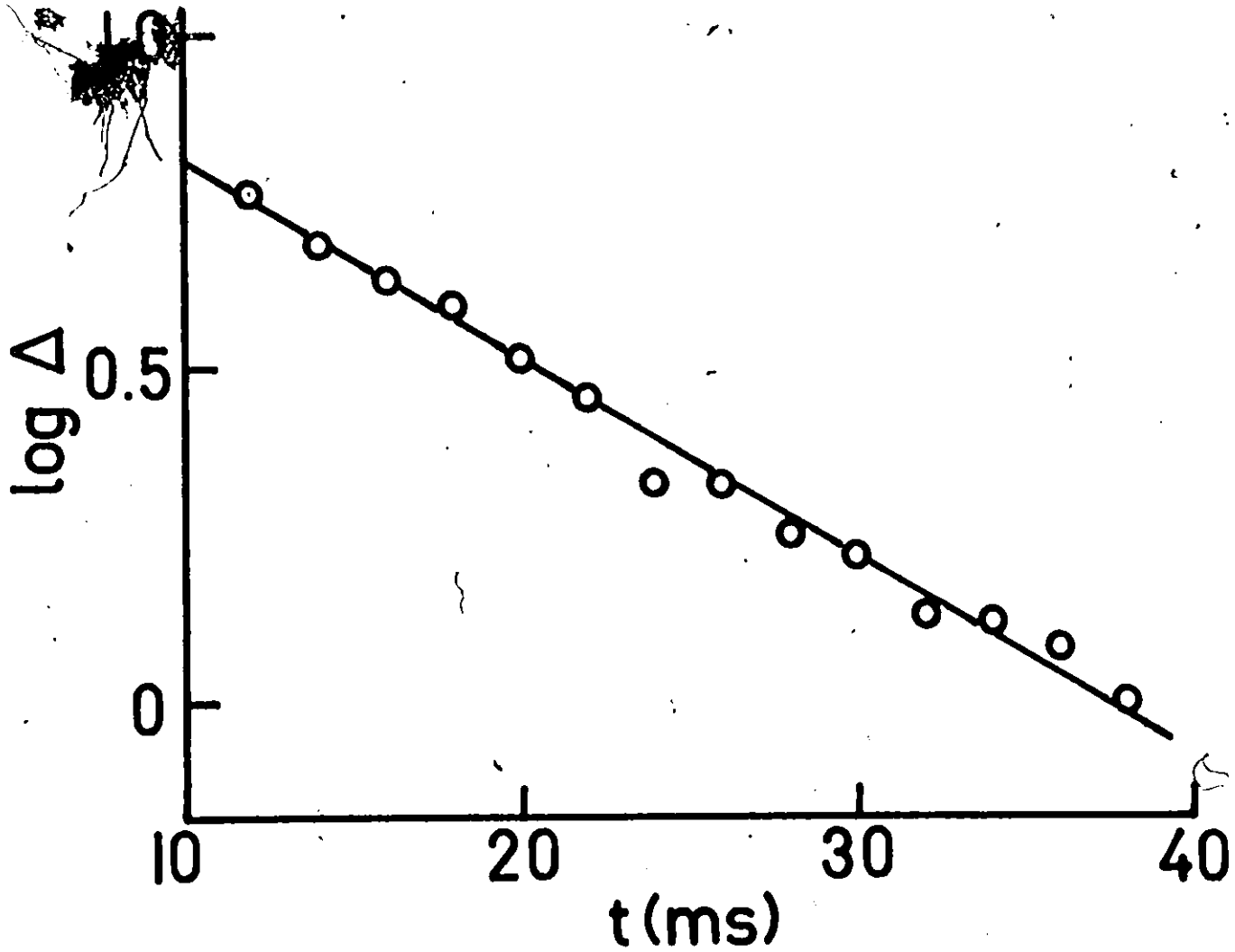
$$(V_{max})\ I = K_{XY} + K_{mX}[Y] \qquad (45)$$

Using two concentrations of Y and equation 44, V_{max} and K_{mY} are readily obtained. Substitution into equation 45 for V_{max} then gives K_{XY} and K_{mX}. For example, at 25°C using beef heart LDH, K_{mX} was calculated to be 0.062×10^{-3}M, K_{mY} was 0.0046×10^{-3}M and K_{XY} was 0.000063×10^{-6} M^2. Hence k_{-4}/k_4 (K_{mX}/K_{XY}), the binding constant for NADH (equation 7 applied to reverse direction) is 9.9×10^5 M^{-1}.

As previously mentioned, λ in the forward direction is obtained by plotting log $(Y - C' - v_{ss})$ against time (equation 25). $(Y - C' - v_{ss})$ is obtained by taking the difference between the straight line in Figure 2 and the observed trace, as exemplified in Figure 6. The λ in the

Figure 6

A plot of log Δ versus time for the pre-steady-state in the forward direction. Δ is equal to $(y - C' - v_{ss})$ (equation 25).


log Δ
1.0
0.5
0
10
20
30
40
t (ms)

reverse direction is obtained by plotting log (1 - B /const.) against time (equation 35). This involves taking the logarithm of the difference between the exponential traces in Figure 3 and the horizontal traces (indicating the endpoint of the reaction). Data from Figure 3 plotted in this way are shown in Figure 7. The variation of λ with substrate concentration is hyperbolic as suggested by equations 27 and 37 and shown in Table II. Hence, as with the steady state data, the pre-steady-state rates are analysed by Wilkinson plots (eg., Figure 8).

D. Denaturation Studies

The denaturation rate was determined by incubating the enzymes in buffer at a concentration of 50 mg/l in a constant temperature bath ($\pm$ 0.05°C). Aliquots were taken at 1 to 10 minute intervals, depending on the temperature, and the resulting enzyme activity was measured at 25°C. The denaturation rate was calculated using the formula

$$E = [E]_o e^{-Dt} \qquad (46)$$

where E is the enzyme concentration, $[E]_o$ is the initial enzyme concentration and t is the incubation time in hours. D was determined from the slope of the line obtained by plotting log (E) against time. Such plots of beef heart and muscle LDH denaturation were completely linear (Figure 9). However, plots of flounder muscle LDH (column purified) denaturation were curved, so the initial slope was taken, as indicated by the solid line in Figure 9.

TABLE II

Affect of Pyruvate Concentration on λ at 8°C

(10 μM NADH ; 14 μM BHLDH)

pyruvate (mM)	λ (sec^{-1})
0.01	17.5
0.05	50
0.25	60
1.00	66
5.00	66

Figure 7

A plot of log Δ versus time for a single turnover experiment in the reverse direction. Δ is equal to (1 - [B]/const.) (equation 35) and is obtained from Figure 3.

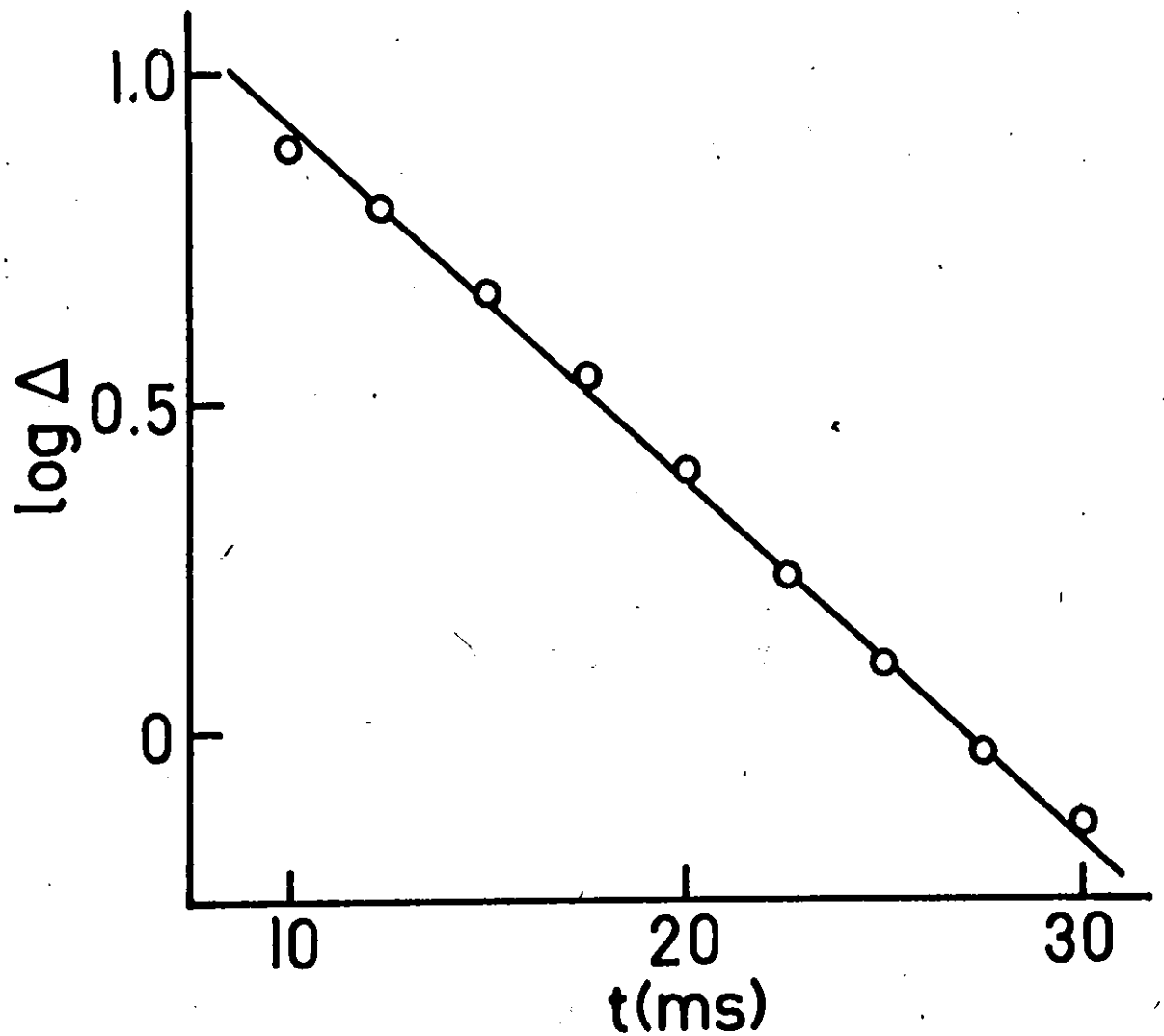

1.0
0.5
0
log Δ
10
20
30
t(ms)

Figure 8

Direct and Wilkinson plots of pre-steady-state rate (λ) in the forward direction as a function of lactate concentration. The maximum value (105 sec^{-1}) is indicated. Temperature: 10°C.

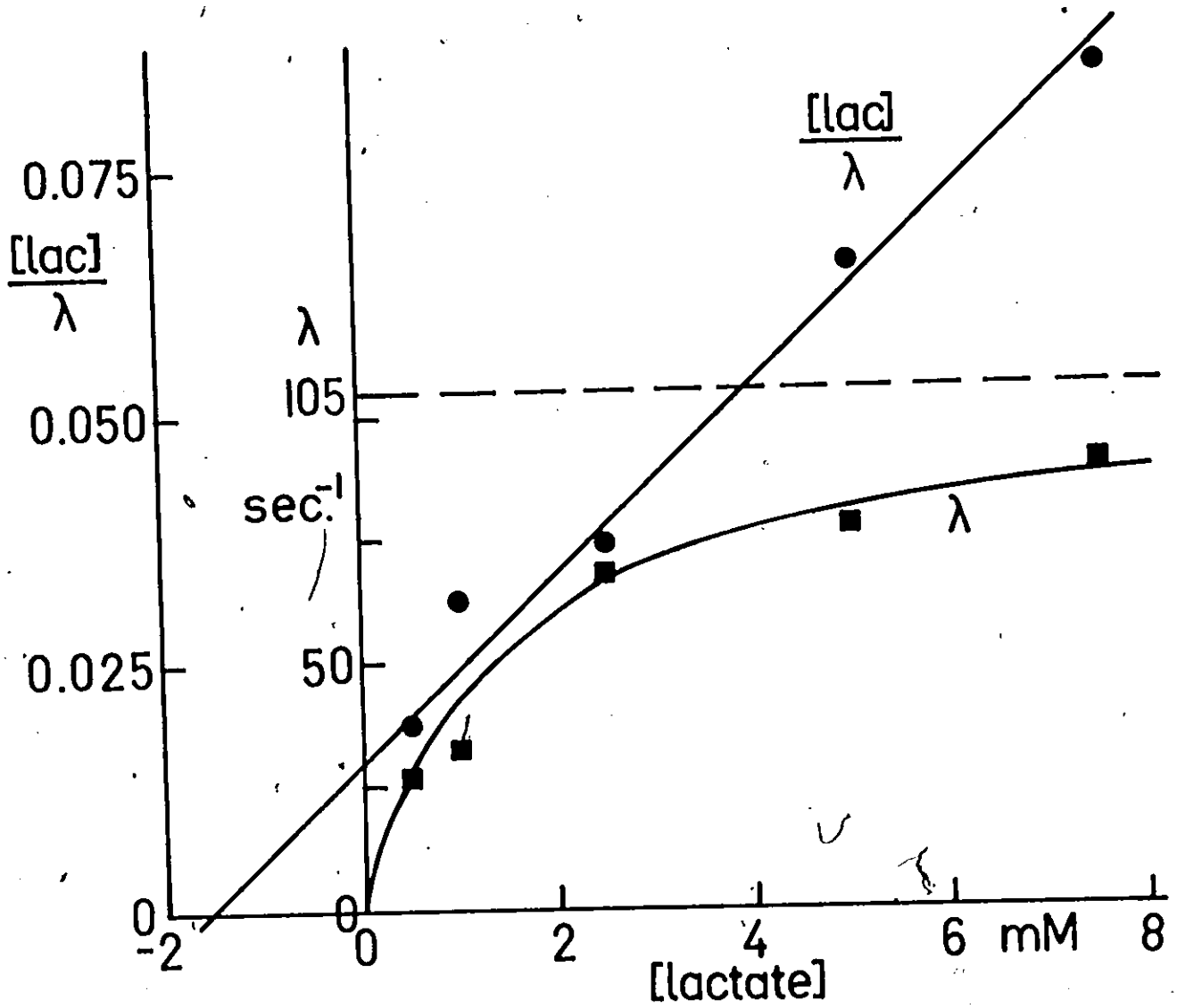
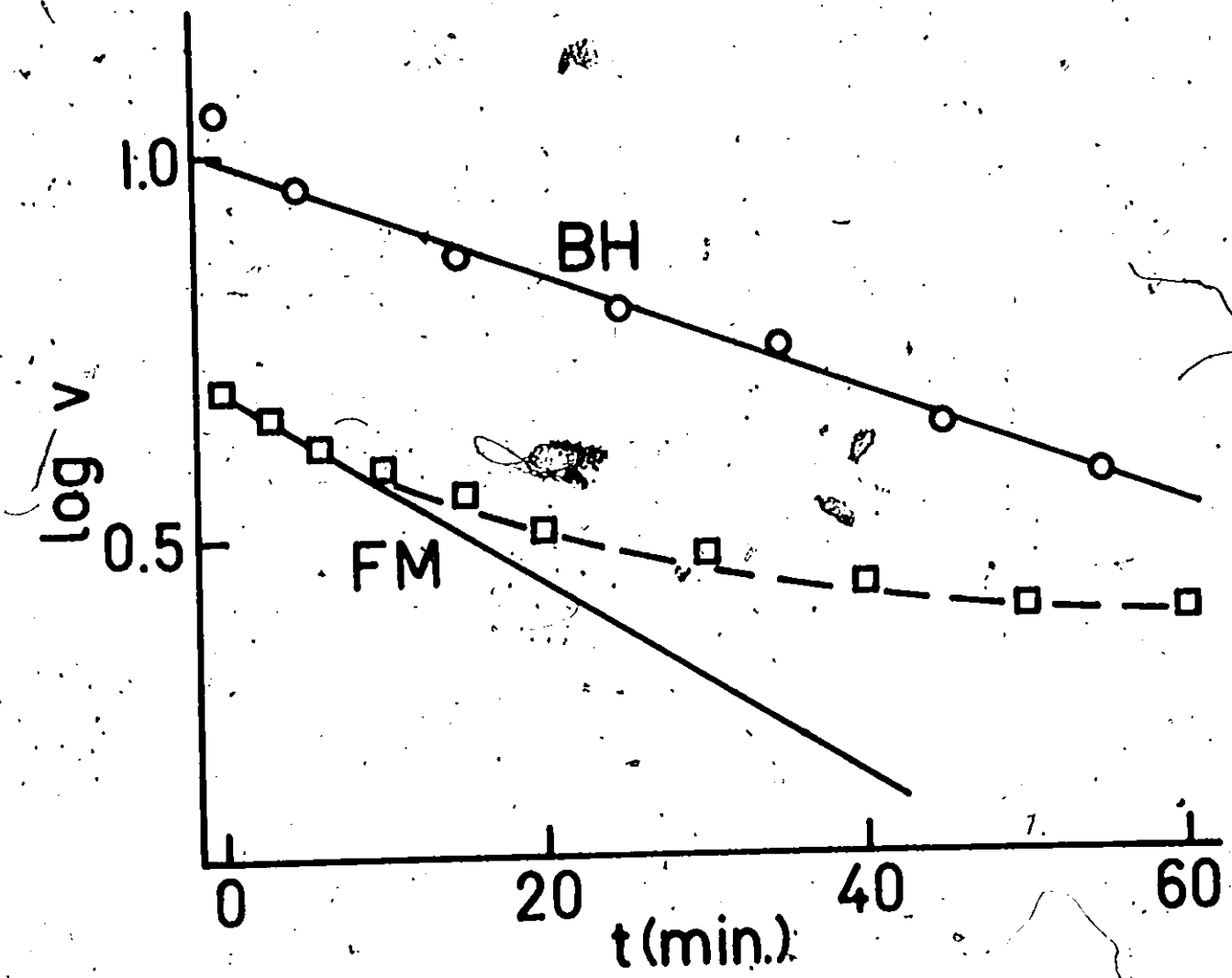

[lac]/λ
0.075
0.050
0.025
0
λ
105
50
0
sec.⁻¹
[lac]/λ
λ
-2
0
2
4
6 mM
8
[lactate]

Figure 9

Denaturation rate of pure beef heart LDH (BH) at 63°C and pure flounder muscle LDH (FM) at 50°C. The denaturation rate is calculated from the slope of the solid lines.


1.0
0.5
log v
BH
FM
0
20
40
60
t (min.)

IV

RESULTS

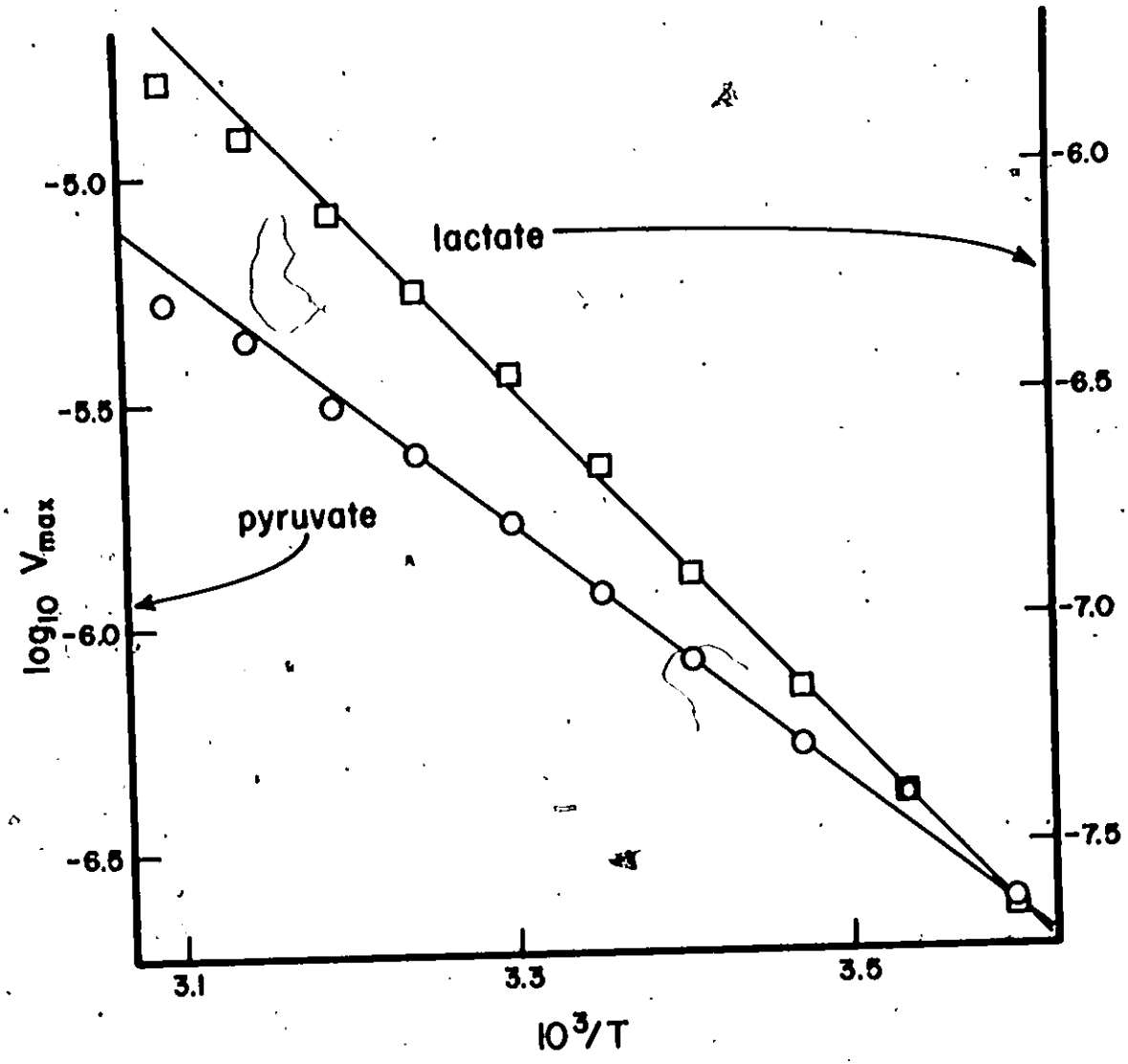
A. The Kinetics of Beef Heart LDH

Beef heart LDH was the only enzyme for which the pre-steady-state could be measured in the forward direction. Perhaps the pre-steady-state is too fast to be observed in the other enzymes using the present techniques. This situation is also observed for pig LDH; the pre-steady-state can be observed in the heart enzyme but not in the muscle enzyme (unless steady-state inhibitors are added) (Bennet and Gutfreund, 1973). As a result, the beef heart enzyme can be studied in greater detail than the other two enzymes and is the only enzyme for which some of the rate constants can be calculated utilizing two independent sets of experimental data, ie., the pre-steady-state in the forward and reverse directions. The beef heart enzyme, therefore, will be examined first, and the validity of the four-step model determined.

Steady-State Kinetics. The maximum steady-state velocities in the two directions, $\vec{V}_{max}$ (in the lactate to pyruvate direction) and $\overleftarrow{V}_{max}$ (in the pyruvate to lactate direction), are shown plotted logarithmically against 1/T in Figure 10. In both cases, straight lines are obtained, which suggests that each velocity is controlled primarily by one rate constant over the temperature range employed. Since a single rate constant is expected to demonstrate a straight line Arrhenius plot (equation 39), a curved plot for V_{max} would have suggested a change in the rate-limiting step with temperature.

Figure 10

Arrhenius plots of the V_{max} values for beef heart LDH for the forward (lactate → pyruvate) and reverse directions; units of V_{max} are M sec^{-1}.


lactate
pyruvate
$\log_{10} V_{max}$
-5.0
-5.5
-6.0
-6.5
-6.0
-6.5
-7.0
-7.5
3.1
3.3
3.5
$10^3/T$

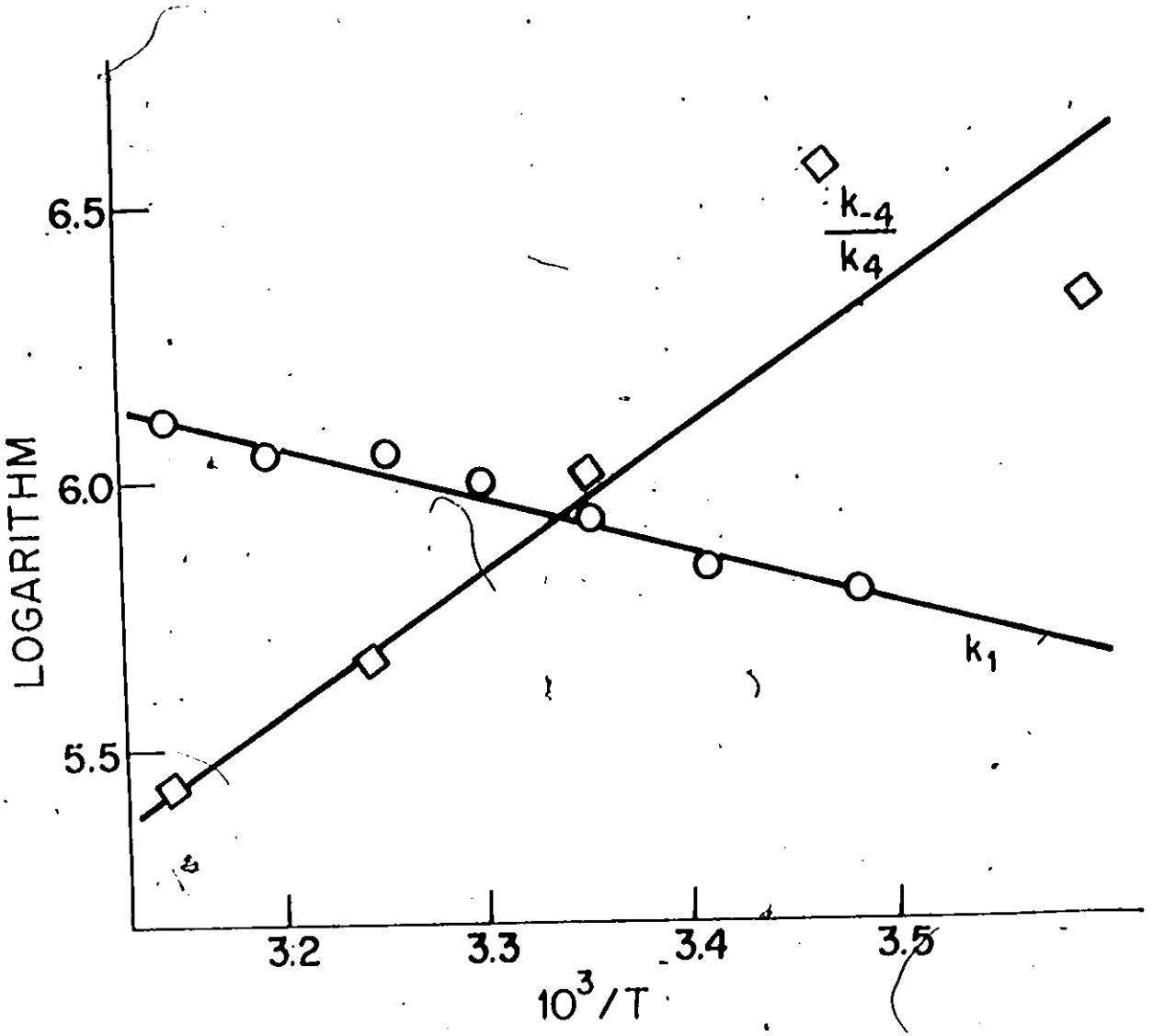
K_{mA} has been obtained from Wilkinson plots (eg., Figure 5) and this permits calculation of k_1 ($= \vec{V}_{max}/K_{mA}$, equation 6). When concentrations of both NAD^+ and lactate are below their respective K_m values, the reaction does not proceed far enough to permit measurement of the initial reaction rate, and K_{AB} therefore cannot be measured.

Fortunately, both K_{mY} and K_{XY} could be calculated from studies in the reverse direction (equations 44 and 45). The values of k_1 and K_{mX}/K_{XY} ($= k_{-4}/k_4$) are shown plotted in Figure 11. The values of k_{-4} calculated from $\vec{V}_{max}/K_{mY}[E]_o$ (equation 6), applied in the reverse direction) produce a curve on an Arrhenius plot, suggesting that more than one step is involved in the binding of NADH to the enzyme. The values of k_{-4} used in the calculation of the enthalpy and entropy of activation were obtained by multiplying k_4 ($\vec{V}_{max}/[E]_o$) by k_{-4}/k_4 ($= K_{mX}/K_{XY}$). It should be noted that this value of k_{-4} is probably a composite value of a rate constant multiplied by an equilibrium constant since, as suggested above, more than one step is probably involved in the binding of NADH, and hence k_{-4}/k_4 calculated above is, in reality, an overall equilibrium constant for two steps.

The values of $k_2k_3/k_{-2}k_{-3}$ (equation 11) plotted logarithmically against 1/T give a straight line (Figure 12). Since k_{-4}/k_4 is also known (Figure 11), k_1/k_{-1} can now readily be obtained from the overall equilibrium constant. k_{-1} can be obtained by dividing k_1 by k_1/k_{-1}. The constants k_1 and k_4 were therefore obtained directly, while k_{-1} and k_{-4}

Figure 11

Logarithmic plots of $\vec{V}_{max}/K_{mA}$ (= k_1) and of K_{mX}/K_{XY} (=k_{-4}/k_4) against 1/T for beef heart LDH. The units are, for k_1, sec^{-1}; for k_{-4}/k_4, M^{-1}.


LOGARITHM
6.5
6.0
5.5
3.2
3.3
3.4
3.5
$10^3/T$
$\frac{k_{-4}}{k_4}$
k_1

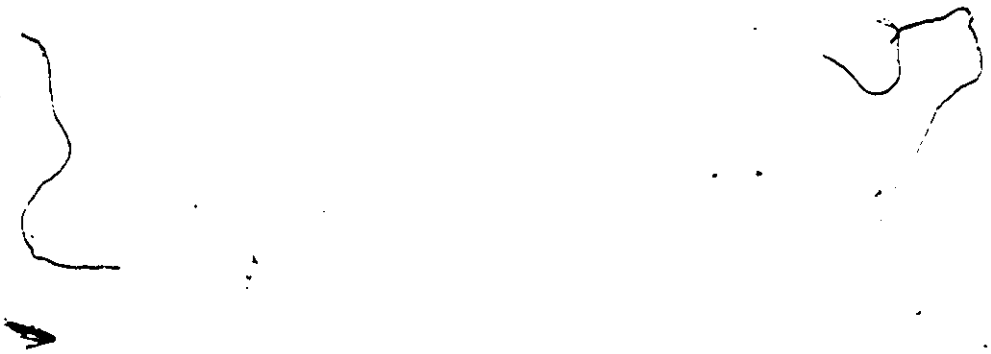
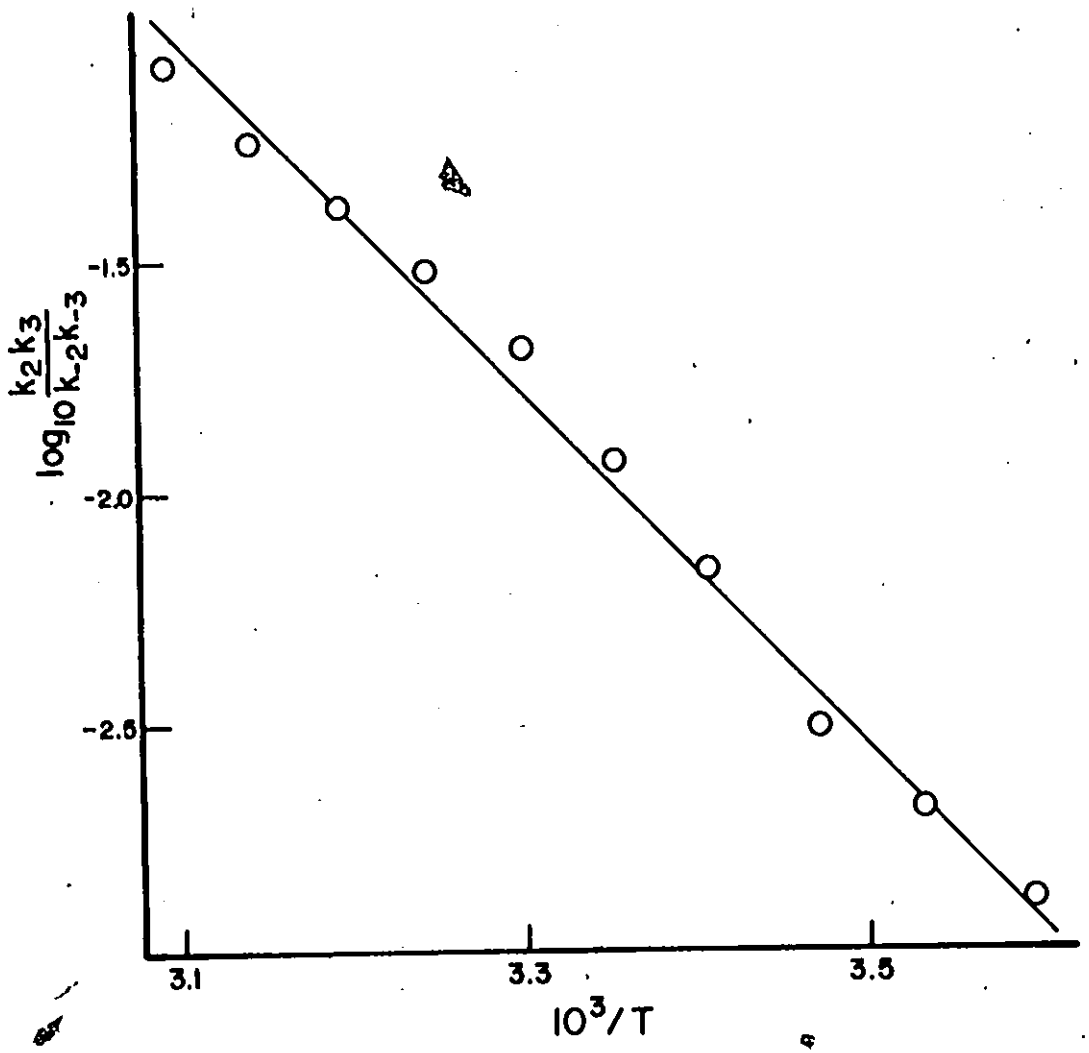


Figure 12

Logarithmic plot of $k_2k_3/k_{-2}k_{-3}$ $(= (\vec{V}_{max}/K_{mB})/(\vec{V}_{max}/K_{mX}))$ against 1/T for beef heart LDH.


$\log_{10}\frac{k_2k_3}{k_{-2}k_{-3}}$
-1.5
-2.0
-2.5
3.1
3.3
3.5
$10^3/T$

were obtained indirectly from k_1 and k_4 and the appropriate equilibrium constant.

The K_m values for lactate and pyruvate are plotted logarithmically against 1/T in Figure 13. The curves fitted to the data were calculated using equations 4 and 10 and values for the individual rate constants shown in Table III. The derivation of the values in Table III will be shown below.

Stopped-Flow Measurements in the Forward Direction. The values of k_3 and k_{-2}/k_2, obtained in the stopped flow studies as explained in the Analysis of Results Section (Materials and Methods), are plotted logarithmical against 1/T in Figure 14. The line drawn through the values of k_{-2}/k_2 was obtained from steady state studies as described below.

Combined Steady-State and Stopped-Flow Studies (Forward Direction). The values of log (C + D) (equation 13) are plotted against 1/T in Figure 15. Values calculated from both $K_{mX}/\vec{V}_{max}$ (equation 13) and $K_{mB}/\vec{V}_{max}$ (equation 12), multiplied by the values of $k_2k_3/k_{-2}k_{-3}$ obtained from the straight line in Figure 12, are shown. The diagram also shows two straight lines, obtained by extrapolation to very high and very low temperatures, one equal to C and the other to D, and their sum is represented by the curve in the diagram. The theoretical expressions ~~for~~ K_{mB} and K_{mX} (equations 4 and 10) suggests that the curvatures in these

TABLE III

Rate Constants at 0 and 50°C based on the Four-Step Model using Pre-Steady-State Data in the Forward Direction

	0°C	50°C
k_1 $(M^{-1}sec^{-1})$	4.00×10^5	1.45×10^6
k_{-1} (sec^{-1})	6.76	1.88×10^3
k_2 $(M^{-1}sec^{-1})$	1.51×10^3	2.59×10^6
k_{-2} (sec^{-1})	8.93×10^{-1}	3.22×10^4
k_3 (sec^{-1})	3.60×10^1	2.20×10^3
k_{-3} $(M^{-1}sec^{-1})$	9.26×10^7	1.75×10^6
k_4 (sec^{-1})	2.53	4.33×10^2
k_{-4} $(M^{-1}sec^{-1})$	1.37×10^7	8.66×10^7

Figure 13

Logarithmic plots of K_m(M) against 1/T for the reactions in forward and reverse directions for beef heart LDH.

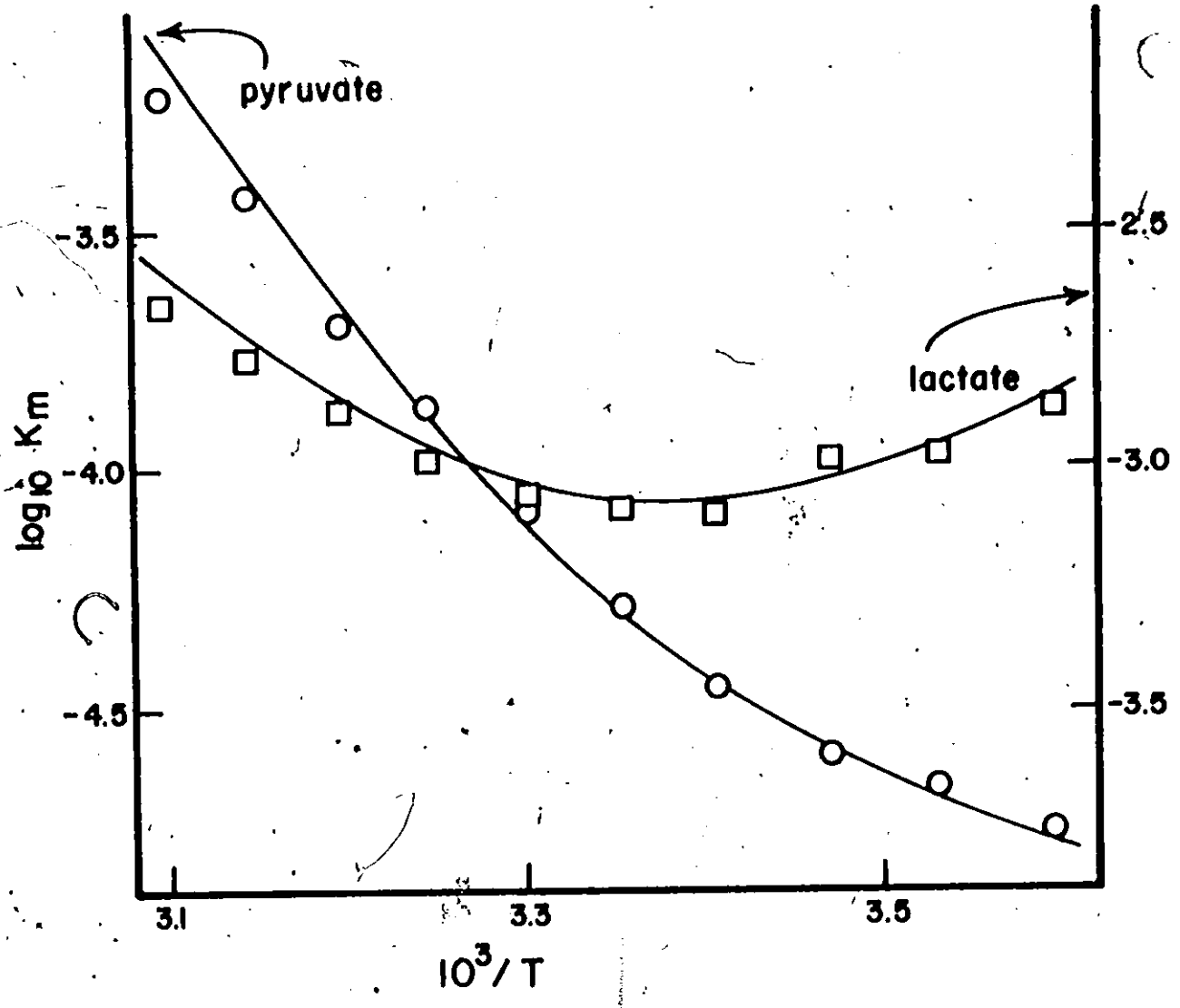

pyruvate
lactate
-3.5
-4.0
-4.5
log10 Km
-2.5
-3.0
-3.5
3.1
3.3
3.5
10³/ T

Figure 14

Arrhenius plots of k_3 (sec^{-1}) and $k_{-2}/k_2(M)$, obtained from the pre-steady-state measurements for beef heart LDH.

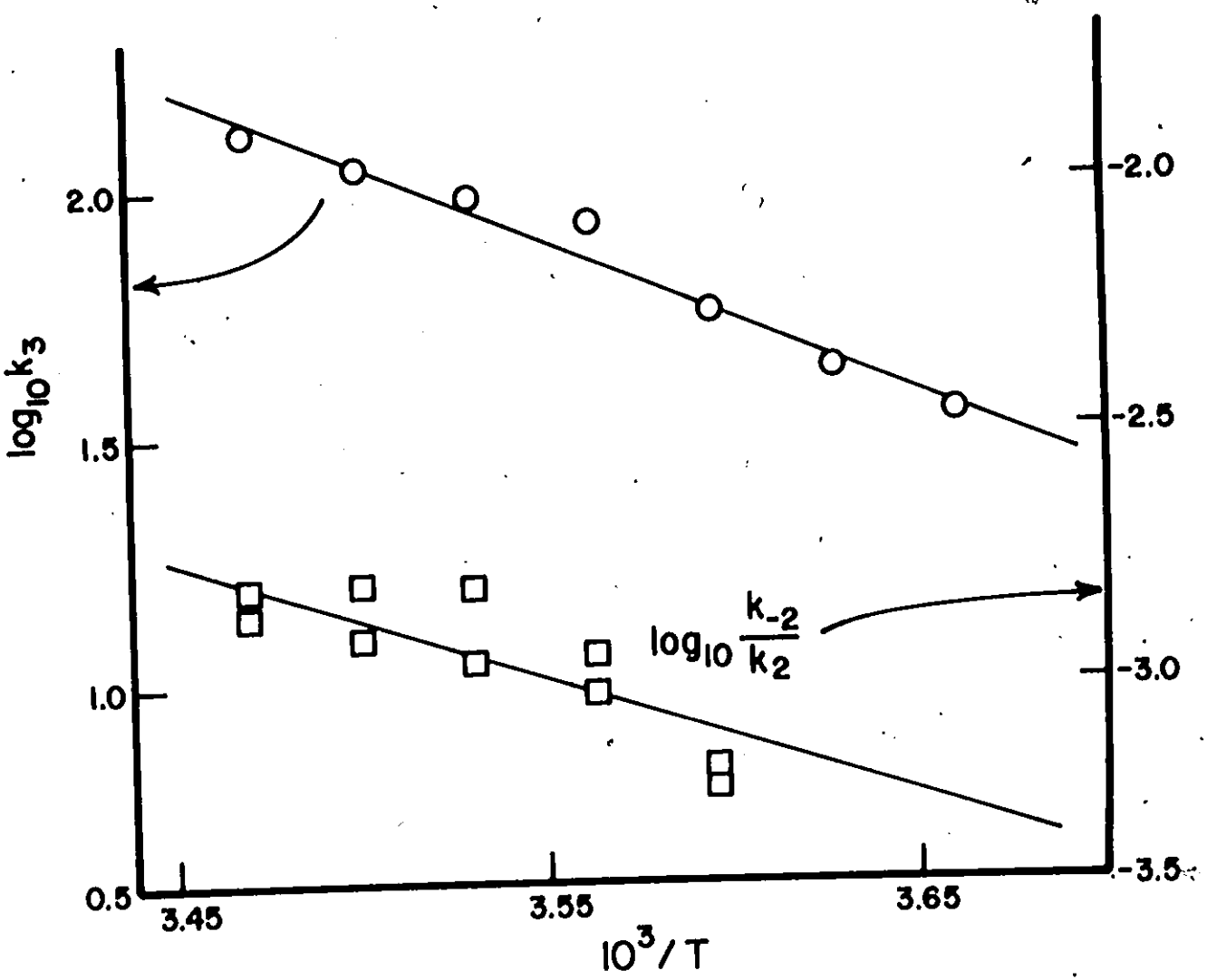
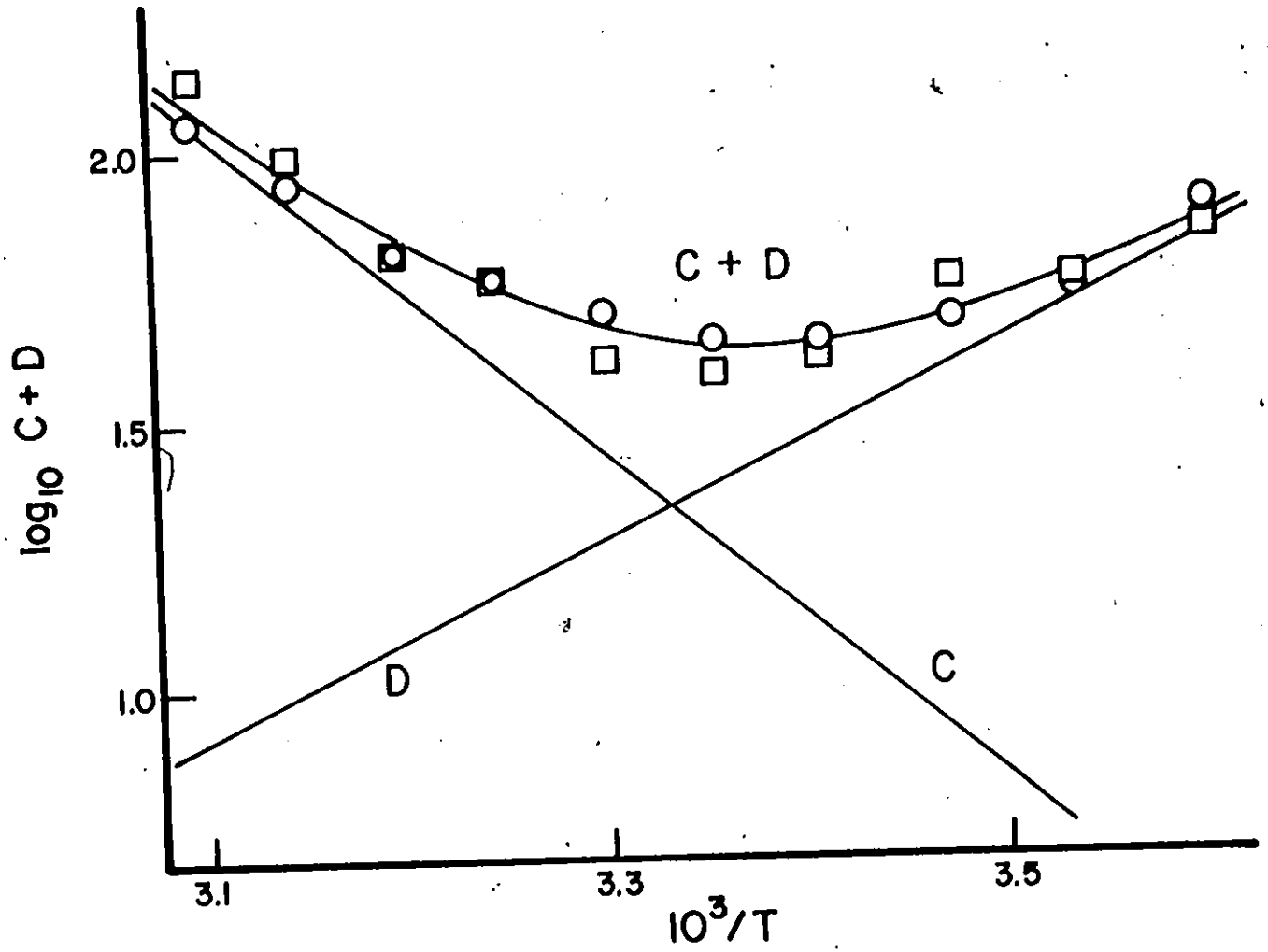

2.0
1.5
1.0
0.5
$\log_{10} k_3$
-2.0
-2.5
-3.0
-3.5
$\log_{10} \frac{k_{-2}}{k_2}$
3.45
3.55
3.65
$10^3/T$

Figure 15

Logarithmic plot of C + D against 1/T for beef heart LDH. The circles are $K_{mX}/\vec{V}_{max}$; the squares are $K_{mB}/\vec{V}_{max}$ multiplied by $k_2k_3/k_{-2}k_{-3}$ (cf. eq. 11).


2.0
1.5
1.0
log10 C+D
C + D
D
C
3.1
3.3
3.5
10³/T

plots result from the summations of the same rate constants; the curvatures for the two sets of points in Figure 15 should, therefore, be the same. The consistency between the two sets of points, and their fit to the calculated curve, supports this interpretation.

If $C = 1/k_{-3}[E]_o$ and $D = k_3/k_{-2}k_{-3}[E]_o$ the value of C/D is equal to k_{-2}/k_3 (equation 14). Multiplication by k_3 (Figure 14) gives k_{-2}; D multiplied by $k_{-2}k_{-3}/k_2k_3$ (reciprocal of Figure 12, equation 11) gives $1/k_2[E]_o$. Multiplication of k_{-2} by $1/k_2[E]_o$ then gives $k_{-2}/k_2[E]_o$. The line drawn in Figure 14 is based on the $k_{-2}/k_2[E]_o$ values obtained in this way, and the temperature dependence agrees satisfactorily with the pre-steady-state values. Comparison of the steady-state ($k_{-2}/k_2[E]_o$) and pre-steady-state (k_{-2}/k_2) values leads to a calculated active enzyme concentration of 5×10^{-9} M. The enzyme concentration calculated from the protein concentration (0.25 mg/l) and a monomer molecular weight of 35,000 g/M (see Introduction) is 7×10^{-9} M. The agreement is satisfactory; the kinetic procedures may have given a slightly lower value since some of the protein may have been catalytically inert.

The alternative assumption that $C = k_3/k_{-2}k_{-3}[E]_o$ and $D = 1/k_{-3}[E]_o$ does not appear to be correct for two reasons. Firstly, the estimate of $k_{-2}/k_2[E]_o$ obtained on this basis has a negative temperature dependence, which is inconsistent with the positive one given by the pre-steady-state data (Figure 14). Secondly, the estimated enzyme concentration

is 50 times less than that given above. This alternative is therefore disregarded.

Use of the value of 5×10^{-9}M for the enzyme concentration leads to values of k_1, k_{-1}, k_2, k_{-2}, k_3, k_{-3}, k_4 and k_{-4} given in Table III for 0°C and 50°C.

Stopped-Flow Measurements in the Reverse Direction. The values of $\overleftarrow{\lambda}_{max}(k_{-2})$ (equation 37), obtained from the single turnover experiments, are shown plotted as an Arrhenius plot in Figure 16. The line drawn through the points is obtained from the steady-state V_{max} (Figure 10) divided by the enzyme concentration. Since the two sets of data coincide, k_{-2} must be the rate-limiting step in the steady-state when the enzyme is saturated with NADH and pyruvate. The value of k_3/k_{-3} (equation 36) is shown in Figure 17.

Combined Steady-State and Stopped-Flow Studies (Reverse Direction). Since k_{-2} is rate limiting in the reverse direction in the steady-state, K_{mX} is given by equation 15. The values of k_3/k_{-3} and K_{mX} are compared in Figure 17. Subtraction of k_3/k_{-3} from K_{mX} gives k_{-2}/k_3, also shown in Figure 17. Dividing $k_2k_3/k_{-2}k_{-3}$ (Figure 12) by k_3/k_{-3} (Figure 17) gives k_2/k_{-2}. k_2/k_{-2} multiplied by k_{-2} (Figure 16) gives k_2. Dividing k_{-2} by k_{-2}/k_{-3} (Figure 17) gives k_{-3}. k_{-3} times k_3/k_{-3} (Figure 17) gives k_3. The values of the four rate constants, k_2, k_{-2}, k_3 and k_{-3}, along with the other four calculated purely from steady-state studies, are shown in Table IV.

Examination of Tables III and IV indicates that estimates of k_2, k_{-2}, k_3 and k_{-3} vary, depending on whether pre-steady-state data in the forward or reverse direction are combined

Figure 16

Arrhenius plot of $\overleftarrow{\lambda}_{max}$ (sec^{-1}) for beef heart LDH; this parameter is equal to k_{-2}. The line through the points is $\overleftarrow{V}_{max}/[E]_o$ obtained from the steady-state measurements.

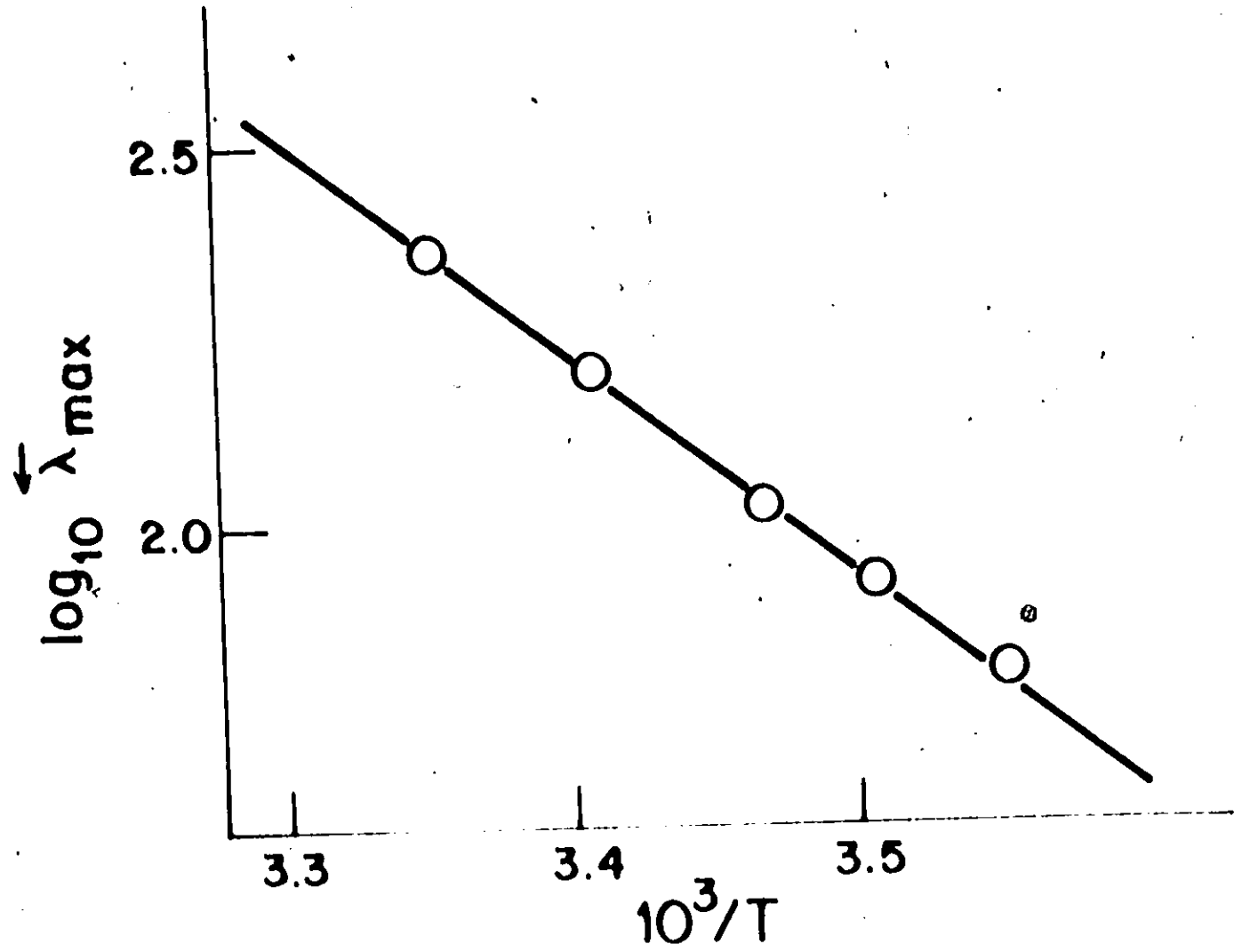

2.5
2.0
log10 λmax
3.3
3.4
3.5
10³/T

Figure 17

Logarithmic plots of $K_{\lambda X}$ (= k_3/k_{-3}, M) and of K_{mX} (=(k_3 + k_{-2})/k_{-3}, M) for beef heart LDH.

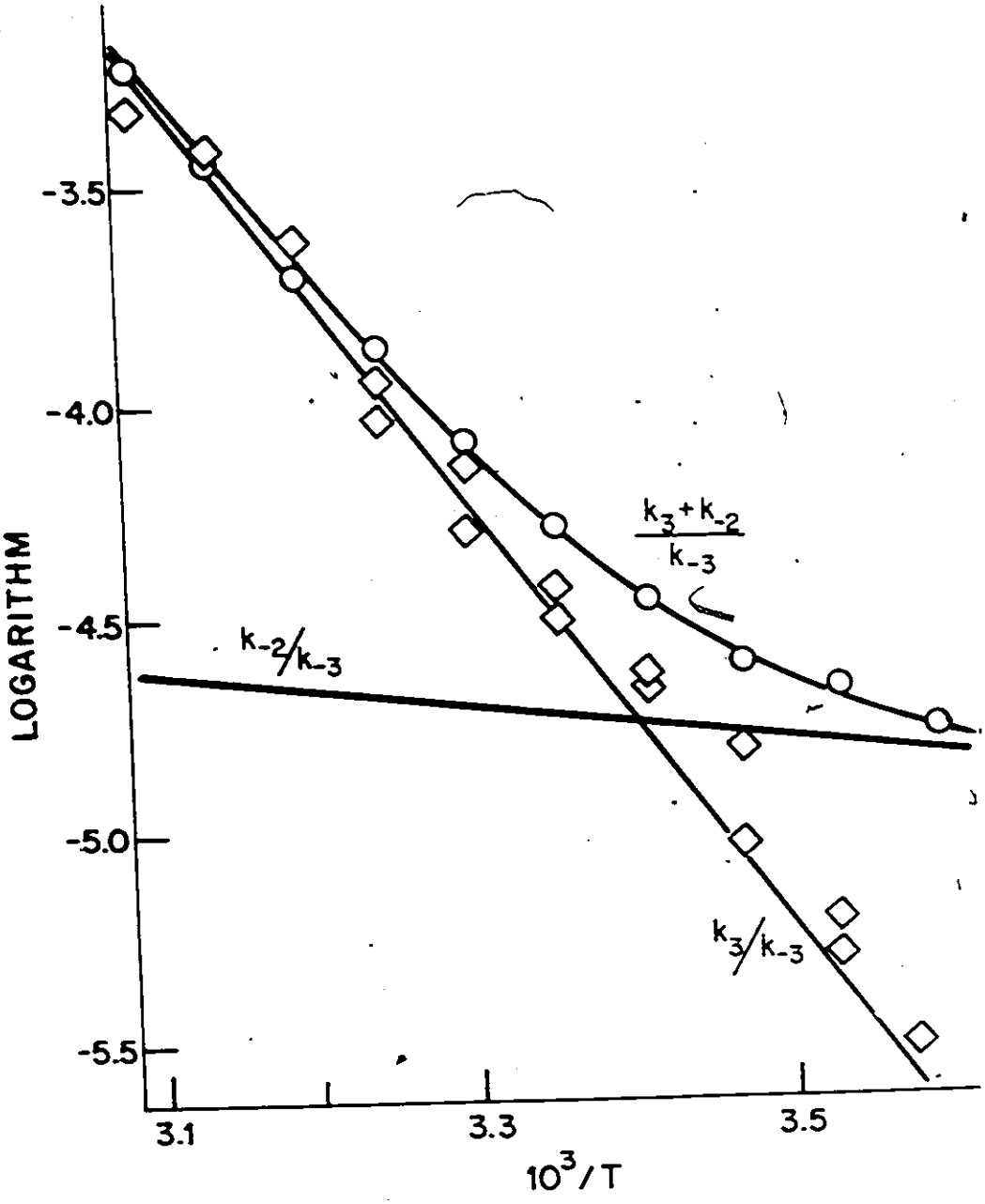

-3.5
-4.0
-4.5
-5.0
-5.5
LOGARITHM
$\frac{k_3+k_{-2}}{k_{-3}}$
k_{-2}/k_{-3}
k_3/k_{-3}
3.1
3.3
3.5
$10^3/T$

TABLE IV

Rate Constants at 0 and 50°C based on the Four-Step Model using Pre-Steady-State Data in the Reverse Direction

	0°C	50°C
k_1 $(M^{-1}sec^{-1})$	4.00×10^5	1.45×10^6
k_{-1} (sec^{-1})	6.76	1.88×10^3
k_2 $(M^{-1}sec^{-1})$	2.04×10^4	2.06×10^5
k_{-2} (sec^{-1})	3.10×10^1	1.27×10^3
k_3 (sec^{-1})	2.14	3.29×10^4
k_{-3} $(M^{-1}sec^{-1})$	2.14×10^6	5.29×10^7
k_4 (sec^{-1})	2.53	4.33×10^2
k_{-4} $(M^{-1}sec^{-1})$	1.37×10^7	8.66×10^7

with the steady-state data for their calculation. Since steady-state data alone are used in determining the other four rate constants; their values are the same in Tables III and IV. Obviously, the simplified four-step model used to describe LDH kinetics to this point is insufficient to explain the discrepancy among some of the rate constants. All the data can, however, be reconciled using a five-step model which will be discussed later.

B. Rate Constants for Beef Heart, Beef Muscle and Flounder Muscle LDH

Since the pre-steady-state for beef muscle and flounder muscle could only be followed in the reverse direction (see Section A), beef heart LDH data in this direction only will be used to compare to the other two enzymes.

All calculations were done in the same manner for all enzymes with the few exceptions stated below. The values of k_1 are shown in Figure 18 and k_{-4}/k_4 in Figure 19. In order to make absolute rates comparable, k_{cat} values ($V_{max}/[E]_o$) will be used to compare the three enzymes instead of V_{max}. k_{cat} values for the forward direction are shown in Figure 20. The equilibrium constant between EA and EY ($k_2k_3/k_{-2}k_{-3}$) is shown in Figure 21. The k_{cat} for the reverse direction is shown in Figure 22. The only k_{cat} which does not produce a straight line Arrhenius plot is that for flounder muscle in the reverse direction. In this case, the catalytic rate constant appears to be a function of two rate constants and is given by k_w at high temperatures

Figure 18

Arrhenius plots of k_1 (sec^{-1}) for beef muscle (BM) and flounder muscle (FM). The k_1 for beef heart (Figure 11) is included for comparison.

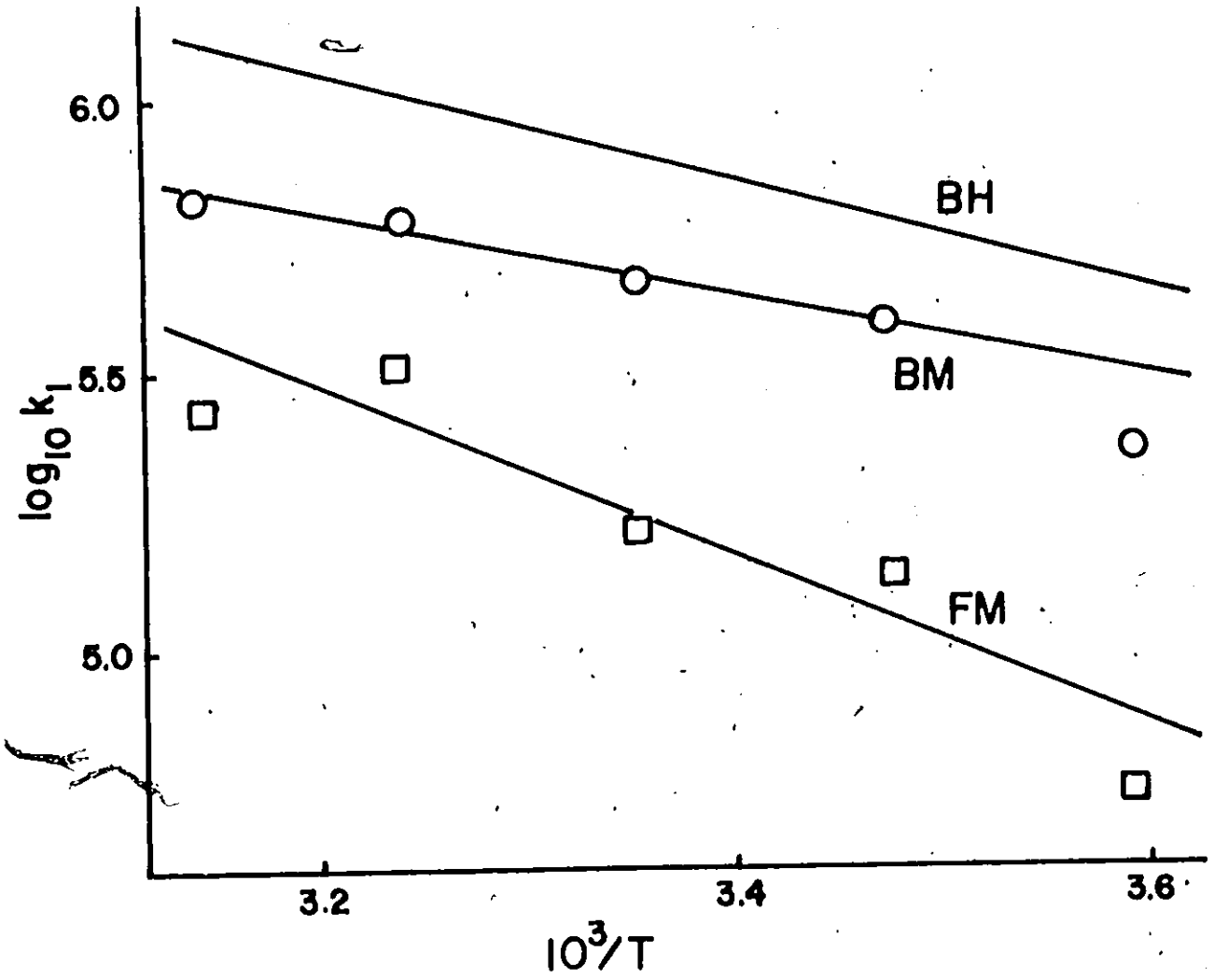

6.0
5.5
5.0
$\log_{10} k_1$
BH
BM
FM
3.2
3.4
3.6
$10^3/T$

Figure 19

Logarithmic plot of k_{-4}/k_4 (M^{-1}) against 1/T for beef and flounder muscle. The value for beef heart (Figure 11) is included for comparison.

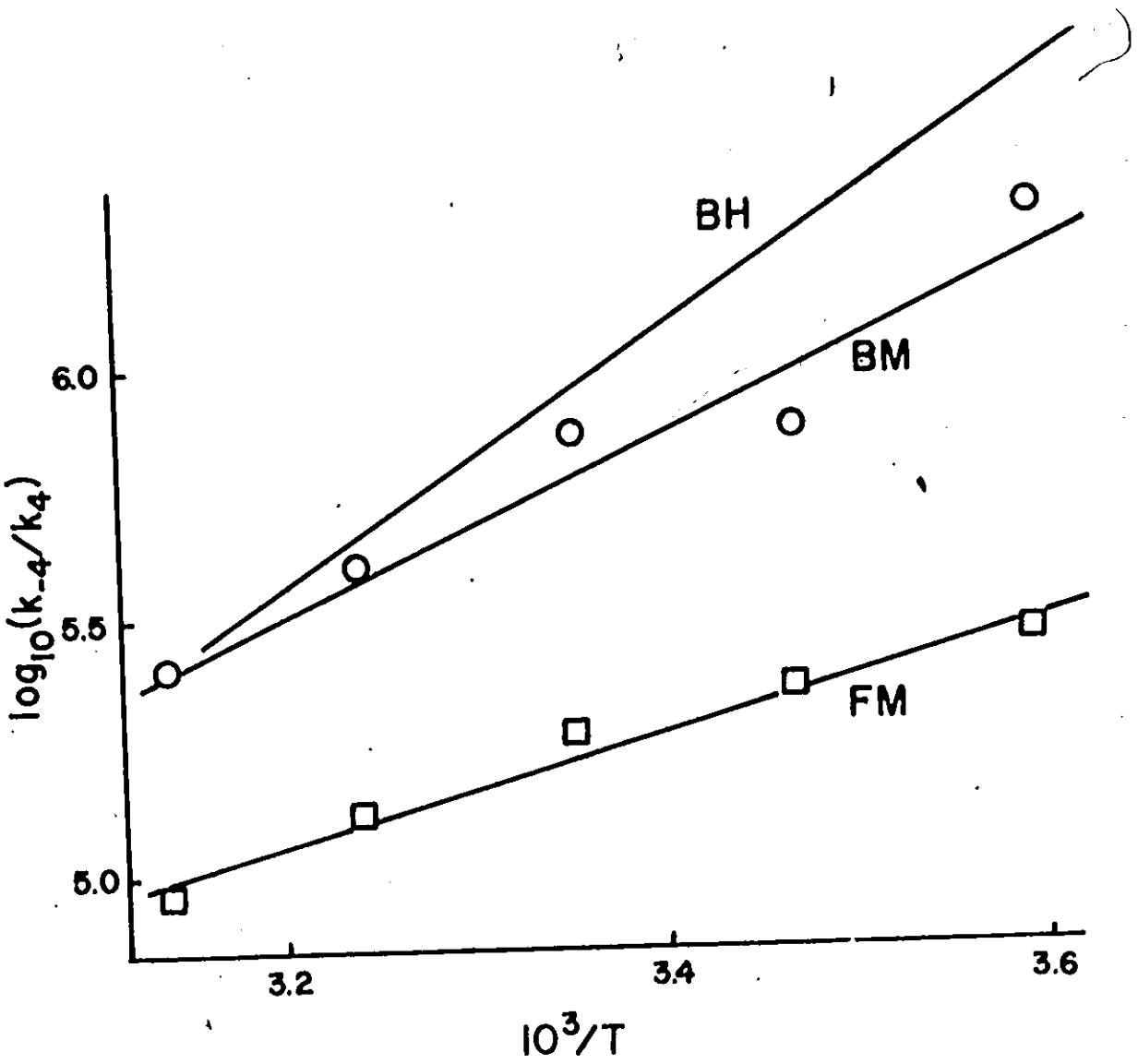

BH
BM
FM
6.0
5.5
5.0
$\log_{10}(k_{-4}/k_4)$
3.2
3.4
3.6
$10^3/T$

Figure 20

Arrhenius plots of k_{cat} (sec^{-1}) in the lactate to pyruvate direction for beef and flounder muscle. The k_{cat} for beef heart (Figure 10) is included for comparison.

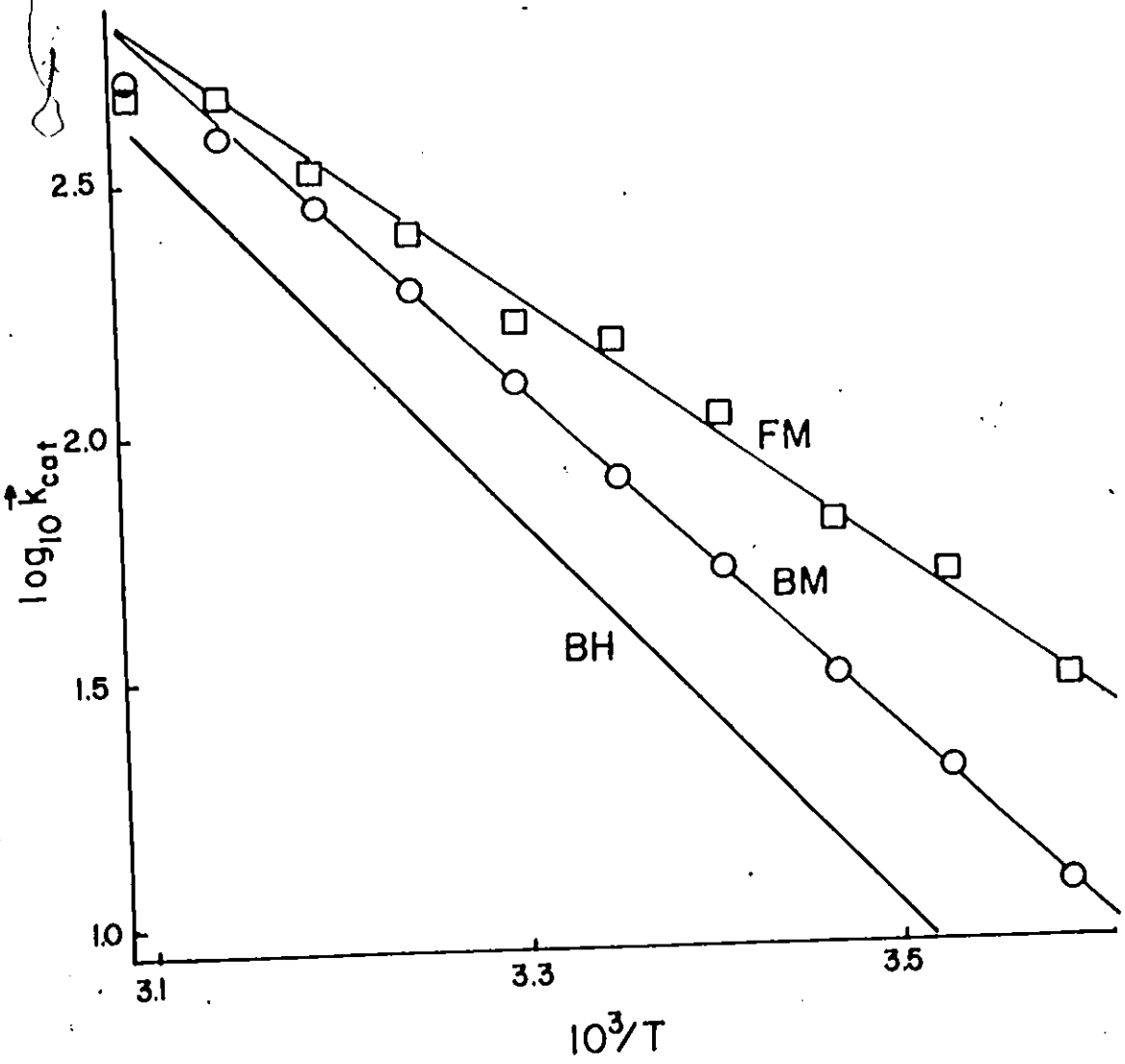

2.5
2.0
1.5
1.0
$\log_{10} k_{cat}$
FM
BM
BH
3.1
3.3
3.5
$10^3/T$

Figure 21

Logarithmic plot of $k_2k_3/k_{-2}k_{-3}$ against 1/T for beef and flounder muscle. The value for beef heart (Figure 12) is included for comparison.

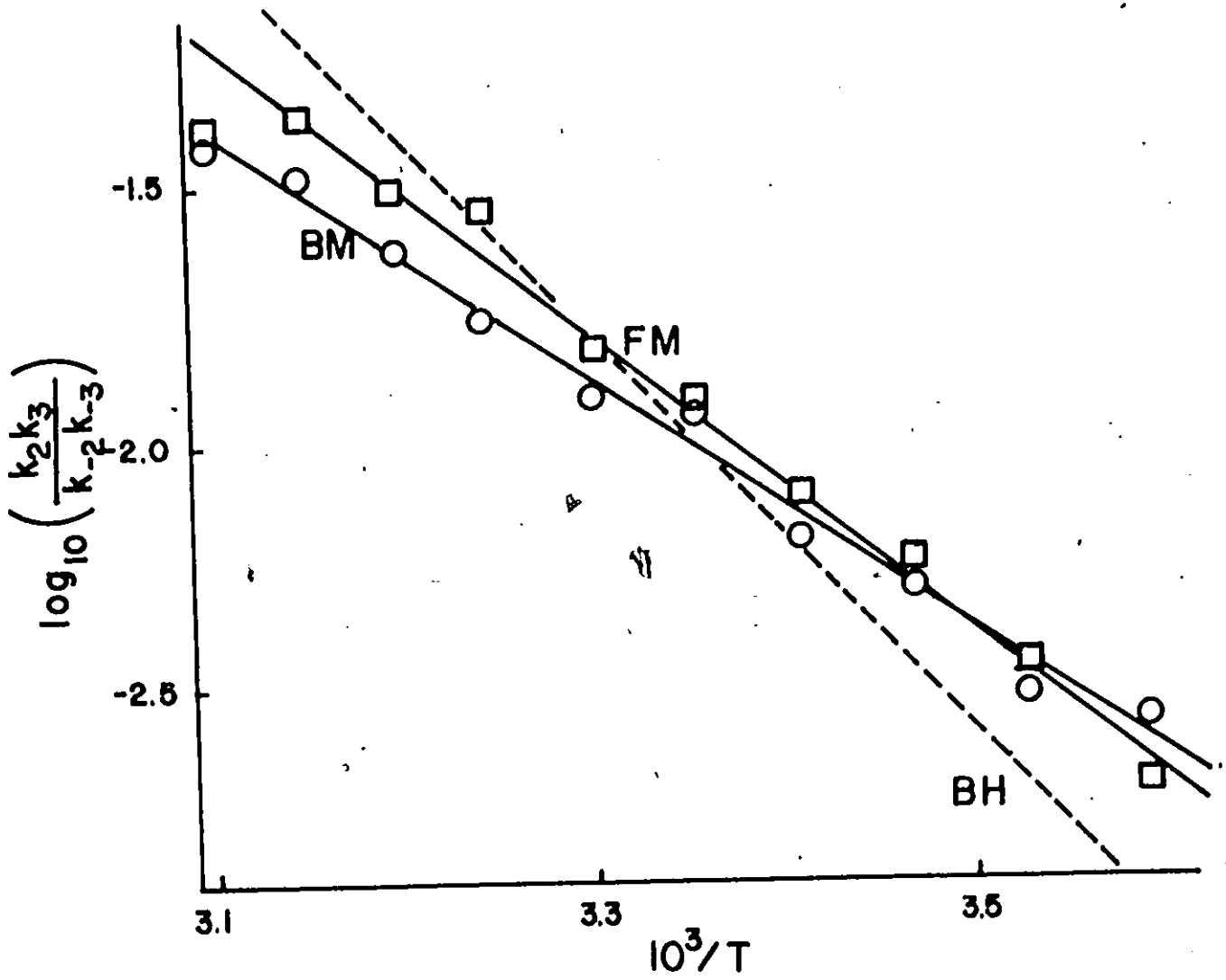
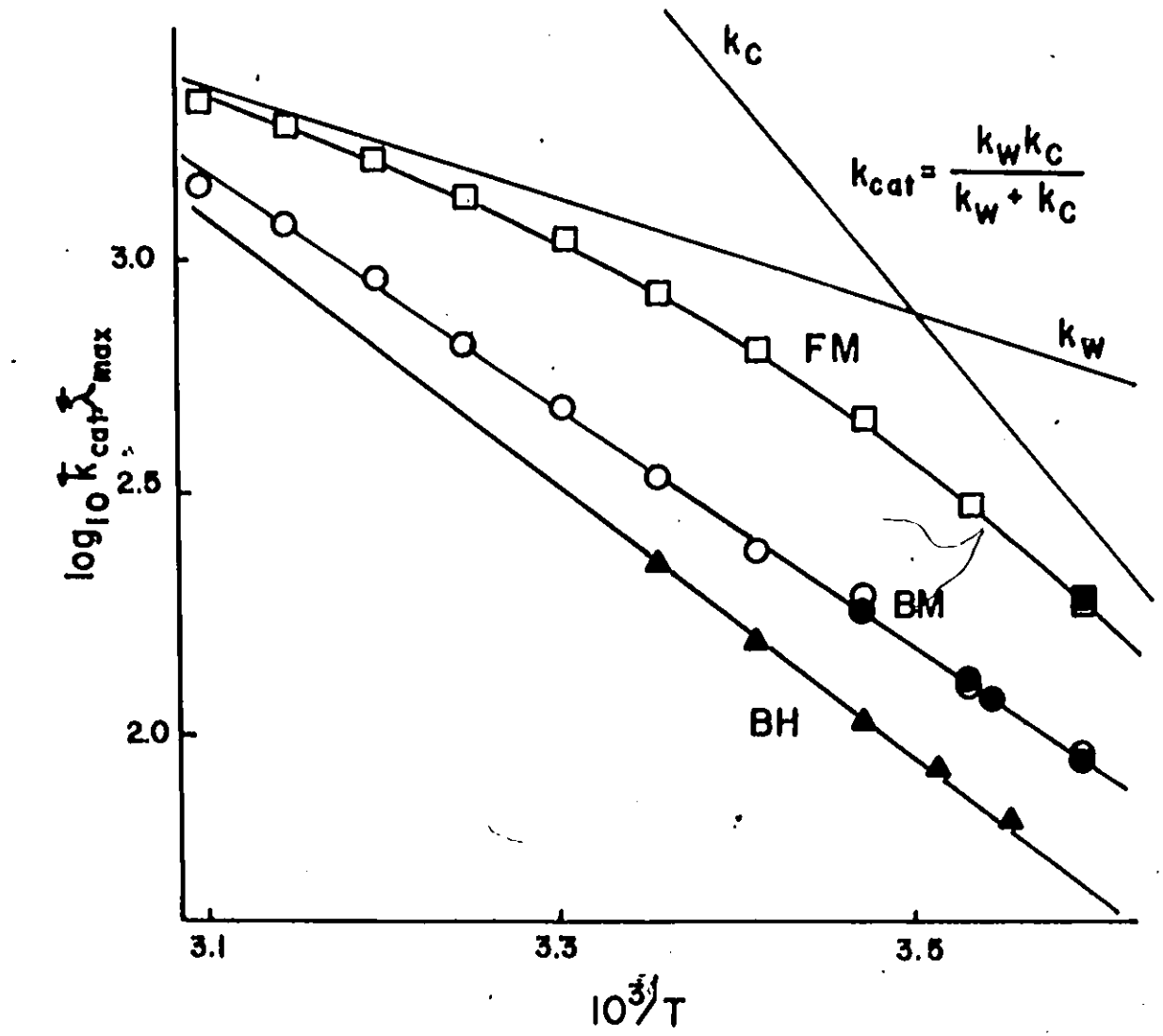

-1.5
-2.0
-2.5
BM
FM
BH
3.1
3.3
3.5
$10^3/T$
$\log_{10}\left(\frac{k_2k_3}{k_{-2}k_{-3}}\right)$

Figure 22

Arrhenius plot of k_{cat} and λ_{max} (sec^{-1}) in the pyruvate to lactate direction for beef and flounder muscle. The values for beef heart (Figures 10 and 16) are included for comparison. The k_{cat} for flounder is a curve calculated from $k_{cat} = k_w k_c/(k_w + k_c)$. Open symbols: k_{cat}; solid symbols: λ_{max}.


k_c
$k_{cat} = \frac{k_w k_c}{k_w + k_c}$
k_w
FM
BM
BH
3.0
2.5
2.0
$\log_{10} k_{cat}/k_{max}$
3.1
3.3
3.5
$10^3/T$

and k_c at low temperatures (Figure 22). Even though an apparent switch in the rate-limiting step occurs, the enthalpy and entropy of activation for k_{cat} were determined from k_w for two reasons: a) k_w approximates k_{cat} over a larger temperature range than does k_c and, b) k_w intersects k_{cat} for beef muscle and beef heart at 60°C, ie., the compensation temperature (discussed in detail later) is 60°C, as is seen for k_{cat} in the forward direction (Figure 20) and most other rate constants as well.

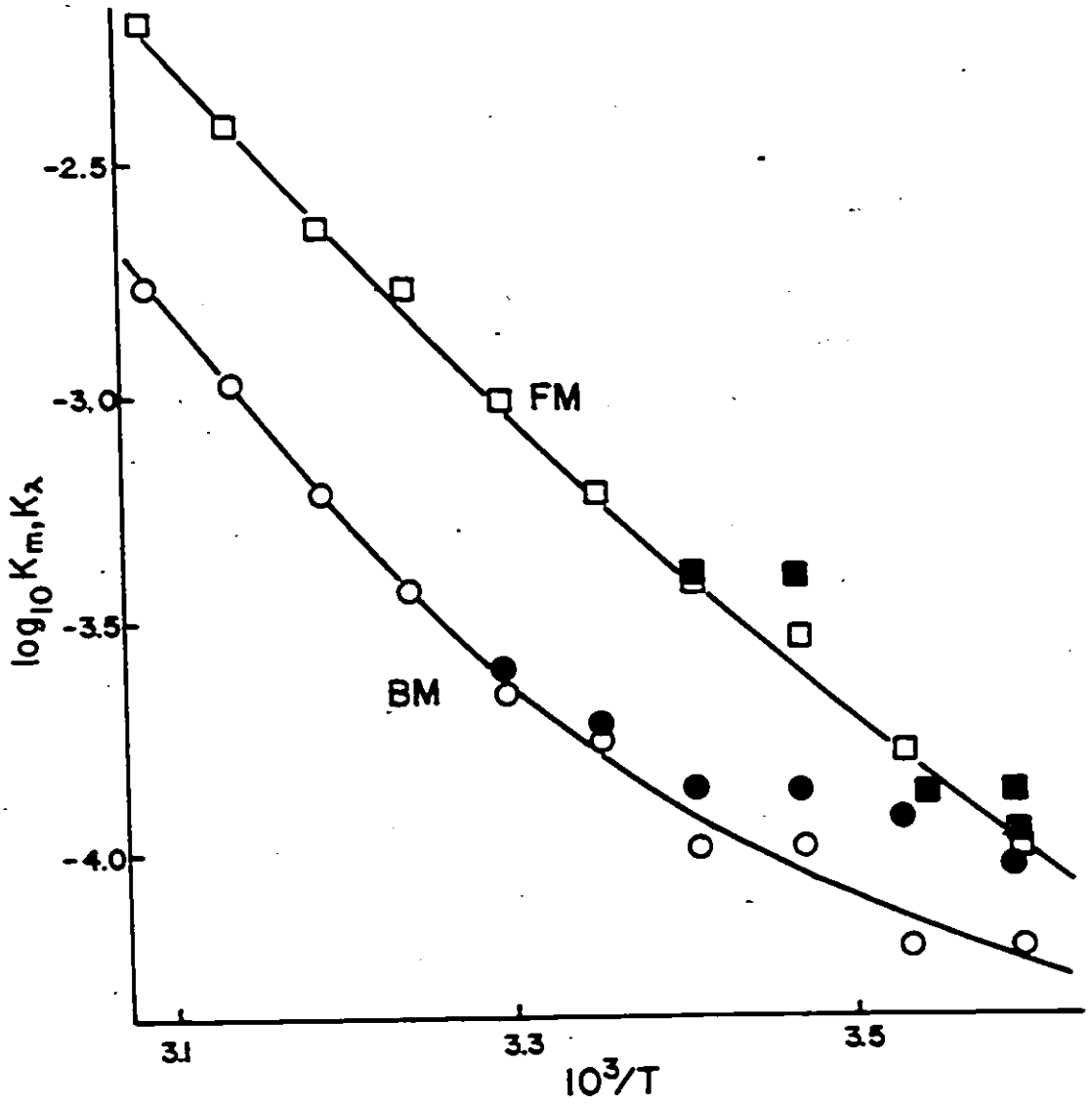
Maximal pre-steady-state rates ($\lambda_{max} = k_{-2}$, equation 36) for all three enzymes in the pyruvate to lactate direction are equal to k_{cat}, and hence the catalytic rate constant in the reverse direction is k_{-2} in each enzyme. For beef heart LDH, pyruvate concentrations corresponding to half-maximal pre-steady-state velocities ($K_\lambda = k_3/k_{-3}$) give a linear plot when plotted logarithmically against 1/T and are equal to K_{mX} at 50°C (Figure 17). Hence K_{mX} is equal to k_3/k_{-3} at high temperatures and k_{-2}/k_{-3} at low temperatures (see section A).

For the two muscle enzymes, logarithmic plots of K_λ are not linear, but are equal to K_{mX} (Figure 23). Pre-steady-state studies at low pyruvate concentrations therefore yield no further information. K_{mX} is taken to be equal to k_3/k_{-3} at 50°C, as with the beef enzyme. All other calculations are performed identically in the three enzymes.

Since the catalytic rate constants in the pyruvate to lactate direction can be obtained directly from the pre-steady-

Figure 23

Logarithmic plot of K_{mX} and $K_{\lambda X}$ (M) against 1/T for beef and flounder muscle. Open symbols: K_{mX}; solid symbols: $K_{\lambda X}$.


-2.5
-3.0
-3.5
-4.0
$\log_{10} K_m, K_s$
FM
BM
3.1
3.3
3.5
$10^3/T$

state studies, and since V_{max} is equal to $k_{cat}[E]_o$, the concentration of active enzyme can be calculated.

Table V shows the values of the eight rate constants, determined by the above method, for the three enzymes. The enthalpies $\Delta H^{\neq}$ and entropies $\Delta S^{\neq}$ of activation are given in Table VI. Standard deviations for these enthalpies, as calculated from regression analysis, are about 0.5 kcal for k_{-2} and k_4; 1 kcal for k_1, k_2, k_3 and k_{-3}; and 2 kcal for k_{-1} and k_{-4}.

From these results, it is now possible to analyse differences in the three enzymes which may indicate adaptation at the molecular level between endothermic (e.g., beef) and ectothermic (e.g., flounder) LDHs.

C. Denaturation Rates for Beef Heart, Beef Muscle and Flounder Muscle LDH

Rates of denaturation (D) are shown in Figure 24, plotted logarithmically against the inverse of temperature. Each enzyme was tested in the absence and presence of substrates (2 mM NAD^+ and 10 mM lactate). High substrate concentrations were used, so presumably most of the enzyme is in the form of the ternary complex. Flounder muscle LDH denatures at the lowest temperature and beef heart LDH at the highest temperature. In all cases, the presence of substrates helps reduce the rate of denaturation. The activation enthalpies calculated from regression analysis were 137 and 144 kcal for beef heart, 128 and 127 kcal for beef muscle and 119 and 123 kcal for flounder muscle LDH

TABLE V

Rate Constants for Lactate Dehydrogenases at 0 and 50°C

		Beef Heart		Beef Muscle		Flounder Muscle	
		0°C	50°C	0°C	50°C	0°C	50°C
k_1	$(M^{-1}sec^{-1})$	4.00×10^5	1.45×10^6	2.90×10^5	7.50×10^5	6.00×10^4	4.40×10^5
k_{-1}	(sec^{-1})	6.76	1.88×10^3	3.45×10^1	3.75×10^2	3.47×10^1	8.21×10^2
k_2	$(M^{-1}sec^{-1})$	2.04×10^4	2.06×10^5	1.00×10^5	4.10×10^4	5.00×10^4	2.41×10^4
k_{-2}	(sec^{-1})	3.10×10^1	1.27×10^3	6.15×10^1	1.59×10^3	5.00×10^2	2.33×10^3
k_3	(sec^{-1})	2.14	3.29×10^4	1.46	1.51×10^4	2.73×10^1	2.74×10^4
k_{-3}	$(M^{-1}sec^{-1})$	2.14×10^6	5.29×10^7	1.32×10^6	9.52×10^6	1.82×10^6	4.44×10^6
k_4	(sec^{-1})	2.53	4.33×10^2	6.85	6.65×10^2	2.08×10^1	6.74×10^2
k_{-4}	$(M^{-1}sec^{-1})$	1.37×10^7	8.66×10^7	1.44×10^7	1.40×10^8	7.49×10^6	5.93×10^7

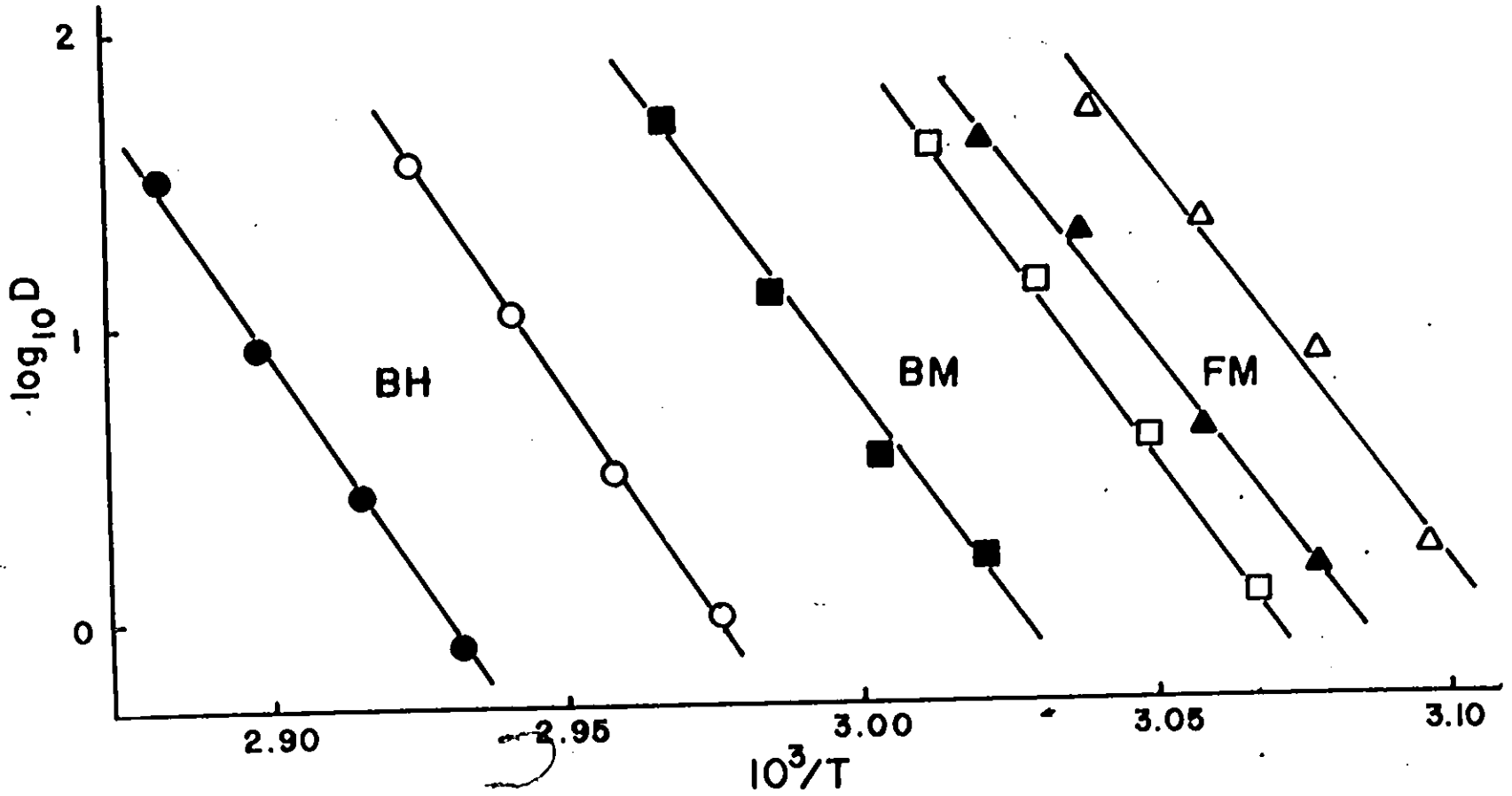
TABLE VI

Enthalpies and Entropies of Activation

	$\Delta H^{\neq}$ kcal.mole^{-1}			$\Delta S^{\neq}$ cal.mole^{-1}deg^{-1}		
	Beef Heart	Beef Muscle	Flounder Muscle	Beef Heart	Beef Muscle	Flounder Muscle
k_1	4.0	2.8	6.4	-18.2	-23.0	-13.0
k_{-1}	19.2	7.8	10.5	15.8	-22.6	-12.6
k_2	7.6	-3.7	-3.1	-10.8	-49.0	-48.2
k_{-2}	12.5	10.9	4.9	-5.8	-10.2	-28.2
k_3	33.3	31.8	23.8	65.0	59.2	35.2
k_{-3}	10.7	6.3	2.6	9.8	-7.0	-20.2
k_4	17.5	15.5	11.7	7.6	2.4	-9.6
k_{-4}	5.9	7.3	6.7	-4.0	1.6	-2.4

Figure 24

Arrhenius plot of denaturation rate "D" of pure enzymes (open symbols) and enzymes in presence of 2 mM NAD^+ and 10 mM Lactate (solid symbols). Units for D are $hours^{-1}$.


2
1
0
log10D
BH
BM
FM
2.90
2.95
3.00
3.05
3.10
10³/T

for the enzyme and enzyme plus substrate respectively. The standard error for these values is about 1.5 kcal; 95% confidence intervals are thus about ± 3 kcal. The differences between the activation enthalpies for enzyme in the pure form and in the presence of substrate therefore does not appear to be significant, especially for the muscle enzymes. For each enzyme, the activation enthalpies in the presence and absence of substrate are averaged and, along with activation entropies, are shown in Table VII. The temperature which results in a denaturation rate of 1.0 hr^{-1} in the presence of substrate is also listed, along with the denaturation rate of the pure enzyme at the same temperature.

TABLE VII

Denaturation Rates and Enthalpy and Entropy of Activation for Denaturation

Enzyme	$T^{o}C$	Denaturation rate, D. ($hrs.^{-1}$)	$\Delta H^{\neq}$ $kcal.mole^{-1}$	$\Delta S^{\neq}$ $cal.mole^{-1}deg.^{-1}$
Beef heart	68.4	25	141	349
Beef heart + 2mM NAD^{+} + 10mM lactate	68.4	1.0	141	343
Beef muscle	57.4	15	129	326
Beef muscle + 2mM NAD^{+} + 10mM lactate	57.4	1.0	129	321
Flounder muscle	51.3	4.3	114	285
Flounder muscle + 2mM NAD^{+} + 10mM lactate	51.3	1.0	114	282

V

DISCUSSION

A. The Kinetics and Thermodynamics of Lactate Dehydrogenase

Beef Heart LDH: A Five Step Model

In the results section, it has been shown that steady-state kinetics combined with pre-steady-state kinetics in either the forward or reverse directions are consistent with the four-step model. However, pre-steady-state kinetics in the two directions are not consistent with each other, as shown by the discrepancies between Tables III and IV. Thus the portion of the model concerned with the pre-steady-state (k_2, k_{-2}, k_3 and k_{-3}) is incomplete.

Pre-steady-state data in the reverse direction have shown that at high temperatures $K_{\lambda X}$ ($= k_3/k_{-3}$) is equal to K_{mX} ($= (k_3 + k_{-2})/k_{-3}$) (Figure 17), and $\overleftarrow{\lambda}_{max}$ is equal to $\overleftarrow{V}_{max}/[E]_o$ (Figure 16). Therefore, $\overleftarrow{V}_{max}/K_{mX}[E]_o$ is equal to $\overleftarrow{\lambda}_{max}/K_{\lambda X}$ ($= k_{-2}k_{-3}/k_3$) at high temperatures and to k_{-3} at low temperatures. Consequently, $\overleftarrow{V}_{max}/K_{mB}[E]_o$ should be equal to k_2 at high temperatures and to k_2k_3/k_{-2} at low temperatures (equation 11). However, pre-steady-state data in the reverse direction indicates that this is not the case.

The term C (equation 13) is equal to $K_{mX}/\overleftarrow{V}_{max}$ at high temperatures (Figure 15). From equation 11 it is known that $\overleftarrow{V}_{max}/K_{mB}$ is equal to $k_2k_3/k_{-2}k_{-3}$ multiplied by $\overleftarrow{V}_{max}/K_{mX}$ ($= 1/C$ at high temperatures). In the results section, it was shown that C is equal to $1/k_{-3}[E]_o$. Hence, at high temperatures, $\overleftarrow{V}_{max}/K_{mB}$ is equal to $k_2k_3[E]_o/k_{-2}$. $\overleftarrow{V}_{max}/K_{mB}[E]_o$ is, therefore, equal to $\overleftarrow{\lambda}_{max}/K_{\lambda B}$ at high, and not at low temperatures.

The simplest way of reconciling the above results is to include an additional step, the interconversion of the two ternary complexes, in the reaction mechanism. A five-step scheme can therefore, be proposed:

$$E \underset{k_{-1}}{\overset{k_1[A]}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2[B]}{\rightleftharpoons}} EAB \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} EXY \underset{k_{-3}[X]}{\overset{k_3}{\rightleftharpoons}} EY \underset{k_{-4}[Y]}{\overset{k_4}{\rightleftharpoons}} E$$

Although more complicated mechanisms have been proposed, all of the results can be explained using this model.

As mentioned above, V_{max}/K_m is equal to λ_{max}/K_λ in either direction at high temperatures (50°C). $\vec{V}_{max}/K_{mB}$ divided by $\vec{V}_{max}/K_{mX}$ is equal to the equilibrium constant between EA and EY, and hence is given by $k_a k_a k_3/k_{-2}k_{-a}k_{-3}$. Consequently, at 50°C, $\vec{\lambda}_{max}/K_{\lambda B}$ divided by $\vec{\lambda}_{max}/K_{\lambda X}$ must also be equal to this equilibrium constant. This can be achieved if the rate limiting step in the pre-steady-state in the two directions is the same step. Hence, the most likely possibility is

$$\vec{\lambda}_{max} = k_a \tag{47}$$

$$\vec{\lambda}_{max} = k_{-a} \tag{48}$$

$$K_{\lambda B} = k_{-2}/k_2 \tag{49}$$

$$K_{\lambda X} = k_3/k_{-3} \tag{50}$$

If k_{-2} were rate limiting, then k_{-2} would also be rate limiting in the pre-steady-state. This is not the case since the pre-steady-state in the forward direction is hyperbolically related to the concentration of B (Figure 8). Similarly, k_3 and k_{-3} can be ruled out as the rate-limiting steps. Under saturating

concentrations of coenzyme, the steady-state treatment gives,

$$\frac{\vec{V}_{max}}{K_{mB}[E]_o} = \frac{k_a\, k_2\, k_3}{k_a\, k_3 + k_{-2}k_3 + k_{-2}\, k_{-4}} \qquad (51)$$

and similarly

$$\frac{\vec{V}_{max}}{K_{mX}[E]_o} = \frac{k_{-a}\, k_{-2}\, k_{-3}}{k_a\, k_3 + k_{-2}k_3 + k_{-2}\, k_{-a}} \qquad (52)$$

At 50°C the largest term in the denominators of these expressions must be $k_{-2}k_3$, from which it follows that V_{max}/K_m is equal to λ_{max}/K_λ in either direction. If at 0°C the largest term in the denominator were k_ak_3, then $\vec{V}_{max}/K_{mB}[E]_o$ would be equal to k_2, and k_{-2} could be calculated. The value of k_{-2} calculated in this way is much smaller than k_{-a}, and hence the steady-state would be slower than the pre-steady-state in the pyruvate to lactate direction. Since this is not the case, this possibility can be ruled out. If $k_{-2}k_{-a}$ is the largest term in the denominator at 0°C, the $\vec{V}_{max}/K_{mX}[E]_o$ is equal to k_{-3}, whence k_3 can be calculated (i.e., k_3 and k_{-3} for the five-step model are equal to k_3 and k_{-3} in Table IV). This value of k_3 at 0°C is 2.14 sec^{-1}, and is approximately equal to k_4, the catalytic rate constant in the forward direction. At 50°C k_3 is very much larger than k_4. The assumption that $k_{-2}k_{-a}$ is the largest term at 0°C is therefore reasonable, and the only assumption consistent with the evidence.

Reliable values of k_2 and k_{-2} cannot be obtained, but estimates can be made. The term k_ak_3 is approximately equal

to 77 sec^{-2} at 0°C and 7×10^7 sec^{-2} at 50°C (k_3 is obtained from Table IV and k_a is equal to k_3 from Table III), and is always one of the smallest terms. At 0°, if $k_{-2}k_3$ is larger than k_ak_3, k_{-2} might be about 400 ($k_{-2}k_3$ = 860 sec^{-2}), while at 50°C, k_{-2} might be about 4×10^5 ($k_{-2}k_{-a} = 5 \times 10^8$ sec^{-2}). These estimates would make k_ak_3 the smallest term in the denominator of V_{max}/K_m. The values of all ten rate constants, at 0°C and 50°C, are shown in Table VIII. The values of k_2 and k_{-2} are minimum values only. The values in Table VIII show that k_2 is lower than k_{-3} and hence, the rate of binding of pyruvate is faster than the rate of binding of lactate. This suggests that the estimates of k_2 and k_{-2} are not unreasonably high.

The values shown in Table VIII can be compared with those obtained by Südi (1974) for pig heart. Values for pig heart at 6°C are similar to those observed in Table VIII at 0°C. For the two first-order rate constants, Südi obtained k_2 = 1.27×10^5 (2.26×10^5 for beef heart) and $k_{-3} = 8.33 \times 10^5$ (2.14×10^6 for beef heart). The other values are a little higher for pig than for beef heart; k_{-2} = 1200 (400 for beef), k_a = 636 (36 for beef), k_{-a} = 204 (31 for beef), and k_3 = 222 (2.1 for beef). The equilibrium constant k_a/k_{-a} therefore differs only by a factor of about 3. It appears that both lactate and pyruvate bind comparatively more tightly to the beef than pig enzyme (i.e., k_2 and k_{-3} are larger; k_{-2} and k_3 are smaller). For both enzymes, k_3 is the smaller rate constant in the forward direction and k_{-a} in the reverse direction. Hence, the above calculations do not appear un-

TABLE VIII

Rate Constants for the Five-Step Model at 0 and 50°C

	0°C	50°C
k_1 $(M^{-1}sec^{-1})$	4.00×10^5	1.45×10^6
k_{-1} (sec^{-1})	6.76	1.88×10^3
k_2* $(M^{-1}sec^{-1})$	2.26×10^5	3.78×10^7
k_{-2}* (sec^{-1})	4.00×10^2	4.00×10^5
k_a (sec^{-1})	3.60×10^1	2.20×10^3
k_{-a} (sec^{-1})	3.10×10^1	1.27×10^3
k_3 (sec^{-1})	2.14	3.29×10^4
k_{-3} $(M^{-1}sec^{-1})$	2.14×10^6	5.29×10^7
k_4 (sec^{-1})	2.53	4.33×10^2
k_{-4} $(M^{-1}sec^{-1})$	1.37×10^7	8.66×10^7

*k_2 and k_{-2} are estimates only.

reasonable. Südi did not include estimates of k_1, k_{-1}, k_4 or k_{-4}.

Table IX shows the relationship between the k_2, k_{-2}, k_3 and k_{-3} values obtained from the four-step model, and the k_2, k_{-2}, k_a, k_{-a}, k_3 and k_{-3} values that are involved in the five-step model. For example, the k_{-2} value obtained in the lactate $\rightarrow$ pyruvate direction with the four-step model is actually $k_a k_3/k_{-a}$ in the five-step model. The ternary complex identified from pre-steady-state data in the forward direction is EAB, while that identified from pre-steady-state data in the reverse direction is EXY. If in fact the actual mechanism is more complicated than the five-step model, the rate constants again will be composite constants. As has been discussed, the non-linear Arrhenius plots of $\vec{V}_{max}/K_{mY}$ suggest that at least two steps are involved in the binding of Y (NADH) to the enzyme. Similarly the evidence suggests that the release of NAD^+ (k_{-1}) must involve two steps; k_{-1} is smaller than k_{-2} in Table VIII although it has been shown that k_{-2} is rate limiting. Thus, although the present data have been satisfactorily interpreted in terms of five steps, the indications are that at least seven steps are probably involved. Previous workers have in fact suggested as many as eleven steps (Stinson and Gutfreund, 1971; Bennett and Gutfreund, 1973).

The four-step model still retains its usefulness, since it is the best that one can do if the reaction can only be studied in one direction. For beef muscle and flounder muscle lactate dehydrogenase it is impossible to study the pre-

TABLE IX

Relationship between Four - and Five - Step Models

Rate Constant from Four step model	Equivalent value from five-step model, as compared to the four step model, using only pre-steady-state data in the:	
	Forward Direction (i.e. Table III)	Reverse Direction (i.e.: Table IV)
k_2	$\frac{k_2 k_a k_3}{k_{-2} k_{-a}}$	$\frac{k_2 k_a}{k_{-2}}$
k_{-2}	$\frac{k_a k_3}{k_{-a}}$	k_{-a}
k_3	k_a	k_3
k_{-3}	$\frac{k_{-a} k_{-3}}{k_3}$	k_{-3}

steady-state kinetics in the lactate → pyruvate direction. Therefore, the kinetics of the three enzyme systems must be compared on the basis of the four-step model.

Thermodynamics of the Five-Step Model

The enthalpies and entropies of activation for the ten rate constants are listed in Table X. The parameters corresponding to k_2 and k_{-2} are estimates since only the minimum values of these rate constants could be determined (see page 92). Figure 25 demonstrates the free-energy profile corresponding to the five-step model using the values in Table X. The height of the barrier between EA and EAB is uncertain although we know that it must be below one of the barriers between EAB and EY. When the enzyme is saturated with A or Y, the barriers between EA or EY and the free enzyme disappear, leaving only the three intermediate barriers. At 50°C, the highest barrier is between EAB and EXY, and hence this barrier is detected kinetically. At 0°C, the barrier between EXY and EY is detected. The height of the barrier between EA and EAB cannot be calculated since it is always below one of the other barriers. The barrier between EAB and EY, which is highest, changes with temperature because of the large difference between the activation entropies of reactions -a and 3 (Table X). Since the entropy of activation for reaction -a is very small, the free energy of activation is almost independent of temperature (i.e., $\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$). The free energy of reaction 3 however,

TABLE X

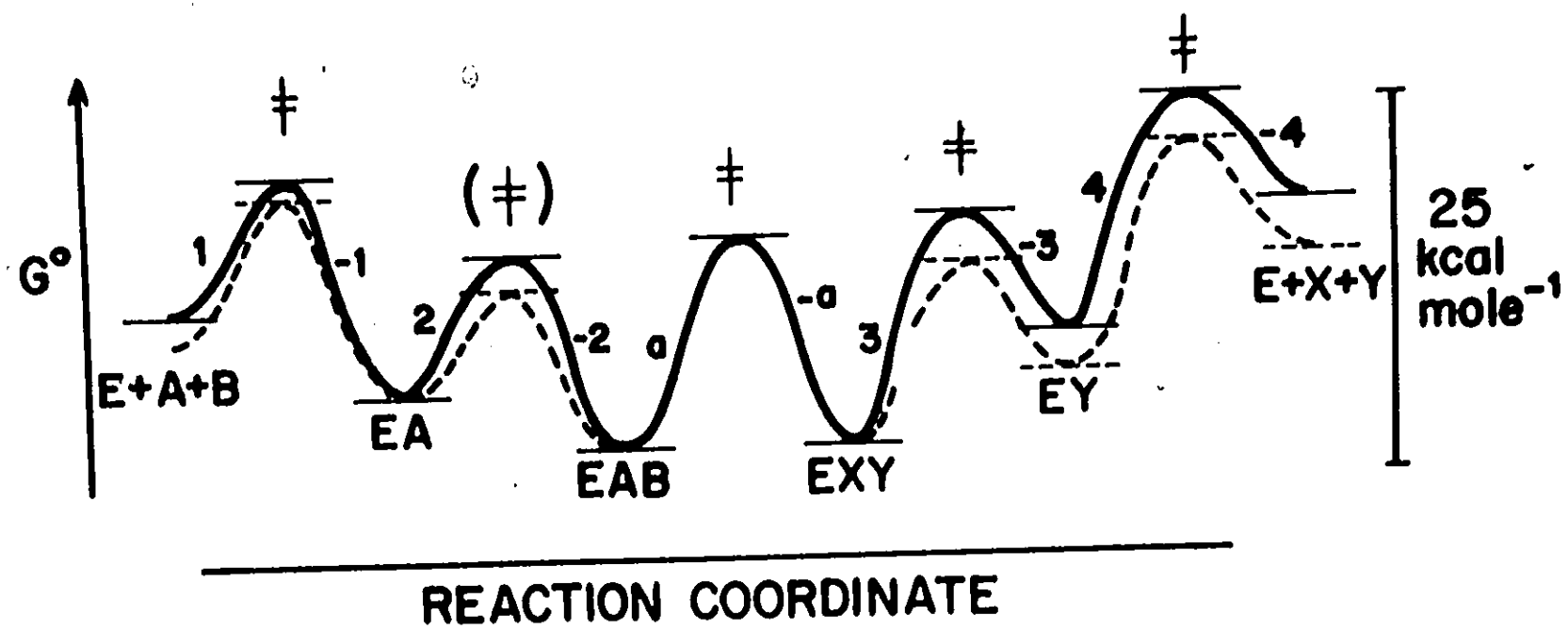
Enthalpies and Entropies of Activation: Five-Step Model

	$\Delta G^{\ddagger}$ at 25°C kcal mole^{-1}	$\Delta H^{\ddagger}$ kcal mole^{-1}	$\Delta S^{\ddagger}$ cal. mole^{-1} deg^{-1}
k_1	9.4	4.1	-18.2
k_{-1}	14.5	19.2	15.8
k_2*	8.6	17.3	28.8
k_{-2}*	11.9	23.6	39.4
k_a	14.0	13.9	-0.2
k_{-a}	14.2	12.5	-5.8
k_3	13.9	33.3	65.0
k_{-3}	7.8	10.7	9.8
k_4	15.2	17.5	7.6
k_{-4}	7.1	5.9	-4.0

*estimates only

Figure 25

Free-energy profile for the five-step model of beef heart LDH. Solid line: 0°C. Dashed line: 50°C.

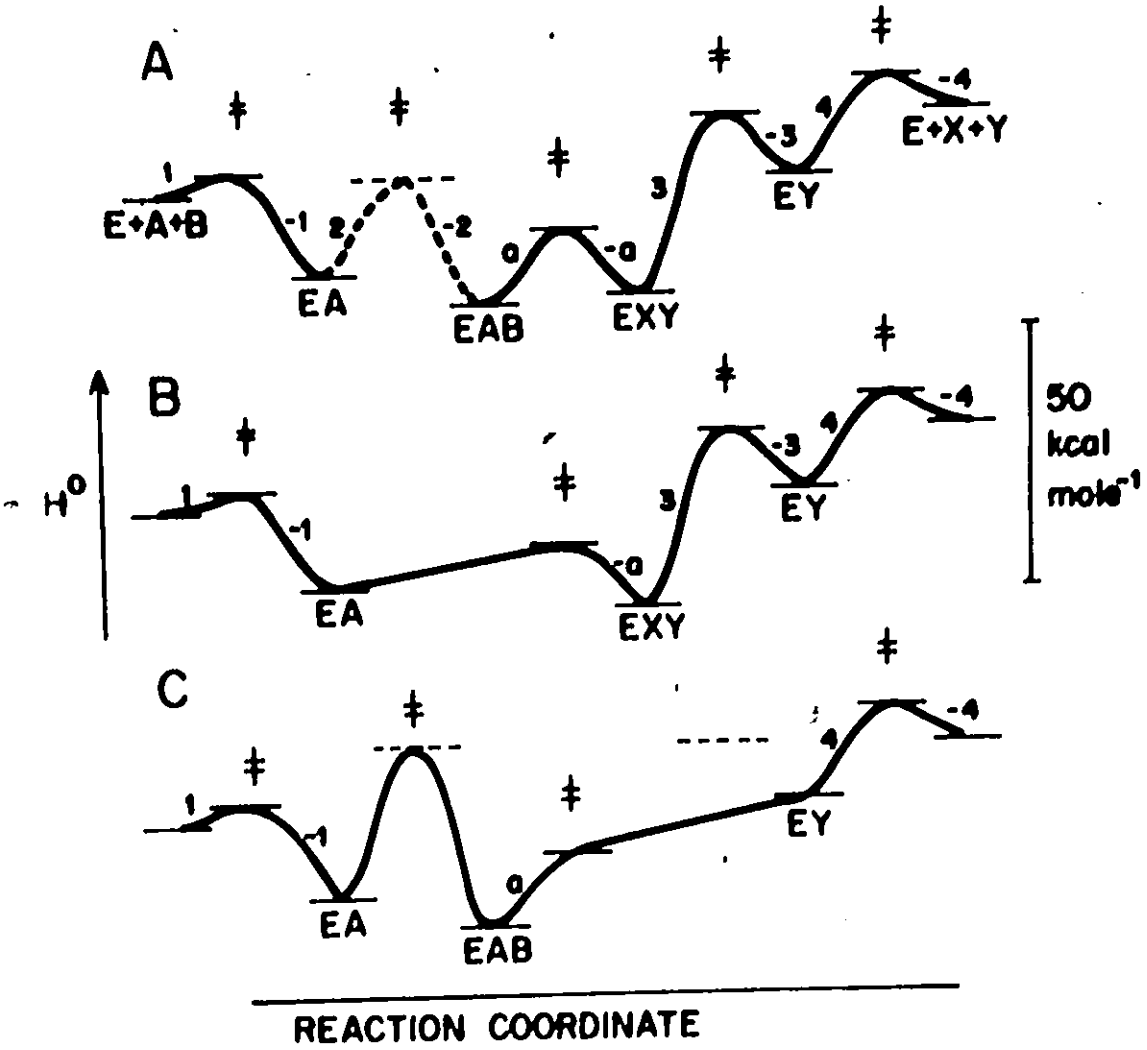

G°
E+A+B
EA
EAB
EXY
EY
E+X+Y
25 kcal mole⁻¹
1
-1
2
-2
a
-a
3
-3
4
-4
REACTION COORDINATE

is strongly dependent on temperature and hence the height of the barrier between EXY and EY drops with an increase in temperature. This change in the highest barrier results in a curved Arrhenius plot for the reaction rate at low pyruvate or low lactate concentrations (i.e., V_{max}/K_m is curved). This in turn results in a curve on the plots of log K_m versus 1/T (Figure 13).

Figure 26A illustrates the enthalpy profile corresponding to the five-step model; again, the height of the barrier between EA and EAB is uncertain. Figure 26B is the analogous enthalpy profile obtained on the basis of the four-step model, based on the data for the reaction in the pyruvate to lactate direction. This profile ignores the pre-steady-state data in the lactate to pyruvate direction. This analysis of the data does not reveal the activated complex between EA and EAB, and ignores the ternary complex EAB. Figure 26C is the enthalpy profile obtained using the four-step model in the lactate to pyruvate direction and ignoring the pre-steady-state data in the pyruvate to lactate direction. Comparison of Figure 26C with 26A shows that interpretation of the lactate to pyruvate data in terms of the four-step model misplaces the barrier that is really between EXY and EY, placing it between EA and EAB. Figure 26B is the one that will be used for comparison with muscle enzyme kinetics, for which pre-steady-state data could not be obtained in the forward direction.

Figure 26

Enthalpy profiles, obtained from the data for the beef heart LDH reaction in the two directions. A: profile for the five-step scheme. B: profile for the four-step scheme, based on the data in the pyruvate → lactate direction but neglecting the pre-steady-state results in the lactate → pyruvate direction. C: profile for the four-step scheme, based on the data in the lactate → pyruvate direction but neglecting the pre-steady-state results in the pyruvate → lactate direction.


A
B
C
E+A+B
EA
EAB
EXY
EY
E+X+Y
1
-1
2
-2
0
-0
3
-3
4
-4
H°
50 kcal mole⁻¹
REACTION COORDINATE

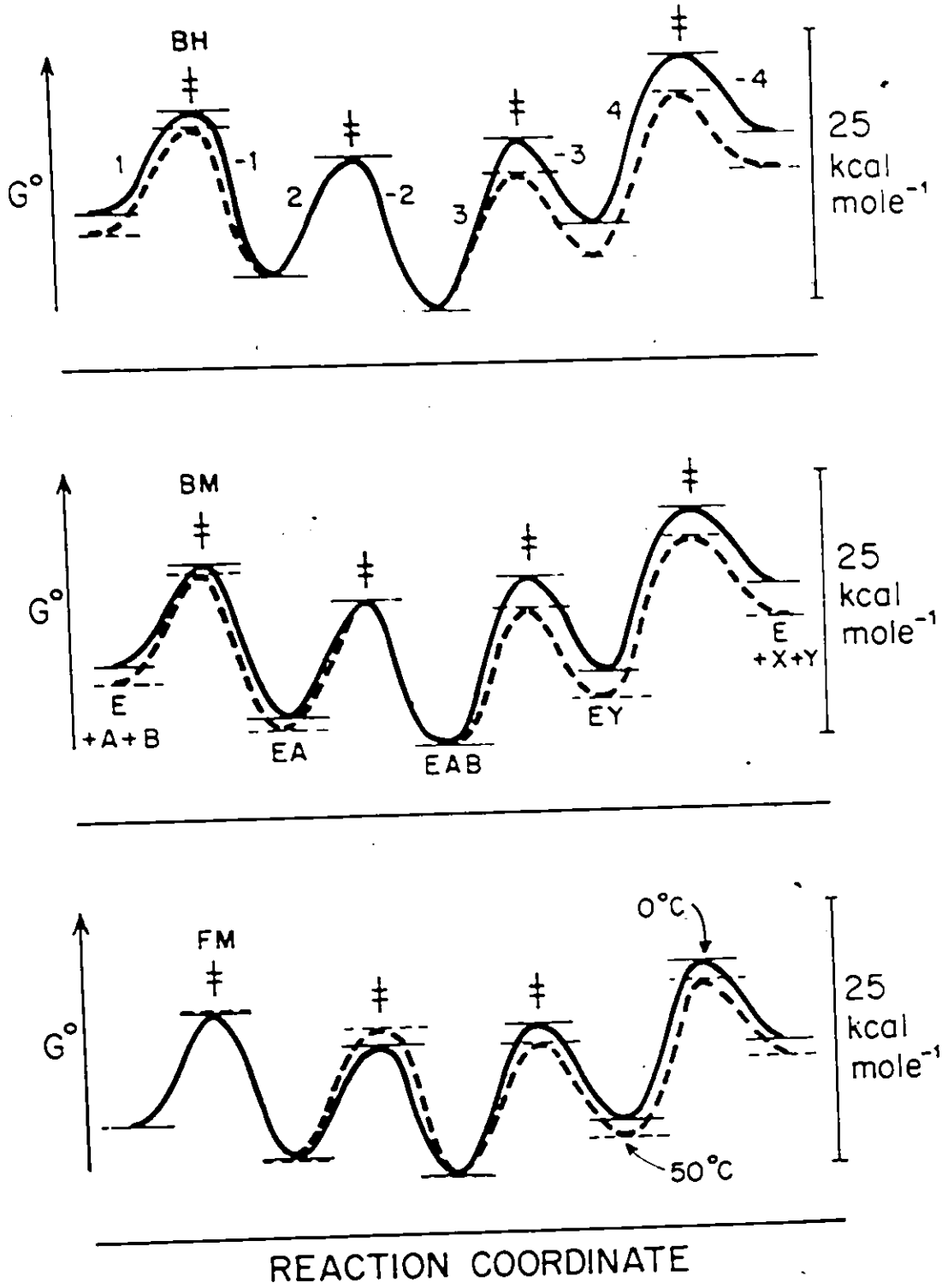
In summary therefore, the four-step model for LDH which will be used to compare the three homologous enzymes is, in reality, a simplification of the complete reaction mechanism which contains at least seven steps. The binding of the coenzyme (NAD^+ or NADH), although probably occurring by at least two steps, is represented as a single step. In this four-step model, the only ternary complex detected (labeled as EAB in previous sections) is probably the enzyme: NADH:pyruvate complex, and the interconversion of the two ternary complexes is a process which cannot be distinguished from the binding of lactate. Hence, many of the rate constants which will be discussed in the next section are, in reality, composite values of true rate constants multiplied by equilibrium constants. Nevertheless, the four-step model is sufficiently detailed to permit a much more detailed comparison of ectothermic and endothermic LDH than has been possible to date, using only steady-state data.

The Comparative Thermodynamics of Beef Heart, Beef Muscle and Flounder Muscle LDH

i. Free-Energy, Enthalpy and Entropy Profiles. The Gibbs free-energy profiles for the three enzymes at 0° and $50^\circ C$ (derived from data in Table V) are shown in Figure 27. For each enzyme, the ternary complex (EAB) is arbitrarily taken to have the same free energy at the two temperatures thus allowing direct comparison of the free energy of activation for k_{-2} and k_3 at the two temperatures. For both the beef

Figure 27

Free-energy profiles for the three enzymes, at 0°C (firm line) and 50°C (dashed line). BH = beef heart; BM = beef muscle; FM = flounder muscle.


BH
G°
1
-1
2
-2
3
-3
4
-4
25
kcal
mole⁻¹
BM
G°
E
+A+B
EA
EAB
EY
E
+X+Y
25
kcal
mole⁻¹
FM
G°
0°C
50°C
25
kcal
mole⁻¹
REACTION COORDINATE

heart and beef muscle enzymes, the free energy of activation $\Delta G^{\neq}_{-2}$ is practically unaffected by temperature, whereas $\Delta G^{\neq}_{3}$ is strongly affected and is higher than $\Delta G^{\neq}_{-2}$ at 0°C and lower at 50°C (Figure 27). Consequently, at low temperatures, k_{-3} corresponds to the rate-limiting step for pyruvate reduction at low pyruvate concentrations. At higher temperatures the rate is controlled by $k_{-3}k_{-2}/k_3$. It is this shift in relative heights of the free-energy barriers that results in the non-linearity of plots of log V_{max}/K_m against 1/T (see theoretical section). For flounder muscle LDH both $\Delta G^{\neq}_{-2}$ and $\Delta G^{\neq}_{3}$ are affected by temperature, but in opposite directions; the net effect is thus the same as for the beef enzymes.

For each of the three enzymes, the ternary complex has the lowest free energy of all the forms of the enzyme (see Figure 27). Relative to the reactants and products, the ternary complex has a lower free energy in the beef heart enzyme than in the muscle enzyme and has the highest free energy in the flounder enzyme (i.e., the lowest drop from reactants to EAB). Further, for the beef enzymes, the free energy of the ternary complex, relative to that of the reactants, is lower at 0°C than at 50°C. For the flounder muscle enzyme, however, the free energy of EAB is the same at the two temperatures, relative to the reactants (E + A + B). Therefore, the free energy drop for formation of EAB from E + A + B, and the effect of temperature on this drop decrease in the order

beef heart > beef muscle > flounder muscle

This suggests that the strength of binding between enzymes and substrates follows the same order. This is also more directly evident from the equilibrium constant for formation of EAB ($k_1k_2/k_{-1}k_{-2}$) at 25°C, which is 2.2×10^6 for beef heart, 8.4×10^5 for beef muscle and 3.1×10^4 for flounder muscle.

The enthalpy profiles (from data in Table VI) are illustrated in Figure 28. As with the free energies, the enthalpies are lowest for the ternary complex for each enzyme. For the beef heart enzyme, there is a large drop in enthalpy for formation of EAB. This drop is smaller for the muscle enzymes, especially flounder muscle. Hence the decrease in enthalpy for formation of EAB is parallelled by the decrease in free energy. Similarly, the size of the drop in enthalpy for formation of EA is largest in the beef heart and smallest in the flounder enzyme.

Entropy profiles for the three enzymes are represented in Figure 29 (from data in Table VI). The beef heart enzyme exhibits a large drop in entropy until the ternary complex is reached; then entropy increases for formation of the activated complex between EAB and EY. The low entropies indicate that the beef heart intermediates, EA and EAB, and the activated complexes between E, EA and EAB, are highly ordered. On the other hand, for the muscle enzymes, especially flounder muscle, the complexes EA and EAB have

Figure 28

Enthalpy profiles for the three enzymes. The notation is as in Fig. 27.

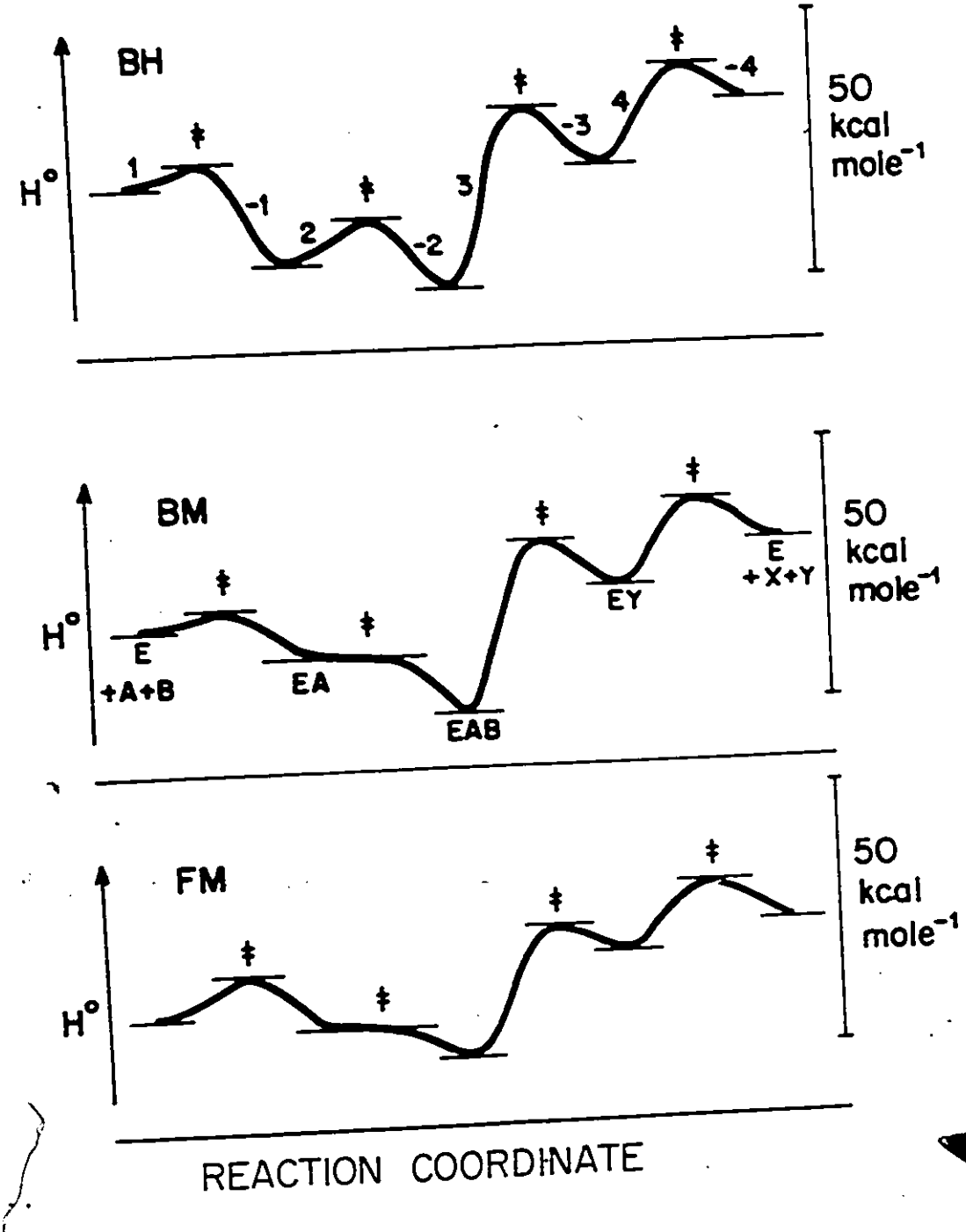
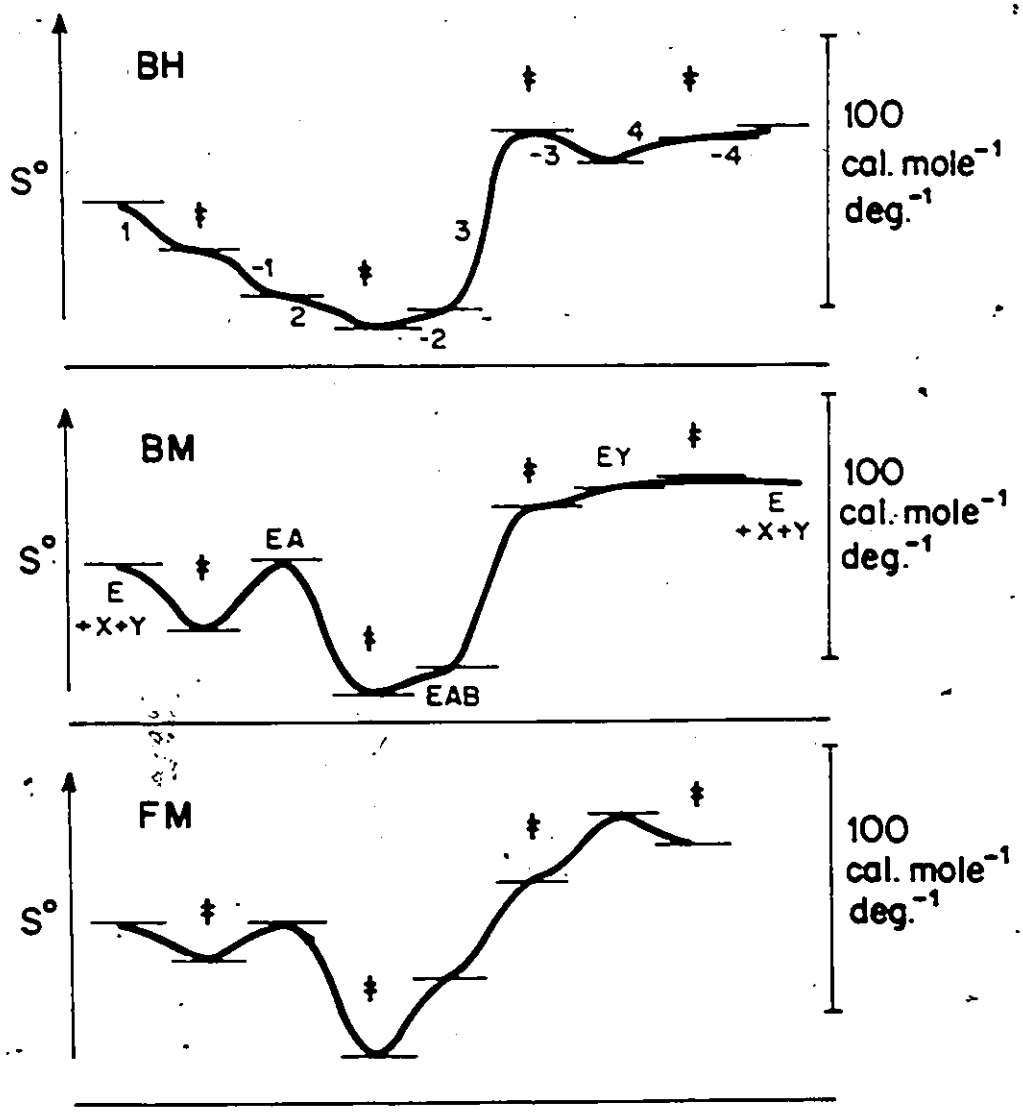

BH
H°
1
-1
2
-2
3
-3
4
-4
50 kcal mole⁻¹
BM
H°
E +A+B
EA
EAB
EY
E +X+Y
50 kcal mole⁻¹
FM
H°
50 kcal mole⁻¹
REACTION COORDINATE

Figure 29

Entropy profiles for the three enzymes. The notation is as in Fig. 27.


BH
S°
1
-1
2
-2
3
-3
4
-4
100
cal. mole⁻¹
deg.⁻¹
BM
S°
E
+X+Y
EA
EAB
EY
E
+X+Y
100
cal. mole⁻¹
deg.⁻¹
FM
S°
100
cal. mole⁻¹
deg.⁻¹
REACTION COORDINATE

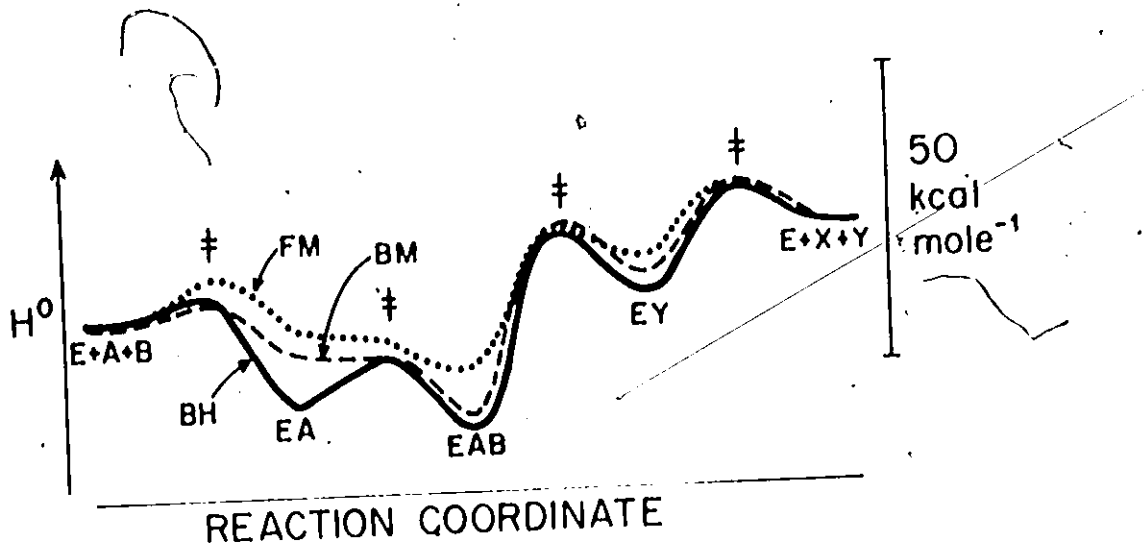
entropies closer to those of the reactants E + A + B. The complexes EA and EAB are therefore less ordered for the muscle enzymes. The decreases in entropy for formation of EA and EAB appear to parallel the decreases in enthalpy and free energy.

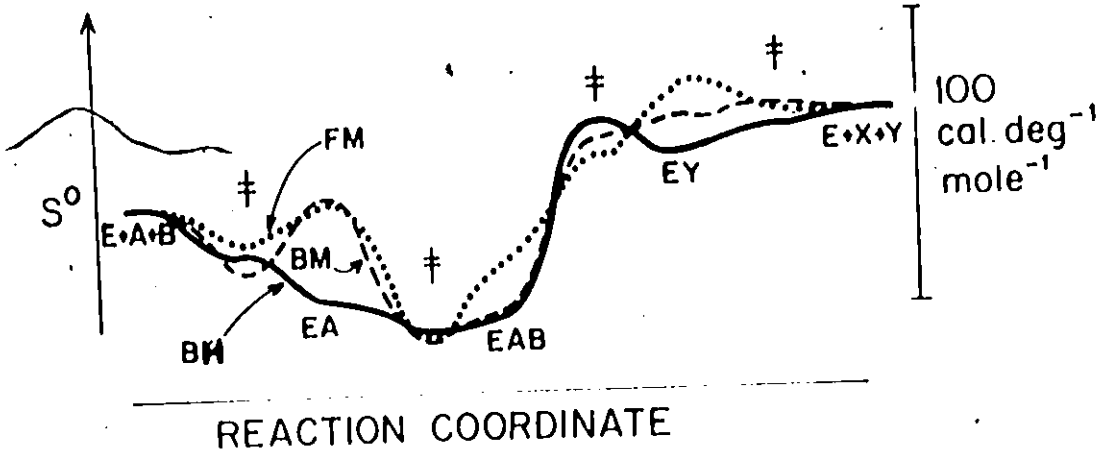
An interesting feature of these results is that the differences in the thermodynamics are reflected much more in the stable complexes (EA, EAB and EY) than in the activated complexes. This is illustrated by Figure 30, in which profiles for the three enzymes are superimposed. The diagram indicates that the differences are smaller in the enthalpy and entropy maxima, corresponding to the activated complexes, than in the minima, corresponding to the stable binary and ternary complexes. Figure 30 also clearly demonstrates compensation between enthalpy and entropy for the stable complexes (i.e., high enthalpy is accompanied by high entropy; e.g., flounder). This compensation effect is discussed in detail below.

ii. Compensation Temperatures. In experiments with homologous reactants, some degree of compensation between enthalpy and entropy is commonly observed. This applies both to thermodynamic quantities relating to overall reactions, and to those relating to activation processes. If, for a series of homologous reactions, $\Delta H^{\ddagger}$ is plotted against $T\Delta S^{\ddagger}$ (see Laidler and Eyring, 1940; Laidler, 1959), the result is typically a straight line of approximately unit slope. A line of exactly unit slope implies that, at the temperature

Figure 30

Enthalpy and entropy profiles; superimposed for the three enzymes. BH = beef heart; BM = beef muscle; FM = flounder muscle.


H°
E+A+B
FM
BM
BH
EA
EAB
EY
E+X+Y
50
kcal
mole⁻¹
REACTION COORDINATE


S°
E+A+B
FM
BM
BH
EA
EAB
EY
E+X+Y
100
cal. deg⁻¹
mole⁻¹
REACTION COORDINATE

in question, the free energies $\Delta G^{\ddagger}$ (= $\Delta H^{\ddagger} - T\Delta S^{\ddagger}$), and hence the rates, are the same. Alternatively, $\Delta H^{\ddagger}$ can be plotted against $\Delta S^{\ddagger}$ (e.g., Low and Somero, 1974), and the slope of such a plot, T_c, is called the compensation or isokinetic temperature. At T_c, differences in $\Delta H^{\ddagger}$ will be exactly compensated for by differences in $\Delta S^{\ddagger}$, cancelling any difference in $\Delta G^{\ddagger}$ or the rate. Similar plots can be made for equilibrium constants (i.e., ΔH versus ΔS) and in this case, T_c is the temperature at which the equilibrium constants are the same.

This type of compensation has frequently been observed (see, e.g., Laidler and Eyring, 1940; Leffler, 1955; Blackadder and Hinshelwood, 1958) and the theoretical basis has been discussed (see, for example, Leffler, 1955; Laidler, 1959; Lumry and Rajender, 1970). Compensation effects have been observed for k_{cat} for numerous enzyme systems; e.g., muscle lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and muscle glycogen phosphorylase-b from ectothermic and endothermic animals (Low and Somero, 1974). Here, compensation temperatures of 333°K have been reported. Cohen et al., (1970) have made a study on chymotrypsin with a series of substrates, some of them amides, and have observed a compensation for the parameters relating to K_m (believed to be close to the true binding constant for the substrate of this enzyme) and to k_{cat}. The compensation temperature for K_m was about 295°K and for k_{cat} was about 285°K (see Lumry and Rajender, 1970).

Compensation plots for the present results are given in Figures 31 and 32 (data from Table VI). Figure 31 shows plots for k_1, k_{-1}, k_{-2}, k_3, k_4 and k_{-4}; all of the points lie on lines corresponding to a compensation temperature of about 333°K. Figure 32 shows plots for k_2 and k_{-3} which give compensation temperatures of approximately 285°K. The equilibrium constant between EA and EY (equation 11) illustrated in Figure 21 is very similar for the three enzymes at 25°C (298°K). The compensation temperature is therefore about 298°K. The reason why the compensation temperatures turn out to be 285, 298 or 333°K is not known.

It is not possible to give a simple and satisfactory interpretation of compensation effects and of compensation temperatures. However, a tightening of the structure of an activated complex, or loosening of the structure of the reactants, leads to a lowering of $\Delta H^{\neq}$, and sometimes to a roughly equal lowering of $T\Delta S^{\neq}$. From an examination of the effects which may lead to a change in structure, Laidler (1959) has concluded that subtle differences in covalent bonding during activated-complex formation will result mainly in changes of $\Delta H^{\neq}$, and not $\Delta S^{\neq}$, with no compensation effect. Laidler (1959) has suggested that compensation will be produced primarily by solvent effects. Similarly, Cohen et al. (1970) have interpreted their compensation effects with chymotrypsin in terms of solvation effects, amide substrates having more water of solvation than non-amide substrates. Formation of an enzyme-substrate complex is

Figure 31

Plot of $\Delta H^{\neq}$ against $\Delta S^{\neq}$ for several constants for the three enzymes.

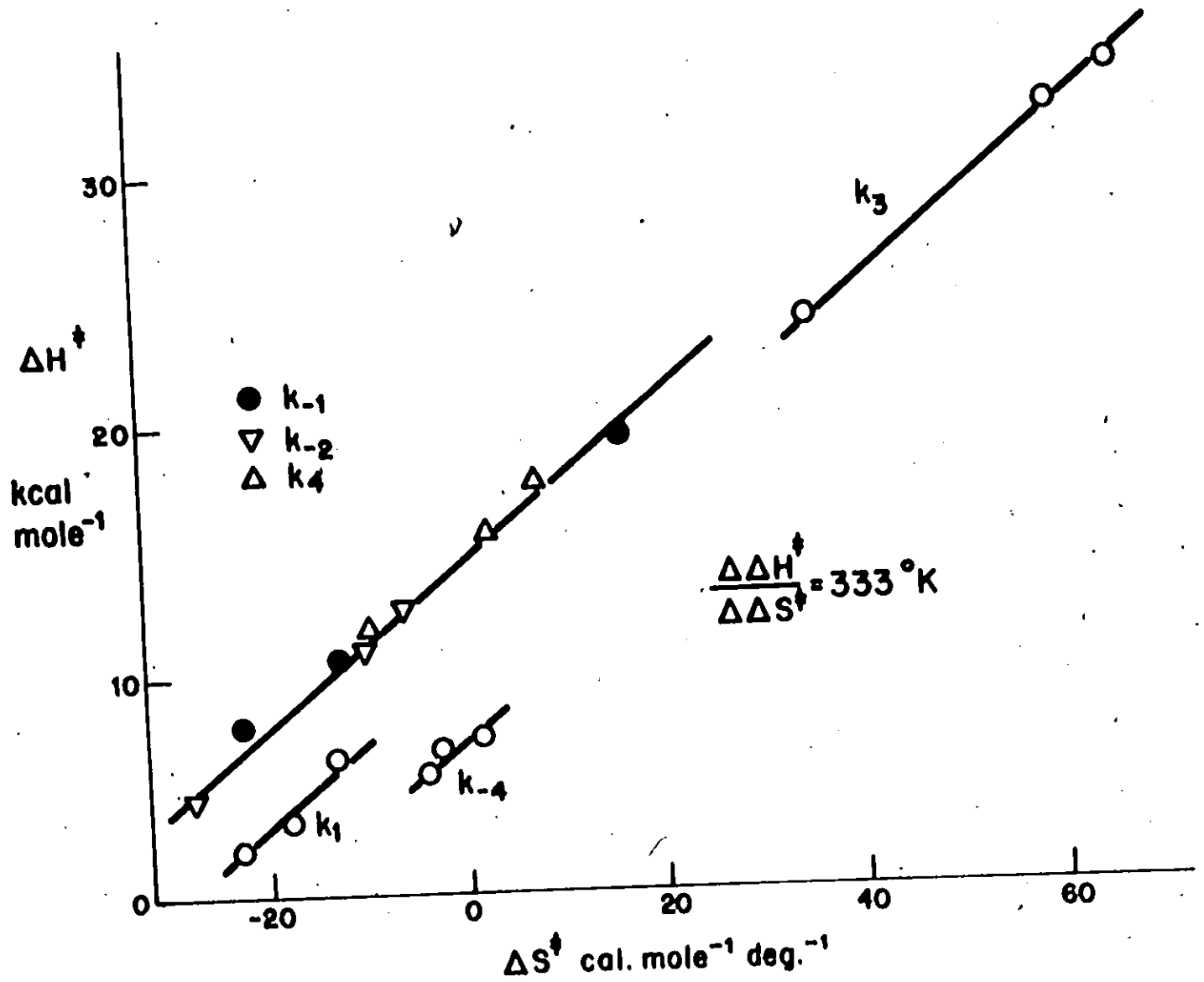
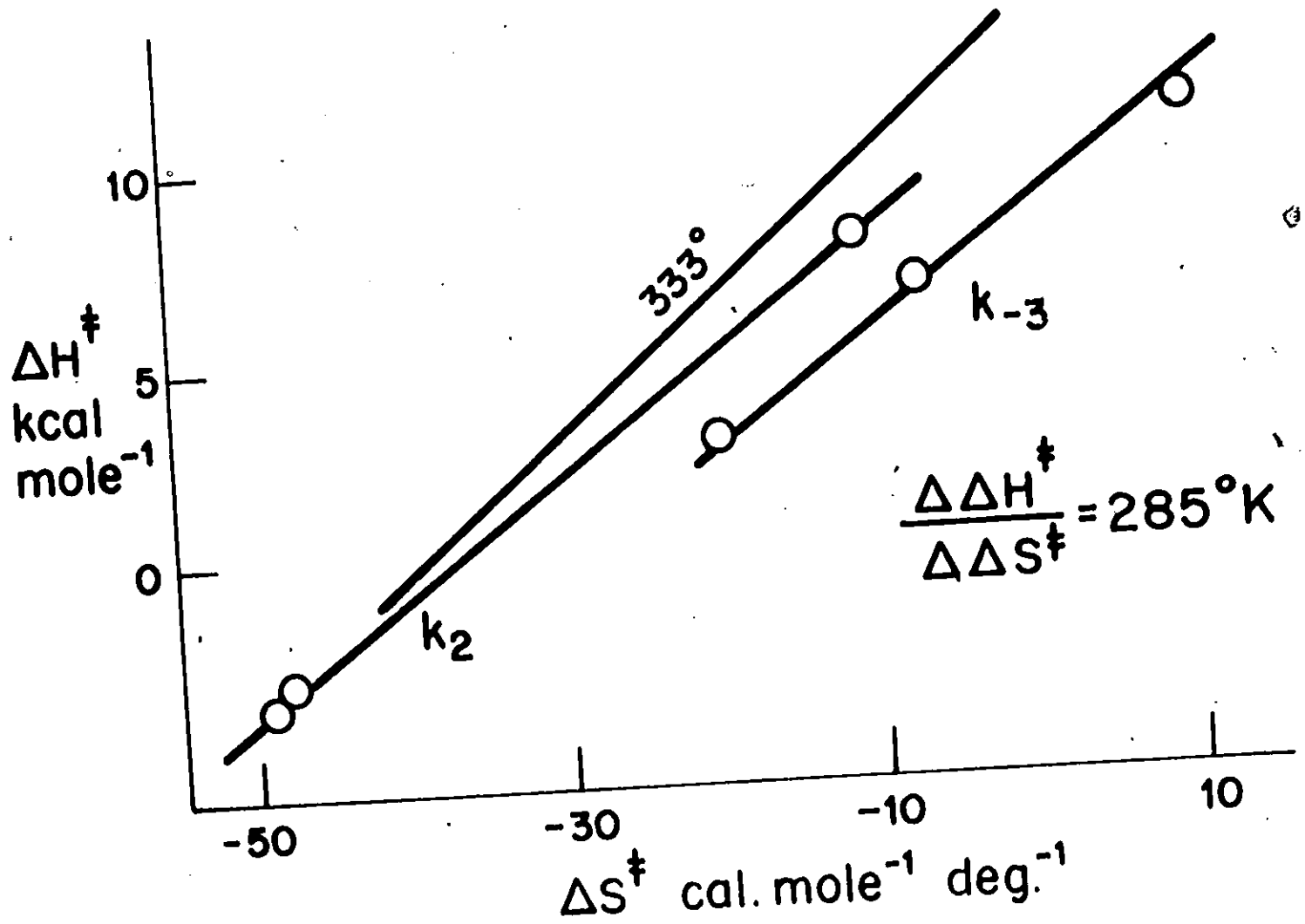

30
20
10
0
ΔH‡
kcal
mole⁻¹
k₋₁
k₋₂
k₄
k₃
k₁
k₋₄
ΔΔH‡/ΔΔS‡ = 333 °K
-20
0
20
40
60
ΔS‡ cal. mole⁻¹ deg.⁻¹

Figure 32

Plot of $\Delta H^{\neq}$ against $\Delta S^{\neq}$ for k_2 and k_{-3}, for the three enzymes. A straight line of slope 333°K is included for comparison.


10
5
0
$\Delta H^{\ddagger}$ kcal mole^{-1}
333°
k_{-3}
k_2
$\frac{\Delta\Delta H^{\ddagger}}{\Delta\Delta S^{\ddagger}} = 285°K$
-50
-30
-10
10
$\Delta S^{\ddagger}$ cal. mole^{-1} deg.$^{-1}$

accompanied by a high ΔH if water must be removed from the substrate (i.e., in amides), and there is a correspondingly high ΔS. For k_{cat}, $\Delta H^{\neq}$ is lower with the amide substrates; this is believed to be due to solvation of the activated complex, which also leads to a lower $\Delta S^{\neq}$.

Alternatively, Low and Somero (1974) have explained compensation effects for k_{cat} in terms of the number of weak bonds formed during the process of activation. The breaking of each weak bond (e.g., hydrogen bonds) is assumed to be accompanied by a ΔH of 4 kcal and a ΔS of 12 $cal.deg.^{-1}$ (T_c = 4000/12 = 333°K) (Stearn, 1949). Lower $\Delta H^{\neq}$ and $\Delta S^{\neq}$ values are therefore associated with the breaking of fewer weak bonds or the making of more weak bonds during formation of the activated complex. The results for LDH (Table VI), and those enzymes discussed by Low and Somero (1974), tend to demonstrate both a lower $\Delta H^{\neq}$ and a lower $\Delta S^{\neq}$ for k_{cat} for the enzyme from the ectothermic than from the endothermic animals. Further, the compensation temperature appears to be similar (333°K). However, the other rate constants in Table VI show no consistent pattern as to whether the ectothermic or endothermic enzyme has the higher $\Delta H^{\neq}$. Also, two of the rate constants (Figure 32) have a different compensation temperature (285°K). From the results presented in this report, it is not possible to determine whether compensation is caused by solvation effects, formation of different numbers of weak bonds in the activation processes, or other causes.

iii. Weak Bond Formation. The compensation data can be analysed on the basis of weak bond formation, although it must be borne in mind that this may include the formation of hydrogen bonds in solvation processes or that other processes may also be involved. If a drop in activation energy of 4 kcal is assumed for each weak bond formed, the relative number of weak bonds formed during each step can be calculated for the three enzymes. Table XI lists such values, rounded off to the nearest half bond, with beef heart LDH used as the standard of comparison. For example, during the activation process of step 1, one half bond more is formed with beef muscle and one half bond less with flounder muscle, as compared to beef heart. The data in Table XI are presented in profile in Figure 33. The horizontal line is the standard for beef heart LDH. The striking feature of this figure is that the total number of bonds present in the activated complexes differs little (not by more than a half bond) from enzyme to enzyme. This results from the similarities in the enthalpies and entropies of the activated complexes as shown in the enthalpy and entropy profiles (Figure 30).

The differences between the enzymes therefore, lie in the stable enzyme-substrate complexes (EA, EAB and EY) rather than the activated complexes. The complexes EA, EAB and EY have a lower number of weak bonds in the muscle enzymes than in the beef heart enzyme (Figure 33). This is especially true of the flounder muscle enzyme. This phenomenon is also reflected in the entropy profiles (Figure 29): e.g.,

TABLE XI

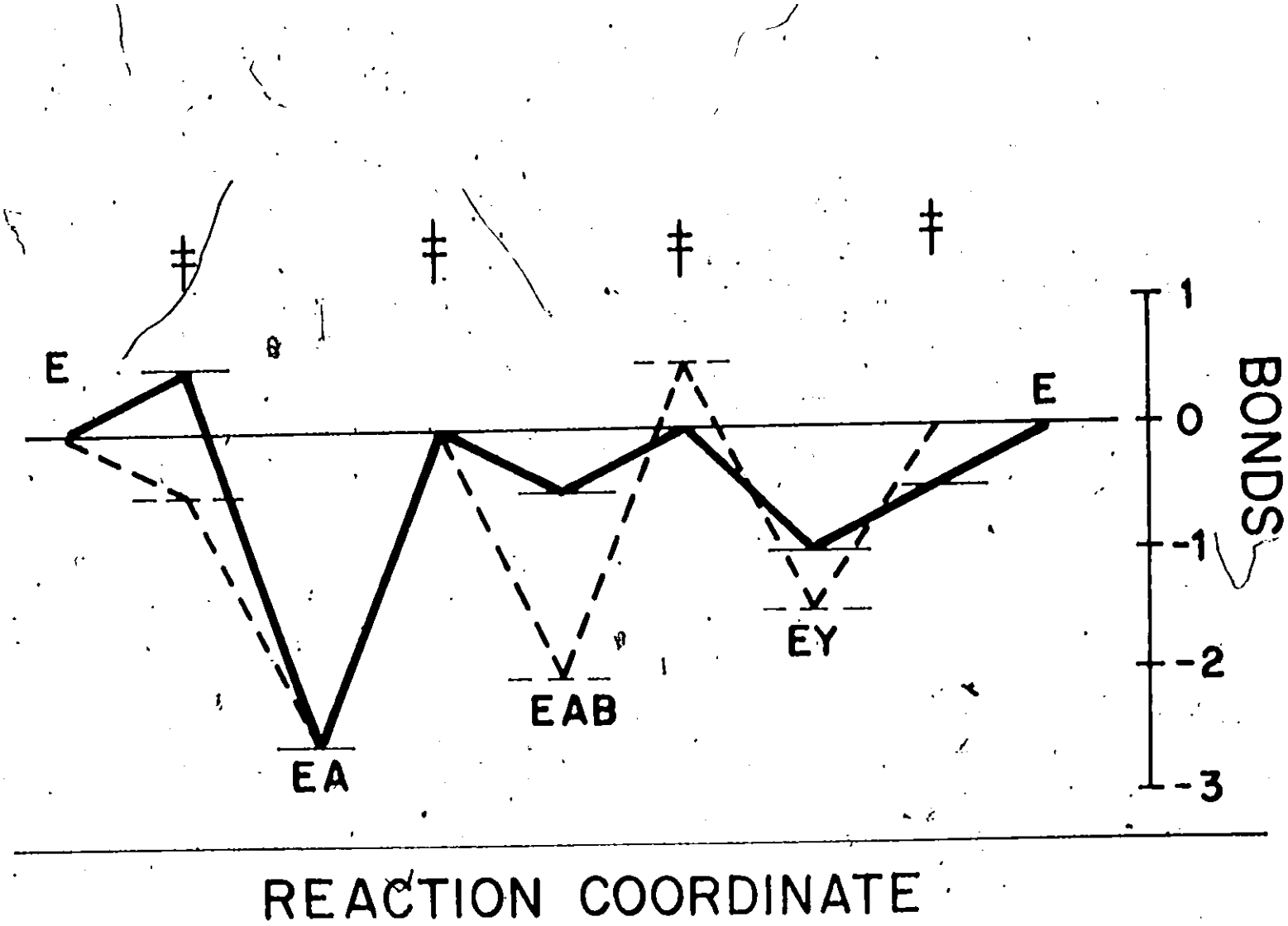
Relative Numbers of Weak Bonds Formed for each Reaction Step*

	Beef Heart	Beef Muscle	Flounder Muscle
k_1	0	½	-½
k_{-1}	0	3	2
k_2	0	2½	2½
k_{-2}	0	½	2
k_3	0	½	2½
k_{-3}	0	1	2
k_4	0	½	1½
k_{-4}	0	-½	0

*Assuming 4 kcal and 12 cal deg^{-1} per bond; rounded off to the nearest half bond.

Figure 33

Profile of the relative numbers of bonds present in each complex. Beef heart is the standard and is represented by the horizontal line. Dashed line-flounder muscle; solid line-beef muscle.


E
EA
EAB
EY
E
1
0
-1
-2
-3
BONDS
REACTION COORDINATE

the muscle enzymes, especially the flounder enzyme, demonstrate higher entropies for the stable complexes. The generally lower $\Delta H^{\ddagger}$ values for the muscle enzymes thus appear to result from decreased bonding in the stable complexes rather than from differences in bonding among the activated complexes. In all cases there are substantial enthalpy drops in going from E + A to EA (see Table VI and Figure 28), but the enthalpy changes in going from E + A to the activated complexes for k_2 are very similar for the three enzymes (BH, -7.6; BM, -8.7; FM, -7.2 kcal.). This again suggests roughly equivalent bond numbers for the activated complexes, with differences for the stable complexes. Similar conclusions can be drawn from the data of Cohen et al. (1970), for which the same enzyme was used but the substrate varied. A large drop in enthalpy for bonding of the substrate is usually accompanied by a roughly equal corresponding rise in enthalpy of activation for k_{cat}, so that there is similar bonding in the activated complexes for k_{cat}.

A comparative analysis of the thermodynamics of the three homologous enzymes has therefore established that most of the differences occur in the stable binary and ternary complexes. There is weaker bonding between the enzyme and its substrates in the muscle enzymes, especially the flounder enzyme. The weaker bonding in the ectothermic enzyme, especially in the ternary complex (Figure 33), may be a sign of molecular adaptation to temperature. The way in which the varying strength of bonding between enzyme and substrates may be of

advantage to the organisms adapted to different temperatures will be discussed in the next section.

B. Adaptation in Ectothermic and Endothermic LDH

The kinetic and thermodynamic differences between beef heart, beef muscle and flounder muscle LDH have been discussed in detail in the previous section. Two observations were noted. Firstly, the degree of bonding within the activated complexes is very similar, suggesting that evolutionary adaptation does not involve these complexes. Secondly, the bonding in the stable binary and ternary enzyme-substrate complexes varies from enzyme to enzyme, with the strongest bonding between enzyme and substrates occurring in the beef heart enzyme and the weakest bonding occurring in the flounder muscle enzyme. The biological significance of the variation in bonding in the stable complexes will now be discussed. The original rate-temperature curves will be reexamined, along with information on heat denaturation, and this will be compared to the degree of bonding.

Adaptation in Flounder Muscle LDH

As discussed in the previous section, the lower enthalpy of activation for k_{cat} in flounder muscle LDH is ascribed to the weaker bonding between enzyme and substrate. Since the compensation temperature is 333°K (60°C), this results in a greater catalytic activity for the flounder at lower (below 60°C) temperatures (see for example, Figure 20). The activation enthalpy and catalytic rate constant at 0°C for

the three enzymes in the forward direction are summarized in Table XII (calculated from Figure 20). At the lower temperatures at which the ectothermic enzyme must function in nature, the enzyme and substrate molecules possess a lower kinetic energy. There is a compensation for this disadvantage since, as seen in Table XII, the activation enthalpy is lower, thereby enhancing the reaction rate, especially at Low temperatures. Further, the resulting lower temperature dependence of k_{cat} for the ectothermic enzyme helps reduce the variability in the reaction rate which could result if environmental temperatures fluctuate. Similar reciprocal relationships between catalytic efficiency and $\Delta H^{\ddagger}$ have been observed for many enzymes (Hochachka and Somero, 1973; Low et al., 1973; Low and Somero, 1974). It is therefore possible that the decreased bond strength and rigidity of the enzyme-substrate complexes may be a general feature of ectothermic enzymes when compared to the homologous enzymes from endothermic animals.

The second parameter (after V_{max}, or k_{cat}) most frequently used in analysing steady-state kinetics is K_m. The K_m for the three enzymes for lactate and pyruvate are compared in Figures 34 and 35. The "U" shaped K_m-temperature plot for lactate (Figure 34) and the "J" shaped plot for pyruvate (Figure 35) are similar to K_m plots for a great many other enzymes (Hochachka and Somero, 1973; Somero and Hochachka, 1971). As mentioned in the introduction, a minimum in K_m is often observed for ectothermic enzymes near the

TABLE XII

k_{cat} at 0°C and the Enthalpy of Activation for k_{cat} for the Lactate to Pyruvate Direction

Enzyme	k_{cat} at 0°C $sec.^{-1}$	$\Delta H^{\ddagger}$ $kcal\ mole^{-1}$
Beef heart	2.53	17.5
Beef muscle	6.85	15.5
Flounder muscle	20.8	11.7

Figure 34

Plot of $\log_{10} K_m$ for lactate against 1/T. K_m in moles per liter. FM = flounder muscle, BM = beef muscle, BH = beef heart.

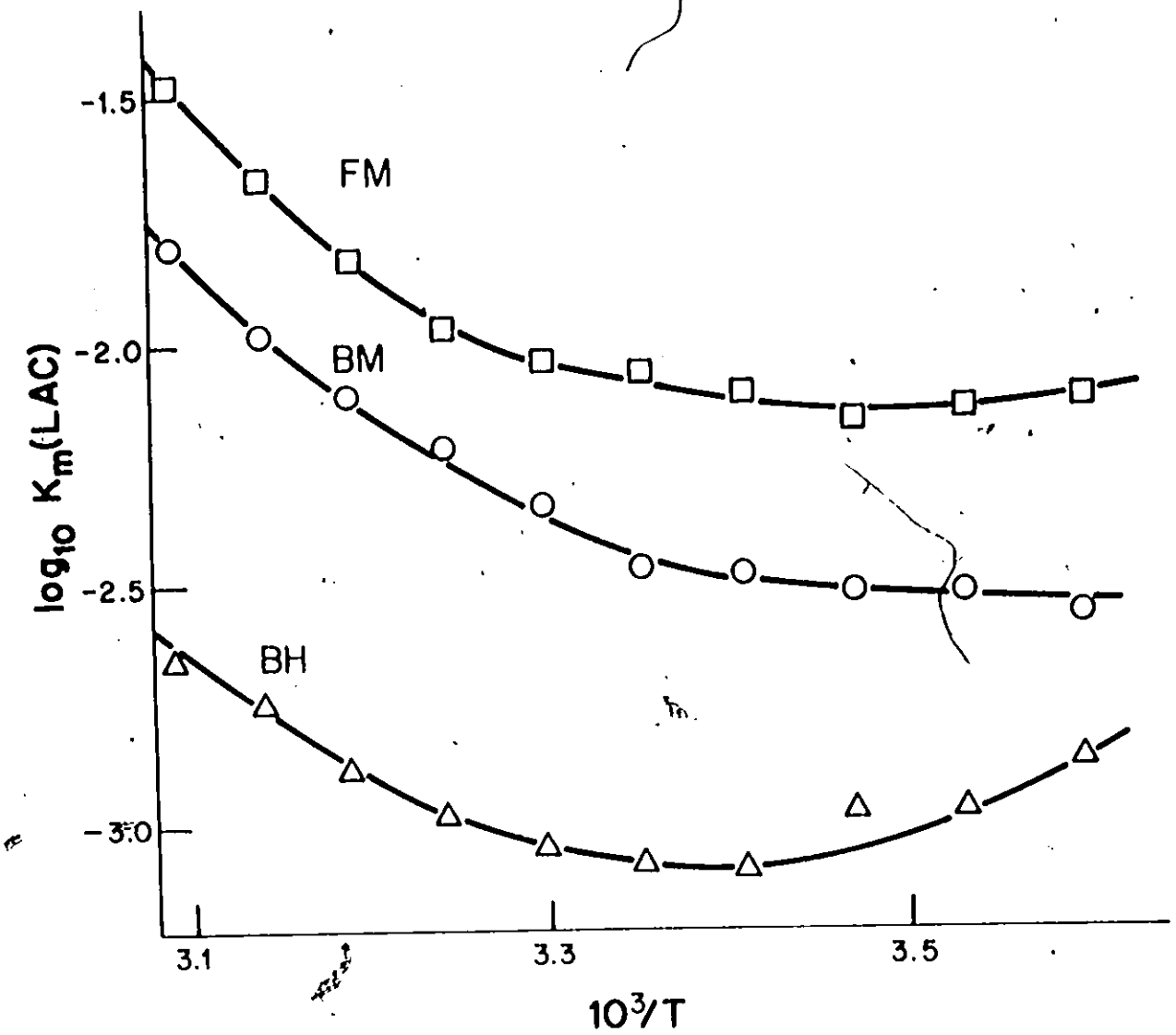
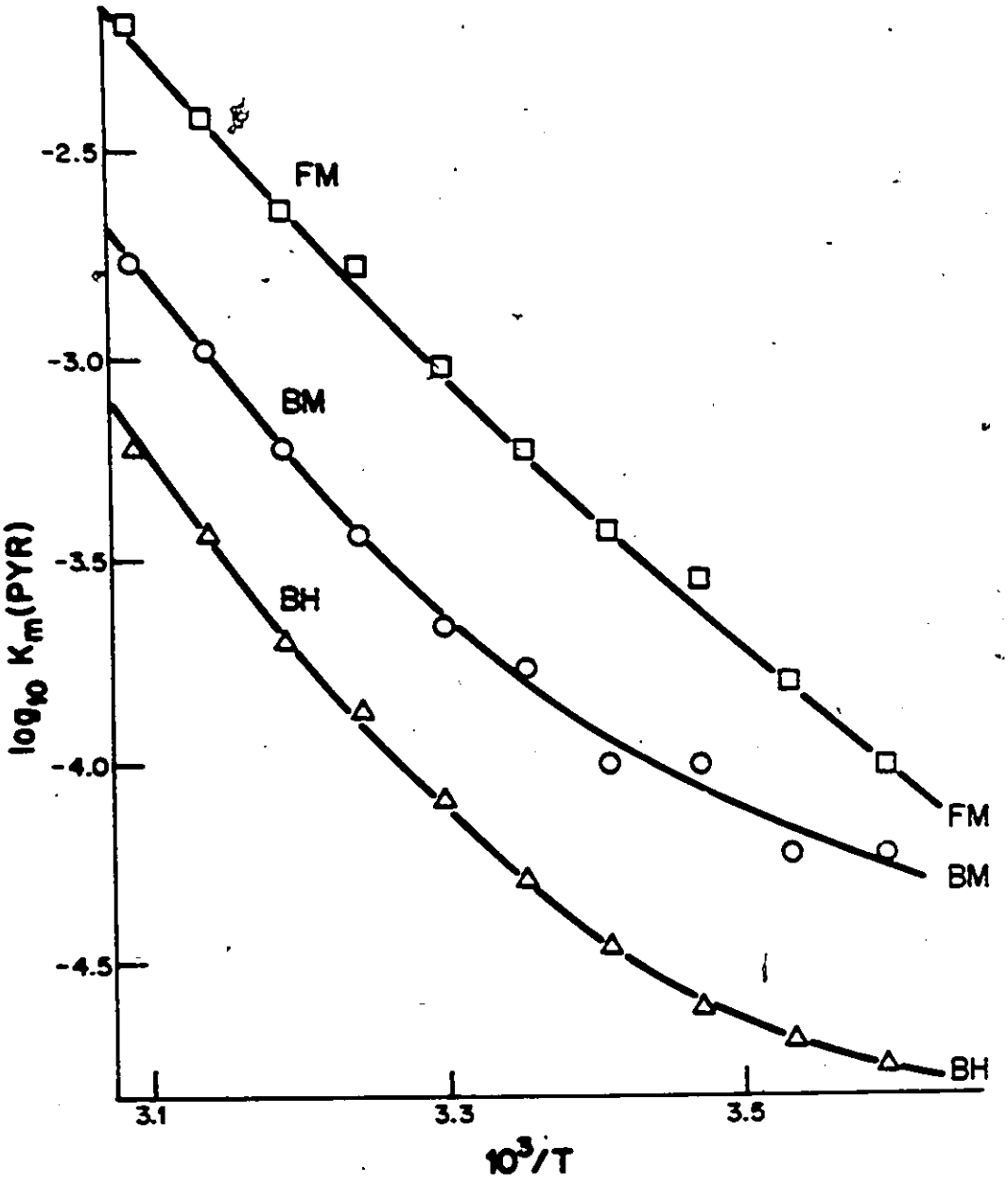

-1.5
-2.0
-2.5
-3.0
$\log_{10} K_m$(LAC)
FM
BM
BH
3.1
3.3
3.5
$10^3/T$

Figure 35

Plot of $\log_{10} K_m$ for pyruvate against 1/T. Notation as in Figure 34.


-2.5
-3.0
-3.5
-4.0
-4.5
FM
BM
BH
FM
BM
BH
$\log_{10} K_m$(PYR)
3.1
3.3
3.5
$10^3/T$

temperature to which the organism is adapted. Although this has been reported for a number of fish lactate dehydrogenases (Hochachka and Somero, 1968), no such correlation is obvious in Figures 34 and 35. In fact, when the K_m values for lactate for the two muscle enzymes are compared, it appears that beef muscle has a minimum K_m at a slightly lower temperature than does flounder. Also, the flounder muscle K_m is higher at all assay temperatures when compared to the endothermic enzymes. These observations are contrary to what would be predicted from previous studies (Hochachka and Somero, 1973).

Unless the enzyme is saturated, a decrease in K_m will give an increase in the reaction rate, all other parameters being equal. Since the reaction velocity at very low substrate concentrations is proportional to k_{cat}/K_m, and k_{cat} usually has a positive temperature dependence, the maximum in k_{cat}/K_m will be observed at a higher temperature than the minimum in K_m. The difference between the two will depend on the sensitivity of k_{cat} to temperature. Figures 36 and 37 illustrate that the maxima in k_{cat}/K_m are at a higher temperature for the endothermic enzymes, as would be expected if this parameter is a sensitive index of thermal adaptation. Since k_{cat} often obeys the Arrhenius law, a logarithmic plot of k_{cat}/K_m against 1/temperature will have the same degree of curvature as a similar plot of K_m. The curvature will be inverted and a correction for differences in k_{cat} will be made. Viewed in this light, the ecto-

thermic enzyme shows adaptation to a lower temperature through both a lower enthalpy of activation for k_{cat} and a maximum in k_{cat}/K_m at a lower temperature relative to the endothermic enzyme.

The term k_{cat}/K_m also has a greater theoretical significance and usefulness than does K_m. In general, when models are used to investigate enzyme kinetics, k_{cat}/K_m is usually a simpler function than K_m (see theoretical section; Harbison and Fisher, 1973). Here, k_{cat}/K_m is a rate constant whereas K_m is a ratio of two rate constants (the rate at saturating substrate concentrations divided by the rate at very low substrate concentrations). In order to express this relationship more clearly, some authors have changed the symbols and use β instead of $k_{cat}[E]_o$ and α instead of $k_{cat}[E]_o/K_m$ (Harbison and Fisher, 1973 a & b; 1974; Lumry, 1959) ($[E]_o$ = total enzyme concentration). For our purposes, k_{cat}/K_m is very useful not only because of its biological significance but because it can be related directly to bonding in a way similar to that used to relate differences in activation enthalpies for k_{cat} to the degree of bonding in the enzyme substrate complexes.

Under saturating concentrations of coenzyme (NAD^+ or NADH for our enzymes), the enthalpy of activation for k_{cat}/K_m is equal to the difference in enthalpy between the enzyme-coenzyme complex and an activated complex. The activated complex need not be the same one that controls the catalytic rate, and in fact the curvature in Figures 36 and 37 indicates

Figure 36

Arrhenius plot of k_{cat}/K_m in the forward direction at saturating NAD^+ concentrations (K_m is for lactate). Notation as in Figure 34. Units in $M^{-1}sec^{-1}$.

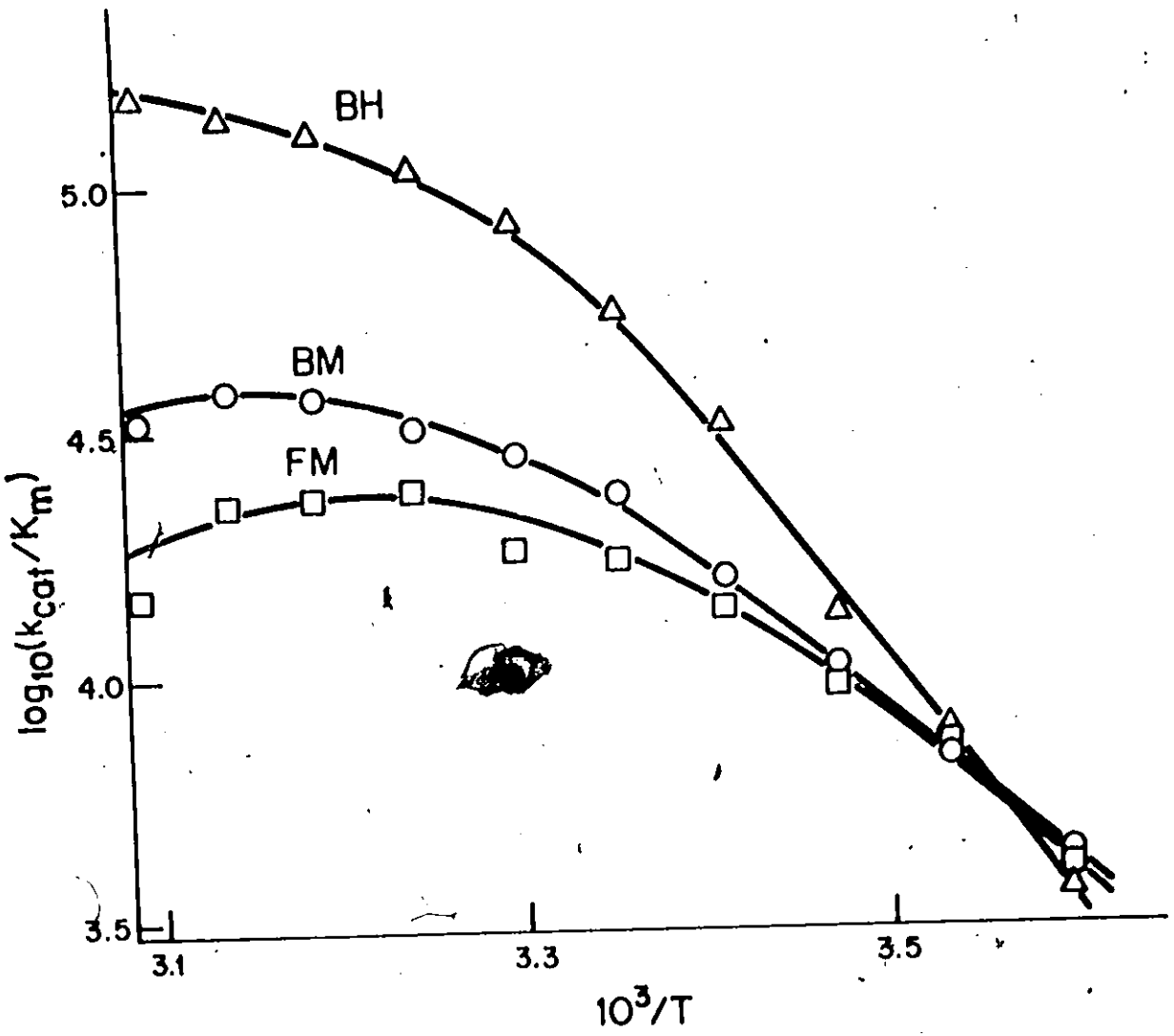
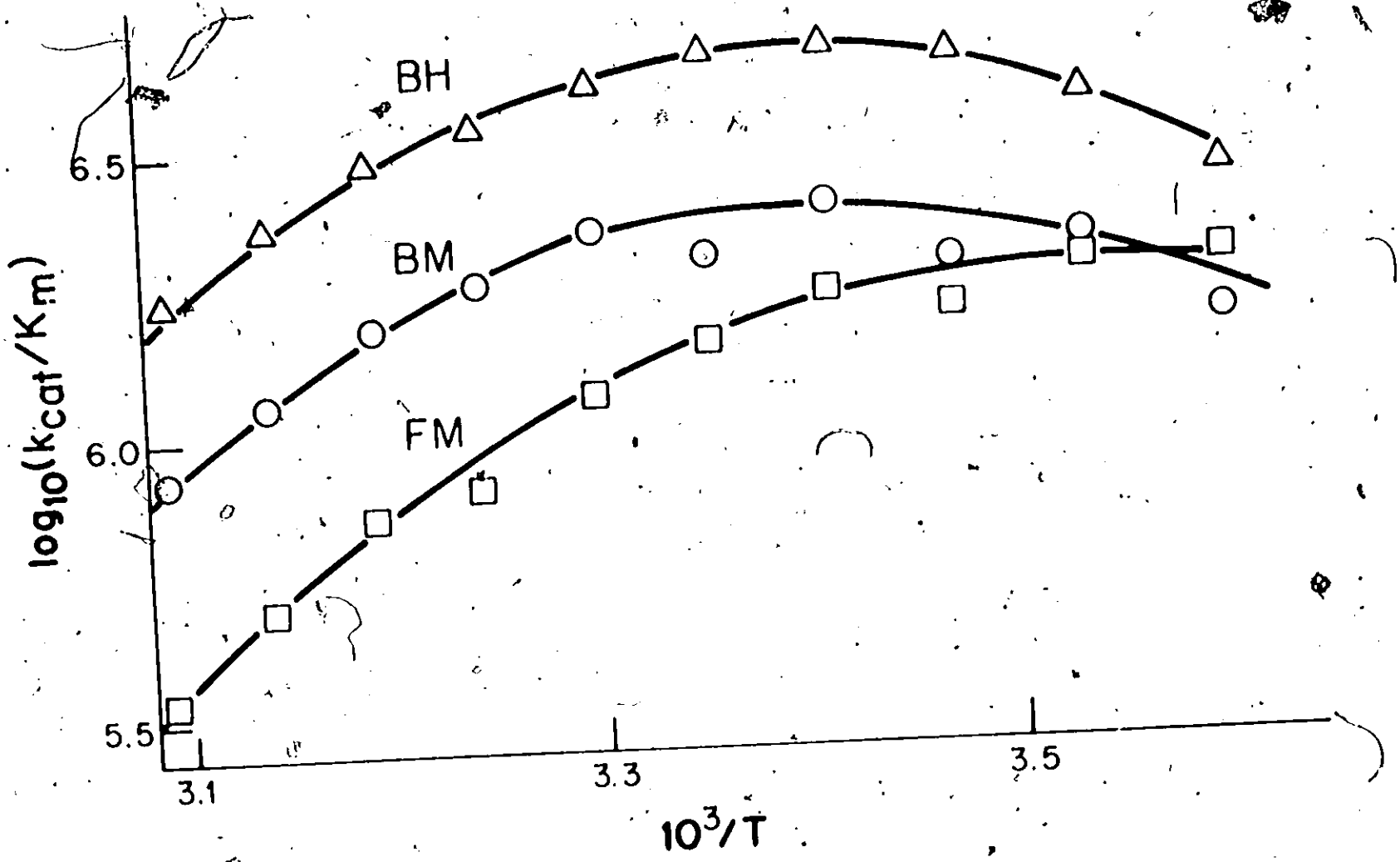

BH
BM
FM
5.0
4.5
4.0
3.5
$\log_{10}(k_{cat}/K_m)$
3.1
3.3
3.5
$10^3/T$

Figure 37

Arrhenius plot of k_{cat}/K_m in the reverse direction at saturating NADH concentrations (K_m is for pyruvate). Notation as in Figure 34. Units in $M^{-1}sec^{-1}$.


BH
BM
FM
6.5
6.0
5.5
$\log_{10}(k_{cat}/K_m)$
3.1
3.3
3.5
$10^3/T$

that the activated complex controlling the rate at low substrate concentrations changes with temperature. However, as mentioned earlier, the degree of bonding in homologous activated complexes is very similar in the three enzymes. The more negative temperature dependence of k_{cat}/K_m (see Figures 36 and 37) in the ectothermic enzyme therefore results from the weaker bonding between enzyme and coenzyme in the enzyme-coenzyme complex. Again, as in the case of k_{cat}, a weaker bonding between the enzyme and substrates (in this case the coenzyme) results in a more negative temperature dependence (i.e., usually a lower temperature dependence, except for k_{cat}/K_m for the catalysis of pyruvate to lactate).

In the ectothermic LDH, therefore, adaptation to a lower and more variable temperature is achieved by weaker bonding between enzyme and substrates; this results in a lower temperature dependence, a maximum in k_{cat}/K_m at a lower temperature, and a greater catalytic efficiency (higher k_{cat}).

Adaptation in Beef Lactate Dehydrogenase

Since the body temperature of the endothermic organism is higher and more constant than that of the ectotherm, there is less of an advantage to a low activation energy, either for k_{cat} or for k_{cat}/K_m. However, even at these high temperatures the catalytic efficiency of the ectothermic enzyme is still greater than the beef LDHs *in vivo*. The

only kinetic advantage of greater bonding between enzyme and substrates in the endotherm is an increase in k_{cat}/K_m at higher temperatures. At physiological substrate concentration the rate will be related to both k_{cat}/K_m and k_{cat}. The increased value of k_{cat}/K_m for beef observed in Figures 36 and 37 could be overshadowed by the decrease in k_{cat}, especially at substrate concentrations above K_m. There must be some other advantage to increased bonding between enzyme and substrate in endothermic enzymes. This advantage may be resistance to heat denaturation. It has been suggested that the presence of substrates may protect enzymes from denaturation (Dixon and Webb, 1958) and on the basis of this suggestion, Hebb et al. have pointed out that a high affinity of an enzyme for its substrates may be a means of stabilizing enzyme structure. The ability of substrates to reduce heat denaturation is therefore examined below.

The rate of heat inactivation for the three enzymes is illustrated in Figure 24. Activation enthalpies for denaturation for the three enzymes are similar and are listed in Table VII. The presence of substrate has no measurable effect on the activation enthalpy. The beef heart enzyme is the most heat resistant and the flounder enzyme the least heat resistant. This is shown by both absolute rates of denaturation as well as activation enthalpies. More significant, however, is the effect that substrates have on reducing heat sensitivity. The beef heart enzyme, which has the strongest bonding between enzyme and

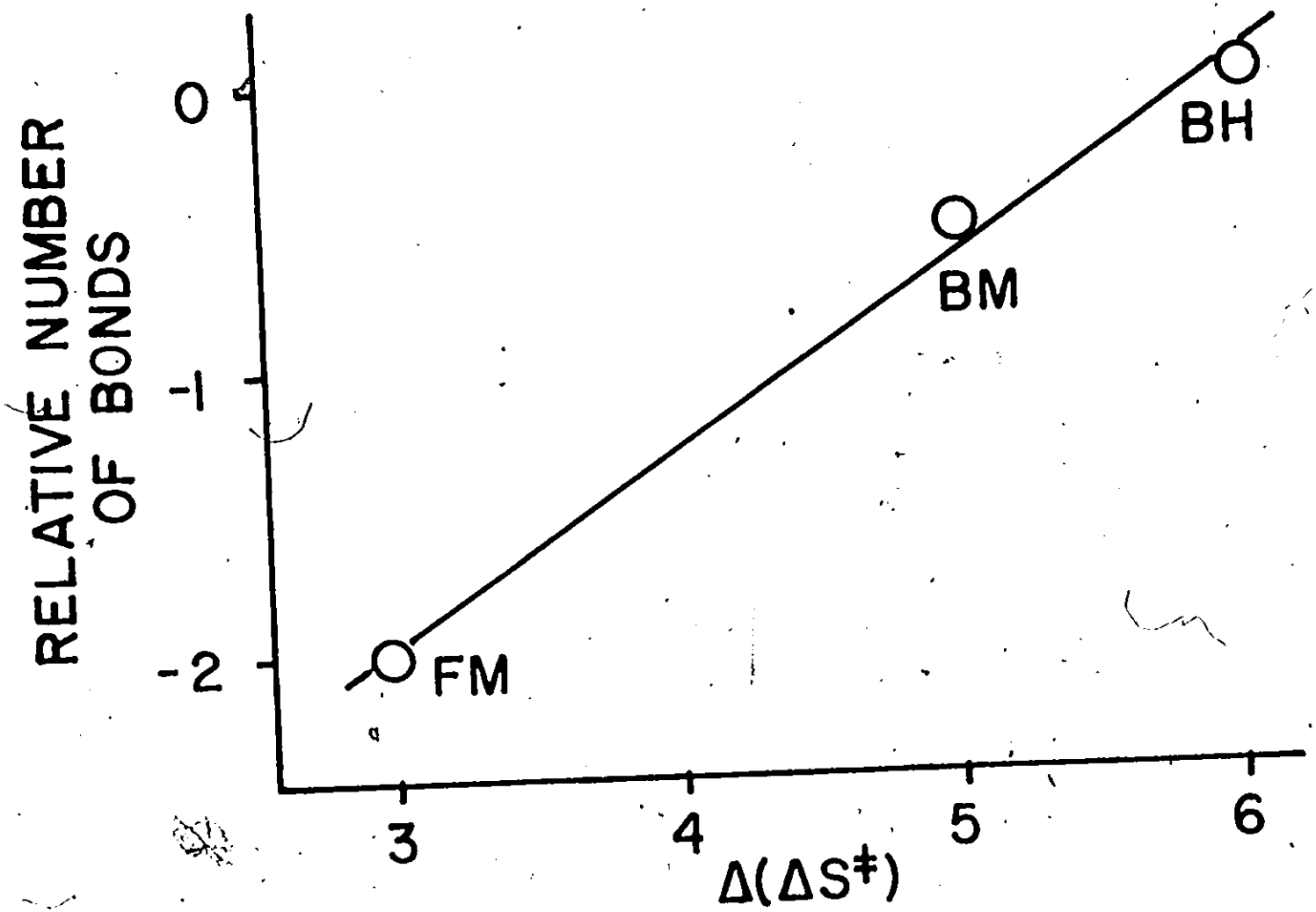
substrate, is benefited more by the presence of substrates (a 25-fold decrease in denaturation rate) than is the flounder muscle enzyme with the weakest bonding between enzyme and substrates (a 4.3-fold decrease in denaturation in the presence of substrate). The beef muscle is intermediate. From Table VII it can be seen that the presence of substrates decreases the entropy of activation by 3 cal $mole^{-1}$ deg^{-1} in the flounder muscle, 5 cal $mole^{-1}$ deg^{-1} in the beef muscle and 6 cal $mole^{-1}$ deg^{-1} in the beef heart enzyme.

It was calculated that if one weak bond (e.g., hydrogen bond) involves a change in enthalpy of 4 kcal and change in entropy of 12 cal $mole^{-1}$ deg^{-1} (Low and Somero, 1974), the flounder enzyme possesses 2 less and the beef muscle 1 less bonds in the ternary complex (enzyme-substrate-coenzyme) when compared to the beef enzyme (Figure 33). There appears to be a direct relationship between the degree of bonding in the ternary complex and the decrease in entropy of activation for denaturation in the presence of substrate (Figure 38). The increased bonding in the beef enzymes therefore, reduces the degree of heat denaturation in these enzymes which function at high <u>in vivo</u> temperatures.

Although the larger difference in the degree of bonding and the degree of reduction in denaturation by substrates is encountered when comparing flounder muscle to beef muscle, a difference also exists between beef heart and beef muscle. Since the beef heart and beef muscle enzymes would function at very similar temperatures, this difference, unlike the one

Figure 38

Plot of relative number of bonds in the ternary (enzyme-substrate-coenzyme) complex against decrease in activation entropy caused by addition of substrates ($\Delta(\Delta S^{\neq})$). $\Delta(\Delta S^{\neq})$ is obtained from Table VII.


RELATIVE NUMBER OF BONDS
0
-1
-2
FM
BM
BH
3
4
5
6
Δ(ΔS‡)

between flounder and beef muscle, cannot be due to adaptation to temperature. The tighter bonding between substrates and the beef heart enzyme is probably related to its decreased K_m and increased substrate inhibition as compared to the beef muscle enzyme, and is related to the different *in vivo* roles played by these two enzymes (Kaplan et al., 1968).

From Table VII there also appears to exist a relationship between the activation enthalpy and activation entropy of denaturation of the free enzyme and the degree of bonding between enzyme and substrates. Perhaps a more rigid structure in the beef enzymes, especially the beef heart, reduces the denaturation rate of the free enzyme; consistent with this is that the activation enthalpy and entropy required for denaturation are highest in these enzymes. This rigidity may be partly responsible for the increased bonding between enzyme and substrates in the endothermic enzymes (Hochachka and Somero, 1973; Low and Somero, 1974).

As mentioned above, adaptation in the ectothermic enzyme was observed through the lower activation enthalpy, a maximum in k_{cat}/K_m at a lower temperature, and greater catalytic efficiency. This results from a weaker bonding between substrates and enzyme, which in turn, is probably caused by a less rigid structure in the flounder enzyme, especially in the enzyme-substrate complexes. This same difference in rigidity and degree of bonding simultaneously leads to the greater resistance to heat denaturation observed in the beef enzymes.

VI

SUMMARY AND CONCLUSIONS

The results obtained in the pre-steady-state experiments agree well with those obtained by other workers using pig heart and muscle LDH. In the forward direction, an initial burst is seen in the beef heart enzyme, just as has been observed for pig heart (Holbrook and Gutfreund, 1973; Sudi, 1974; Whitaker et al., 1974). This is not observed in either beef muscle, flounder muscle, or pig muscle LDH (Bennett and Gutfreund, 1973). In the reverse direction, the maximum pre-steady-state velocity is equivalent to the catalytic rate constant for beef heart and muscle, flounder muscle and also the pig enzymes (Stinson and Gutfreund, 1971). Comparison of the five-step model for beef heart LDH with an equivalent model for pig heart (Sudi, 1974) leads to the same conclusions with regard to the slowest steps occurring between the EA and EY complexes. Curvature in the Arrhenius plots of $\overleftarrow{V}_{max}/K_{mY}$ (k_{-4}) and unexpectedly low values of k_{-1} in Table V indicate that the five-step model is still incomplete and suggests that k_{-1} and k_{-4} in Table V are composite values consisting of rate constants multiplied by equilibrium constants. Evidence from the pig enzyme also suggest additional steps between EA or EY and the free enzyme (Criddle et al., 1968).

The inability to measure the pre-steady-state in the forward direction for the muscle enzymes necessitates the use of a simplified four-step model. Comparison with the five-step model indicates that the rate constants in Table V

are equivalent to those that would be obtained from the five-step model with the exception of k_{-2}, which would be relabeled k_{-a}, and k_2, which in reality represents k_2k_a/k_{-a}. The data presented in Table V appear to be the first for which the activation enthalpies and entropies for all of the steps in a model of LDH are reported, the determination of these values being made possible by use of the stopped-flow apparatus. Also, this appears to be the first time such values have been compared among three homologous enzymes.

A considerable degree of compensation has been observed between the activation enthalpies and entropies. Hence a relationship of the type

$$\Delta H^{\ddagger} = a + b(\Delta S^{\ddagger})$$

is observed. Here b represents the compensation or isokinetic temperature, the temperature at which the rate constants for all three enzymes are approximately equal. The most common compensation temperature observed was about 333°K, with values of 285 and 298° also seen. Compensation temperatures of 333° (Low and Somero, 1974) and 285 and 298° (Cohen et al., 1970) have also been observed for other enzymes. Although no clear and concise explanation can be given for such compensation, it is postulated that the bonding in the enzyme-substrate complexes is stronger in the beef enzymes, especially beef heart. The degree of bonding, whether affected by hydrogen bonds, solvent effects

or other types of forces, is believed to affect $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ in the same direction and to a similar degree.

The differences in the kinetics of the homologous enzymes is explained in the following manner. The increased activation enthalpies and entropies for denaturation of the pure beef enzymes, as compared to the flounder enzyme, suggest that the beef enzymes, especially the beef heart, have a more rigid structure. This increased resistance to thermal denaturation is, in itself, an adaptation to a higher temperature, as experienced by the beef enzymes *in vivo*. This increased rigidity may be responsible for the increased bonding between enzyme and substrate and/or coenzyme. The increased bonding in the enzyme substrate complexes in the beef enzymes has the following results:

1) The decrease in the denaturation rate observed upon addition of substrates is greatest in the beef enzymes. This represents a further adaptation to high temperatures.

2) The activation enthalpy for k_{cat} is lowest in the ectotherm, representing adaptation to a lower and more fluctuating temperature. This has also been observed for other enzymes (Hochachka and Somero, 1973; Low and Somero, 1974).

3) The activation enthalpy for k_{cat}/K_m is lowest in the ectotherm, again representing adaptation to a lower and more fluctuating temperature.

4) The maximum in k_{cat}/K_m is observed at a lower temperature in the ectotherm.

5) The k_{cat} is largest in the ectotherm, especially at low temperatures. This again represents adaptation to a lower temperature and has been observed among many other enzymes (Hochachka and Somero, 1973; Low and Somero, 1974).

The relationship between the activation enthalpy for k_{cat} and the denaturation rate of the enzyme has also been noted by Johnston et al. (1973) for ATPase; the relationship between molecular rigidity, catalytic efficiency (i.e. size of k_{cat}) and resistance to thermal denaturation has been suggested by Hochachka and Somero (1973) and Low and Somero (1974).

One discrepancy with previous observations can be noted. The degree of "positive thermal modulation" appears to be similar in both beef and flounder LDH, and no adaptation is observed in the plots of K_m for pyruvate and lactate as a function of temperature. Adaptation can only be observed if k_{cat}/K_m is examined.

VII

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