A COMPARATIVE STUDY OF PYRUVATE KINASE AS A REGULATORY ENZYME IN HIBERNATING AND NORMOTHERMIC *MYOTIS LUCIFUGUS*

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ABSTRACT

Pyruvate kinase (PK) is an important regulatory enzyme in the glycolytic pathway. Study of this enzyme was undertaken to determine its role in the annual cycle of an obligate hibernant, Myotis lucifugus, the little brown bat. Breast (pectoralis major) muscle and liver PK isozymes were isolated from bats in two physiological ~~states~~: the homeothermal state and the heterothermal (hibernating) state. Corresponding to each state, electrophoretically and kinetically distinct forms of PK were discovered in each of the bat tissues which are particularly well-suited to the physiological states alternatively encountered in the bat.

The four major aspects of the kinetic study can be summarized in this manner:

When comparing the normothermic and hibernating enzyme, the lowest thermal sensitivity (as determined by lowest E_a-values for V_{max} and V_{max}/K_m, reduced Q_{10}-values and decreased K_m(PEP) values in the temperature range of 34° to 16°C) was recorded for NL-PK whereas in the thermal range of 5° to 16°C, the lowest thermal sensitivity was found in HL-PK and HM-PK.

The hibernating forms of PK demonstrated the higher specific activities.

Lowered critical temperatures (T_c-values) were recorded for the Arrhenius plots of hibernating PK compared to the normothermic enzyme. This may prevent

potential metabolic blockage at the lowered temperatures of hibernation.

Fructose diphosphate and alanine appear to exert tighter control over HL-PK activities than those of the NL-enzyme, thus providing optimal control over the glycolytic/gluconeogenic transition.

RESUME

La pyruvate kinase (PK) est une importante enzyme régulatrice dans le cycle glycolitique. L'étude de cette enzyme fut entreprise pour déterminer son rôle dans la période annuelle d'un hibernant obligatoire, Myotis lucifugus, la petite chauve-souris brune. Les isozymes du PK de muscle de la poitrine (le pectoral majeur) et du foie furent isolées des chauve-souris dans deux états physiologiques: l'état "homéothermal" (non-hibernant), et l'état "hétérothermal" (hibernant). Des formes du PK electrophorétiques et kinétiques bien-adaptées aux états physiologiques rencontrés alternativement dans le chauve-souris furent découvertes dans chacun des tissus, selon chaque état.

Les quatre aspects majeurs d'étude kinétique peut récapituler dans cette manière:

Quand on compare les enzymes non-hibernant et hibernant, dans la zone de températures de 16° à 34°C, la plus basse sensibilité thermique (déterminée par la plus basse valeur E_a pour V_{max} et V_{max}/K_m, le Q_{10} réduit, le K_m(PEP) diminué) fut enregistrée pour le NL-PK tandis que dans la zone de température de 5° à 16°C, la plus basse sensibilité thermique fut trouvée pour le HL-PK et le HM-PK.

Les formes hibernantes du PK ont manifesté les plus hautes activités spécifiques.

Des valeurs des températeurs critiques (T_c) diminuées furent enregistrées pour la courbe Arrhenius pour le PK hibernant comparé à la forme d'enzyme non-hibernante.

Ceci peut empêcher une obstruction potentielle du métabolism aux températures baisées d'hibernation.

Fructose diphosphate et alanine semblent exercer un contrôle plus serré sur les activités de l'enzyme du HL que celui du NL, et alors fournant un contrôle sur la transition glycolitique/gluconeogénique.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ala	alanine
ATP	adenosine triphosphate
CNS	central nervous system
E_a	activation energy
E.T.C.	electron transport chain
F6P	fructose-6-phosphate
FDP	fructose diphosphate
FDPase	fructose diphosphatase
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
HK	hexokinase
HL	hibernating bat liver
HM	hibernating bat muscle
K_a	activation constant
K_i	inhibition constant
K_m	Michaelis constant
LDH	lactate dehydrogenase
n_H	Hill value, Hill constant
NL	normothermic bat liver
NM	normothermic bat muscle
NST	nonshivering thermogenesis
OXA	oxaloacetate
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase

PFK	phosphofructokinase
PK	pyruvate kinase
RQ	respiratory quotient
ST	shivering thermogenesis
[ ]	substrate or metabolite concentration in μM, mM or M
T_a	ambient or environmental temperature
T_b	body temperature
T_c	critical or transition temperature
V_{max}	maximum catalytic constant under defined experimental conditions

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INTRODUCTION

INTRODUCTION

The little brown bat, Myotis lucifugus (Le Conte) (Chiroptera: Vespertilionidae), must cope with numerous problems in the North Temperate Zone due principally to its small size (see Fig. 1). The energy expenditure of endotherms in general (endotherms are those organisms which obtain the majority of their body heat from their own metabolic processes; Hochachka & Somero, 1973) is dependent upon climate, food habits and most importantly, body size (Mc Nab, 1974). It has been calculated that mammals weighing less than 2.5 gm could not exist in any climate because they simply could not eat enough food to permit the necessary rate of heat production required to maintain endothermy (Pearson, 1948). The basic reason for this lower weight limit for survival is that the smaller the mammal, the greater the exposed surface area relative to its body weight, and the greater the risk of heat loss to the colder environment (Hull, 1973). Death could result, depending upon the degree and duration of heat loss, from hypothermia. Under such environmental conditions it is often not feasible for endothermy to exist since such regulation is energetically very costly (Mc Nab, 1974). Although all species of Pteropodidae (tropical fruit-eating bats) are considered to be endothermic, the body temperature (T_b) of the smallest, Synycteris australis (weighing 15 - 20 gm) declined to 25°C (i.e., became hypothermic) during an exposure of one hour to an

Fig. 1 Myotis lucifugus, the little brown bat (actual size), photographed in flight by H.E. Edgerton. (From Griffin, 1974).

ambient temperature (T_a) of 5°C (Henshaw, 1970). Myotis lucifugus (weighing 7 - 10 gm; Fenton, 1970) and the birchmouse, Sicista betulina (weighing 7 - 16 gm; Johansen & Krog, 1959) are two examples of small mammals which evade the high cost of endothermy through hibernation and diurnal torpor. If the wings of the bat are included in the ratio of body surface: body mass, this ratio is larger for the bat than for any other mammal (Lyman, 1970). In contrast, a large animal would probably not benefit from becoming torpid since the cooling and rewarming phase would be intolerably slow due to its greater thermal capacity (Whittow, 1973).

During hibernation and diurnal torpor, the organism experiences some variation in T_b. In response to temperature or its variation, the physiological systems of such animals have developed many temperature-sensitive and temperature-compensating mechanisms (Riedesel, 1973). Ultimately some adaptation to the alteration in T_b must be reflected to the molecular level. In this study, the enzyme pyruvate kinase (PK) of the pectoral muscle and the liver of hibernating and normothermic (i.e., non-hibernating, active) bats, Myotis lucifugus, was investigated in an attempt to further our present knowledge of the molecular adaptations involved in hibernation and diurnal torpor.

A. Hibernation

The term hibernation, as defined by this study, is found only in mammals and has been the subject of many reviews,

the most recent by Burlington (1972), Mihailovic (1972), Pengelley & Asmundson (1972), Hudson (1973) and Riedesel (1973). Also, it has been discussed in connection with the thermoregulation of bats (Davis, 1970; Henshaw, 1970; Lyman, 1970). Of the variety of terminology used to describe the yearly phases of behaviour in hibernants, that of Morrison & Galster (1975) has been chosen for its clarity. Essentially, the annual cycle is divided into a heterothermal (hibernation) season, during which natural hibernation occurs and a homeothermal (active) season, during which natural hibernation does not occur. Whereas obligate hibernants facing consistently severe conditions invariably resort to hibernation, permissive hibernants may neglect this capability in favourable years. Representatives of obligate hibernants include bats of the family Vespertilionidae* (Fenton, 1970, 1972; Rauch, 1973; Studier, 1974; Olsson, 1975; Moon, 1976) and rodents such as ground squirrels (Atherton & Zimmerman, 1974; Behrisch, 1974; Galster & Morrison, 1975; Hannegan & Williams, 1975), hamsters (Roberts & Chaffee, 1973; Fang & Willis, 1974), woodchucks (Addis et al., 1973; Bito & Roberts, 1974), hedgehogs (Pagels & Blem, 1973; Gutierrez et al., 1974; Olsson, 1975), chipmunks (Pengelley & Asmundson, 1972), dormice (Pengelley & Fisher, 1961), and birchmice (Johansen & Krog, 1959; Menaker, 1964).

1. Hypothermia and hibernation

Hypothermia and hibernation are two different physiological states and, therefore, are not synonomous.

*Note: Not all Vespertilionids are obligate hibernants; also, some species of Rhinolophidae and Molossidae hibernate as well.

Hypothermia is defined as the condition of a homeothermic organism when its T_b drops significantly below the average T_b of the species (Bligh & Johnson, 1973), this drop being uncontrolled by the organism. Hypothermia is frequently a consequence of inadequate energy, or is artificially induced through suppression of heat production by hypoxia and hypercaphia (Hudson, 1973). On the other hand, hibernation is not simply an uncontrolled abandonment of temperature regulation, but an active suppression of a high T_b (Lyman & O'Brien, 1963; Lyman 1970; Mc Nab, 1970). For example, at a T_a below 0^oC, the heart rate increases in Lasiurus borealis and Myotis lucifugus demonstrating control of physiological functions during hibernation (Davis & Reite, 1967). Also, experimental evidence exists showing differences in cortical and subcortical electrical activity of the brain between the states of hypothermia and hibernation (Mihailovic, 1972). Further, a model for control of thermoregulation during hibernation as proposed by Luecke & South (1972) suggests that deep hibernation, in contrast to induced hypothermia, is an active state maintained by an inhibitory channel, the Active Inhibitory Channel, which affects thermosensitive neurons in the hypothalamus. This Active Inhibitory Channel presumably exists only in hibernants. Finally, hibernation is distinguished from hypothermia by the fact that a hypothermic animal cannot initiate spontaneous arousal but requires external heating to regain a normal T_b (Henshaw, 1970; Hudson, 1973). The antelope ground squirrel, Ammospermophilus leucurus, is an example of a mammal which enters

torpor on occasion but cannot arouse spontaneously (Kramm, 1972); i.e., it becomes hypothermic. Hibernators can be forced into hypothermia from which they cannot arouse, and in which they will die after several days, even though that same animal could have entered hibernation at the same time of year if prepared for hibernation, and have lived for months (Henshaw, 1970).

2. Diurnal torpor and hibernation

It is conceivable that diurnal torpor, the frequent reduction of T_b and metabolic rate during the homeothermal season (called diurnation by Hall, 1832), is an exaggeration of the circadian rhythm of T_b. During the course of one day, for example, the average human T_b peaks at about 1600 hours and reaches a minimum at 0400 hours, a difference of $2^{\circ}F$ or $1^{\circ}C$ (Curtis, 1969). For smaller animals, a more dramatic lowering of T_b during the quiet periods of a day could amount to a significant energy savings. In fact, diurnal torpor appears to be more widespread among small mammals than was originally believed (Lyman, 1970). If exposed to cool conditions in the homeothermal season, bats will also undergo diurnation, otherwise they will remain warm throughout the day (Henshaw, 1970; Lyman, 1970). Diurnation is also found in birds; e.g., swifts, caprimulgids and colies (Bartholomew, 1972) and hummingbirds (Hainsworth & Wolf, 1971; Bartholomew, 1972). It would seem then, that small animals other than hibernants may also have the Active Inhibitory Channel described by Luecke & South (1972) which would allow thermoreg-

ulation to be actively shut off and yet permit spontaneous arousal. Although hibernants may not be unique in possessing the Active Inhibitory Channel, biochemical adaptation of mammals to a very low T_b (i.e., hibernation) is a phenomenon peculiar to hibernants.

Is there a physiological difference between hibernation and diurnation? According to Hock (1951, 1965), the answer to this question is "no". He further stated that "those differences that do exist are those of degree, not of kind" (Hock, 1951). However, physiological differences must exist between the two states since it has been demonstrated that during the homeothermal season a hibernant must undergo a period of preparation before hibernation (Menaker, 1962) although it is capable of diurnation. In one study, Myotis lucifugus were exposed to high and low T_a for 4 - 6 weeks and it was found that their thermoregulatory patterns resembled summer and winter-captured bats, respectively (Holyoak & Stones, 1971). In another study (Hurst & Wiebers, 1967), of the thirteen M. lucifugus captured in Indiana from April 1 to October 10, five died during exposure to -3 to -9°C, and the other eight died within five days of the experiment. On the other hand, 20 of 21 bats which were exposed to these same temperatures but were captured from October 11 to March 31, survived to the termination of the experiment (3 weeks or longer). Furthermore, Kayser (1965, p. 264) states that "hibernation may occur at any time of the year (at least in the ground squirrel) so long as the endocrines have the characteristics of winter glands". Thus, it seems that there

are two states for any hibernant: the heterothermal state (in which the animal is capable of hibernation) and the homeothermal state (the state in which the animal, due to lack of preparation, is not capable of hibernation). In nature these states coincide with the heterothermal and homeothermal seasons.

3. Stages of hibernation

The heterothermal season is not made up of a single uninterupted period, but rather is divided into heterothermal periods and intermittent periods of activity, or homeothermal periods. The heterothermal period begins with an entry (or reentry) phase as the animal cools and terminates when arousal is triggered and the animal rewarms (Morrison & Galster, 1975).

The length of the heterothermal period is species-specific (Menaker, 1964). The maximum number of days between arousals for Citellus* tridecemlineatus, C. lateralis, C. columbianus, and Glis glis is 12, 16, 19, and 33 days, respectively (Pengelley & Fisher, 1961). Hamsters (unusual hibernants in that they regularily eat during their homeothermal periods), periodically arouse every 2 - 7 days (Menaker, 1964). In contrast, of the 1023 "bat hibernation days", a total of only 9 days of arousal was calculated for M. lucifugus (Menaker, 1964). Since arousal is an energy-consuming process it would be expected that the fewer the number of arousals, the greater would be the amount of energy conserved. Hence hibernating bats such as Myotis yumanensis,

*Note: "Citellus" has been used throughout in lieu of "Spermophilus".

M. thysanodes and M. lucifugus which spend as much as 192, 163, and 165 days, respectively, in uninterupted torpor (Ewing et al., 1970), appear to conserve more energy during the heterothermal season when compared to other species of hibernants.

4. Physiological changes associated with hibernation

a. Entrance

The heart rate, respiration rate and basal metabolic rate begin to decline prior to the decrement in T_b and thermogenic activity during the entry stage (Lyman & O'Brien, 1963; Bach, 1972; Hudson, 1973). Initially, hibernants respond to a decrease in T_a by increasing heart rate and maintaining T_b at high levels. In the bat, if the T_a remains depressed, the heart rate undergoes wide fluctuations in cycles of about 20 minutes. During one of these cycles, the heart rate continues to fall and hibernation ensues (Lyman, 1970). In addition, the nervous system also undergoes changes. Bito & Roberts (1974) observed that during entry the cerebral cortex undergoes physiological changes similar to those thought to occur in acute corticocerebral shutdown. Further, Harrison (1965) found no response from the posterior colliculus (a portion of the mesencephalon primarily concerned with auditory reflexes) of Myotis lucifugus below a T_b of 12°C, suggesting that bats lose their sense of hearing as they enter hibernation.

b. Deep hibernation

It appears that major body functions during

hibernation proceed at a level just adequate for hibernant survival (Kulzer, 1968; Henshaw, 1970; Lyman, 1970). For example, at a T_b characteristic of deep hibernation (1° to 10°C), the hibernant respiratory centre remains functional while non-hibernant homeotherms cease to breath when their T_b goes below 19°C (Bach, 1972). Also, heart rate (at least in the bat) remains under CNS control (Kulzer, 1968); and nerve conduction remains unimpaired whereas nervous conduction halts when T_b reaches 9°C in non-hibernant homeotherms (Bach, 1972). Even the blood of the hibernant Citellus tridecemlineatus was found to be different from that of non-hibernant homeotherms in that 40 - 50% of the erythrocytes were "folded over" and demonstrated increased resistance to hemolysis (Spurrier & Dawe, 1973).

Another characteristic of the deep stage is the ability of the organism to respond to a further and potentially dangerous drop in T_a. There have been several observed responses to such a temperature change; i.e., metabolic compensation, "supercooling", and arousal (Davis, 1970). For example, the heart rate of Myotis lucifugus in deep hibernation when T_a is 5°C is 24 - 32 beats/minute (Davis & Reite, 1967); as temperatures drop to or below freezing (0° to -5°C), the heart rate increases up to 200 beats/minute (Reite & Davis, 1966) and thermoregulation ensues without arousal (Reite & Davis, 1966; Lyman, 1970; Hudson, 1973). After several hours of such metabolic compensation, if the T_a remains below 0°C, the ability of the hypothalamus to

regulate temperature is lost, the heart rate drops (to about 9 beats/minute in M. lucifugus; Davis, 1970) and rectal temperature drops to T_a. Although Davis (1970) refers to this drop in rectal temperature as "supercooling", it is more likely that this phenomenon is a case of induced hypothermia since the bat loses its ability to spontaneously arouse and must be rewarmed passively by increasing T_a. M. lucifugus in the heterothermal state can survive "supercooling" where T_b or 0^o to -4^oC are encountered, with no apparent complications (Hurst & Wiebers, 1967). Spontaneous arousal is another survival response to subfreezing temperatures, allowing the hibernant the option of finding a more hospitable hibernaculum (Fenton, 1970; Henshaw, 1970).

c. Arousal

There are two types of heat production in the arousing hibernant; non-shivering thermogenesis (NST) which appears to be essential for the initiation of arousal, particularily at near-freezing T_a, and shivering thermogenesis (ST) which has been observed to begin at a T_b of 17^oC in Myotis myotis (Mejsnar & Jansky, 1970). After the initiation of the arousal phase, there is a redistribution of blood such that the capillary blood-flow rates in organs of the anterior body rise more rapidly than do the organs of the posterior (Rauch, 1973). Brown adipose tissue, the unique thermogenic tissue of newborn mammals (except in the miniature pig in which NST is absent; Whittow, 1973), adults exposed to prolonged cold exposure, and hibernants, contributes to NST

(Smith & Horwitz, 1969; Hull, 1973; Whittow, 1973; Chaffee et al., 1975). This tissue is located around major blood vessels and organs of the anterior part of the body and was found to receive about 16% of the total cardiac output in its capillaries during arousal in Eptesicus fuscus (Rauch, 1973). It has been estimated that the greatest amount of heat during arousal is generated by brown adipose tissue (Mejsnar & Jansky, 1970).

Another organ which has been implicated in NST is the liver since propanolol-HCl, an inhibitor of norepinephrine-induced NST, caused a decrease in metabolic heat production in liver as well as a drop in the temperature of this organ at a T_a of 20°C (Stoner, 1973). Thus, brown adipose tissue, liver and heart are heat-producing organs which may cause the anteroposterior temperature gradient seen in small hibernants such as Sicista betulina and E. fuscus; this gradient would shorten the time required for arousal since heat lost via the body surface is minimized (Johansen & Krog, 1959; Lyman, 1970; Henshaw, 1970; Hudson, 1973; Rauch, 1973).

Control of NST appears to be by norepinephrine (Hayward, 1968). By comparing the maximum norepinephrine-induced thermogenesis with the maximum rate of thermogenesis (NST + ST) during arousal, Hayward (1968) established that NST accounts for about 82% of arousal heat production in E. fuscus, the highest NST value reported for any hibernant.

During the initial stages of arousal (NST) while the animal is capable only of slow movements of the head and appendages, T_b rises very slowly (Henshaw, 1970). However,

once ST is initiated, a faster rate of increase in T_b ensues until normothermia is attained (Smalley & Dryer, 1967; Henshaw, 1970). Shivering may not be necessary for arousal in the bat E. fuscus which could arouse even when its skeletal muscles were paralyzed with curare (Hayward & Lyman, 1967). It is not known if other bats are similar in this respect, although the rodents Mesocricetus auratus and Glis glis are unable to arouse at a normal rate when ST is blocked with curare (Hayward & Lyman, 1967).

The brain also undergoes changes during arousal; Heller & Hammel (1972) reported a rapid re-activation of the hypothalamic temperature regulator and a rapid rise in the set-point temperature until the attainment of normothermia. Furthermore, upon arousal from hibernation, electrical activity has been reported to return to different brain areas at different temperatures (Bach, 1972). Thus, arousal is made up of many complex physiological occurrences, other than just a return of the T_b to active levels.

B. Metabolism and Hibernation

The deposition of fat in the summer and fall, and the subsequent utilization of this energy depot for survival during the heterothermal season is a major feature of the annual cycle of hibernating mammals. Understanding the complex interrelationships of lipid, carbohydrate and protein metabolism as they are related to the above events remain a challenge which is being accepted by an increasing number of researchers (Burlington, 1972).

Storage of energy in the form of triglycerides in mammals (especially in hibernants) is essential for at least two reasons: 1. the ability of the different cells of the body to store carbohydrates in the form of glycogen is slight whereas the fat cells of adipose tissue are capable of storing almost pure triglycerides in quantities equal to 80 to 95% of their volume; 2. each gram of fat contains approximately 2¼ times as many calories of energy as each gram of glycogen (Guyton, 1971). However, inadequate blood flow to the muscle during arousal may limit oxygen availability during ST making anaerobic glycolysis essential at this time. It could be expected then, that a hibernant might utilize primarily lipid during the heterothermal period and upon initiation of the arousal phase where ST occurs, switch to glycolysis.

In contrast to glycogen and triglycerides, there are no equivalent storage forms for proteins or amino acids (White et al., 1968). During starvation, however, a certain proportion of tissue proteins are obligatorily degraded into amino acids which are then deaminated and oxidized to release energy (Guyton, 1971). Once carbohydrate and lipid stores have been depleted, oxidation of amino acids, which result from degradation of tissue protein, proceeds rapidly, and cellular functions deteriorate precipitously (Guyton, 1971). It is possible that end products of protein catabolism are the trigger for spring arousal since white fat cells (which would normally spare protein) may become limited by early spring (Dodgen & Blood, 1956); however, this would separate the final spring arousal from spontaneous arousals which

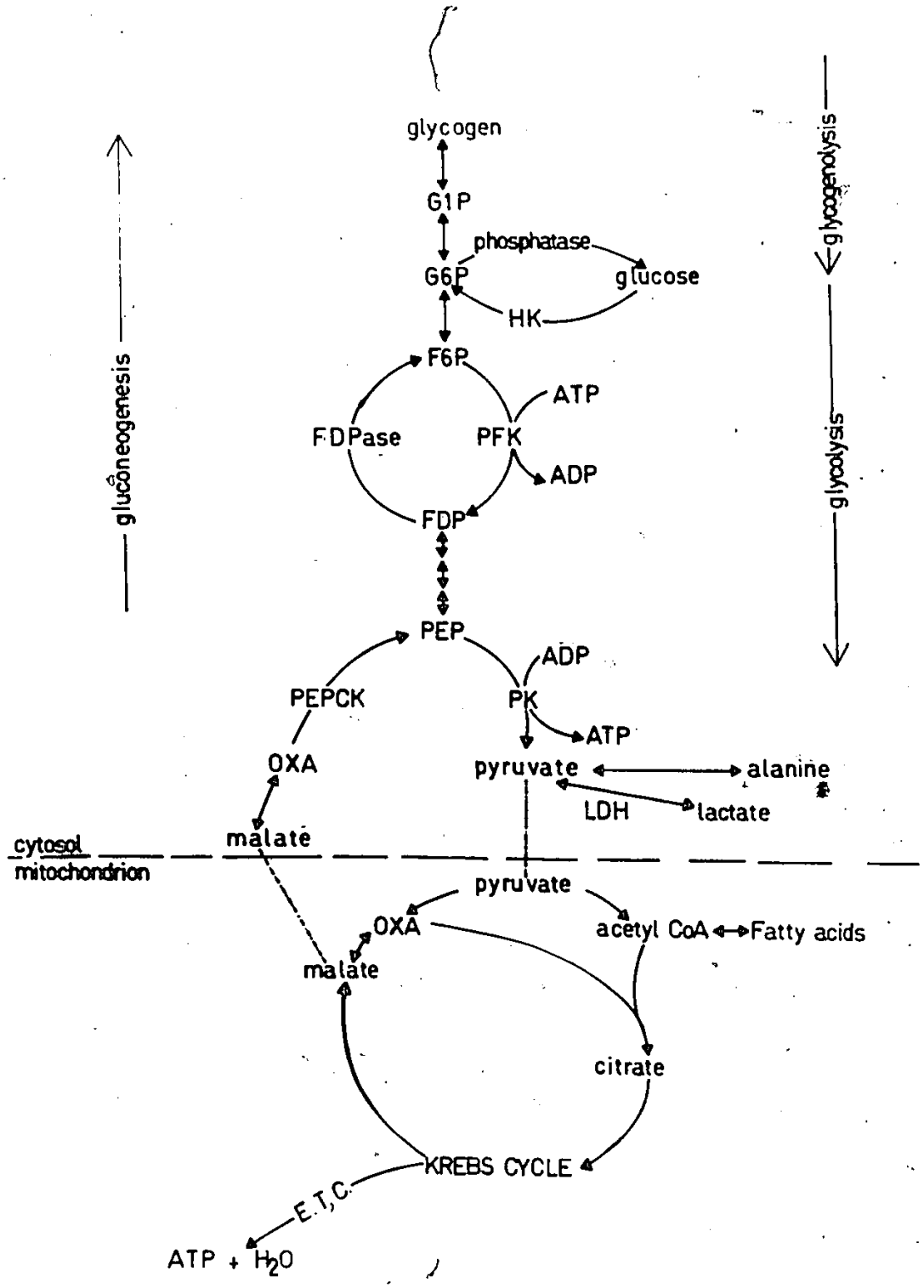
occur throughout the heterothermal season and thus this theory is not widely accepted (Fenton, 1970; Hudson, 1973; Galster & Morrison, 1975). Spontaneous arousals during hibernation may not necessarily be induced by metabolic conditions but rather may be related to water economy such as thirst and the need to urinate (Fenton, 1970; Hudson, 1973).

Fatty acids and glycerol (products of lipid catabolism) and pyruvate (carbohydrate metabolism) are channelled into acetyl Coenzyme A and the Krebs cycle/electron transport chain where they are ultimately oxidized to CO_2 and water, with the liberation of ATP and heat; amino acids from protein catabolism are channelled either into acetyl Coenzyme A or move directly into the Krebs cycle (see Fig. 2). The importance of each energy source during the heterothermal season will be discussed.

1. Carbohydrate metabolism

During hibernation carbohydrate metabolism continues at a decreased but measurable rate. In fasting, hibernating Citellus tridecemlineatus (Burlington & Klain, 1967) and C. undulatus plesius (Galster & Morrison, 1970) a significant decline was observed in blood glucose. Plasma glucose changes can be induced by a variety of experimental conditions including the method of blood withdrawal and handling, and the physiological status of the animal when the blood is withdrawn. For example, blood glucose did not decline in hibernating C. lateralis when supplied with food (Twente & Twente, 1967).

Fig. 2 Metabolic map showing the position of pyruvate kinase (PK) in relation to the glycolytic, gluconeogenic, and glycogenolytic pathways and the Krebs cycle. Abbreviations used here are seen in LIST OF ABBREVIATIONS (p.xiv).


glycogen
G1P
phosphatase
G6P
glucose
HK
F6P
ATP
FDPase
PFK
ADP
FDP
PEP
ADP
PEPCK
PK
ATP
OXA
pyruvate
alanine
LDH
lactate
malate
cytosol
mitochondrion
pyruvate
OXA
acetyl CoA
Fatty acids
malate
citrate
KREBS CYCLE
E.T.C.
ATP + H2O
gluconeogenesis
glycogenolysis
glycolysis

Although the presence of food in the hibernaculum may affect glucose values, investigators agree that food consumption is negligible for most hibernants during the short homeothermic periods of the hibernation season (South & House, 1967). Furthermore, as Hudson (1973) has pointed out, the lowering of blood glucose may result from the reduction in liver glycogenolysis because of a reduction in the blood flow to the liver in the dormant animal. Also, a reduction in the blood flow to the pancreas of the hibernating animal would cause a reduction in glucagon production which would ultimately cause a lowering of blood glucose. In other words, the lowering of blood glucose may be a secondary manifestation of hibernation.

Liver glycogen is depleted during the heterothermic period (Galster & Morrison, 1970; Tashima et al., 1970). Tashima et al. (1970) estimated that the difference in liver glycogen content between active and hibernating C. lateralis could maintain plasma glucose within the normal range for 4.5 days. However, this species is known to remain in continuous hibernation for up to eight days suggesting that gluconeogenesis must be the source of some of the plasma glucose during the heterothermal period.

During arousal, the respiratory quotient (RQ) increases from 0.7 to 1.0 indicating that there may be a switch over to glucose as the major source of energy (South & House, 1967). However, it is also possible that the increase in the RQ results from the release of carbon dioxide as the capacity of the tissues for this gas is reduced at the higher

T_b of the arousing hibernant (Goodrich, 1973). Furthermore, very little glucose is catabolized to CO_2 during arousal according to Tashima et al. (1970). It has been reported that lactate accumulates in the warming muscles of C. undulatus plesius during the arousal phase (Galster & Morrison, 1975) suggesting that this animal might obtain some of its energy from the anaerobic metabolism of glucose or glycogen at this time. The presence of lactate (and other metabolic acids) will push the carbonic anhydrase equilibrium towards CO_2 which would affect the RQ-value. Yet glucose may be a major source of energy during arousal regardless of what causes the increase in RQ, since there is a 15-fold increase in the rate glucose disappears from the plasma during arousal of C. lateralis suggesting an apparent increase in its tissue utilization (Tashima et al., 1970). Also, Daudova & Stepanova (1966) found a markedly increased liver and muscle hexokinase activity in arousing ground squirrels. Similarily, although there is a reduced carbon flux to lactate during hibernation in M. lucifugus, Moon (1976) states that the potential for lactate dehydrogenase catalysis nevertheless is maintained for arousal.

2. Lipid metabolism

Autumn fat deposition was studied in three species of New Mexico Myotis (Ewing et al., 1970). About 72 - 81% of the fatty acids were found to be unsaturated, a common occurrence in animals preparing for hibernation. More specifically, oleic acid was found to be most abundant and

a high content of palmitoleic acid (unusual for mammalian body fat) was reported for these bats.

A RQ-value of 0.7 during hibernation indicates that a major source of energy is lipid catabolism (Lyman & Chatfield, 1955; Kayser, 1961; South & House, 1967; Lyman, 1970). The rate of lipolysis is apparently geared to a reduced but constant supply of energy at the T_a encountered during this period. Further, capacity for synthesis of lipids is markedly decreased during the heterothermal season (Whitten & Klain, 1968); hepatic lipogenesis from C^{14}-acetate is not stimulated *in vitro* until some time after the initiation of arousal in the hamster (Denyes & Carter, 1961). Support for this hypothesis was provided by Whitten & Klain (1969), Klain & Rogers (1970), and Tashima *et al.* (1970), working with various hibernating species.

Essentially, triglycerides are hydrolyzed to free fatty acids and glycerol. The fatty acids released from depots enter the blood stream either to be metabolized in a large number of tissues to carbon dioxide and water, thus providing energy in the form of ATP, or are re-esterified in these tissues with α-glycerol-phosphate to triglyceride, a process which requires energy (Hull, 1973).

Stress normally causes the release of epinephrine and norepinephrine which then activate the hormonal-dependent lipases enhancing triglyceride hydrolysis (White *et al.*, 1968). Thus, with arousal, which is under adrenergic control (Hayward, 1968), lipid metabolism would be expected to change.

In M. lucifugus, Esher et al. (1973) recorded a 2.7-fold increase in serum free fatty acid concentration concurrent with a 0.6-fold decrease in serum phospholipid concentration. Also during arousal, blood glycerol content increased from 1.3 to 25 mg% in Myotis myotis (Heldmaier, 1969). These facts, coupled to a decrease in liver triglycerides (73% decrease) and free fatty acids (90%), support the hypothesis that fatty acids are available during arousal (Esher et al., 1973). Similarily, in Citellus undulatus, plasma free fatty acid concentration increased four-fold during arousal (Galster & Morrison, 1975).

In white adipose tissue the major fraction of free fatty acids is released from the cells and enters the circulation; in brown adipose tissue of newborn rabbits, the major fraction is either oxidized or re-esterified to triglyceride within the cells (Hull, 1973). The heat released from brown adipose tissue may come from triglyceride catabolism (Smalley & Dryer, 1967), but Schenk et al. (1975) report that this does not appear to be the principle mechanism of calorigenesis in this tissue in newborn rabbits. The biochemical mechanisms associated with heat production in brown adipose tissue are thought to involve the presence of unusually dense and large mitochondria and a high fat content (Hudson, 1973); however, how these mitochondria release chemical energy as heat is still under investigation.

3. Protein metabolism

Protein synthesis is drastically reduced during

hibernation (Manasek et al., 1965; Whitten & Klain, 1968). Incorporation of methionine by liver microsomes at 37°C is significantly lowered in tissues from hibernating and aroused ground squirrels (i.e., animals in the heterothermal state) when compared to normothermic squirrels (Whitten & Klain, 1968). These reports also demonstrated that the incorporation of one amino acid, alanine, into glycogen increased in the heterothermal state, suggesting gluconeogenesis at this time.

Changes also occur in polysome content with hibernation. A marked disaggregation of hepatic ribosomes was reported for Citellus lateralis and C. tridecemlineatus in the heterothermal state as compared to the active animals (Whitten et al., 1970a, b). Furthermore, these changes in polysomes were accompanied by a decreased protein synthetic capacity in a highly purified cell-free system (Whitten et al., 1970a,b). An increase in ribonuclease activity during the heterothermal season would result in disaggregation of ribosomes and decreased protein synthesis, but would seem to be counterproductive since energy used in m-RNA synthesis would be wasted. A more plausible reason for decreased protein synthesis may be a reduction in total RNA synthesis. Although not yet reported, it seems probable that protein synthesis is reduced in hibernating Chiroptera.

Significant increases in alanine and leucine catabolism in ground squirrel liver slices during arousal compared to that during normothermia suggests that there is increased protein catabolism during arousal (Whitten & Klain,

1968; Whitten & Burlington, 1971); this increase is probably associated with a breakdown of muscle protein and an increased requirement for 4-carbon intermediates to completely oxidize lipids (Whitten & Burlington, 1971). During early arousal (T_b in the range of 6° to 15°C) no significant increase in hepatic alanine or leucine was noted, in C. lateralis. This is not to say that amino acids could not serve as oxidative substrates for heat production during the later states of arousal (15° to 36°C), since these two amino acids increased significantly when the T_b was increased.

4. General scheme of metabolism during hibernation

During the hibernation period, it appears that the major source of energy is derived from lipid catabolism whereas carbohydrate metabolism may become equally if not more important during arousal, particularily during the early stages when inadequate blood flow may necessitate anaerobic metabolism. Fatty acid oxidation is turned off during anaerobic glycolysis because high lactate concentrations serve to inhibit the modulation of triglycerides by inhibiting the first step in fat catabolism (Hochachka & Storey, 1975). Mobilization of adipose tissue triglycerides is under hormonal control, and this may contribute to the required integration between triglyceride and glycogen metabolism (Hochachka & Storey, 1975). The carbohydrate pool available prior to arousal could possibly be depleted during the early stages, but at the higher T_b adequate oxygen might be available as the blood flow rate returns to normothermic levels. At

this point lipid oxidation would ensue as well as amino acid catabolism which could serve as a source of four-carbon dicarboxylic acid intermediates for lipid oxidation (Whitten et al., 1974) and also as a source of gluconeogenic precursors for maintenance of blood glucose and replenishment of tissue glycogen prior to reentry into hibernation (Whitten et al., 1974; Galster & Morrison, 1975).

C. Biochemical Adaptations in Hibernation

The requirement for biochemical specialization to allow hibernants to survive decreased temperatures which are lethal for most homeotherms, to maintain normal physiological regulatory processes at these decreased temperatures for extended periods when in the heterothermal state, and to produce large quantities of heat over a relatively short time period upon initiation of spontaneous arousal, seems implicit from the preceding discussions. These biochemical adaptations are not outwardly apparent, but allow an organism to maintain its basic metabolic function under the new environmental conditions. An example of one such biochemical modification to changing T_a was observed by Lyons & Raison (1970) for mitochondrial oxidation. Many mitochondrial enzymes of endotherms demonstrate breaks on Arrhenius plots (log rate of oxidation versus the reciprocal of absolute temperature) which is indicative of a phase change in the membrane lipids. For both the rat and the normothermic C. lateralis, a transitional or critical temperature (T_c) of 23°C was reported. The evidence indicates that a lipid phase

change renders the membrane less fluid at temperatures lower than the T_c, thereby decreasing the rate of turnover of mitochondrial oxidases very rapidly as the T_a is further reduced. Since ectotherms (animals whose major source of body heat is environmental) have a T_b which could be either above or below 23°C, a T_c in this range would be disadvantageous, and indeed Arrhenius plots for analogous systems of ectotherms are quite linear (Lyons & Raison, 1970; Hochachka & Somero, 1973). Since North Temperate bats also encounter temperatures well below 23°C during hibernation, a phase change in membrane lipids would be detrimental and thus, a shift from the endothermic to ectothermic thermal patterns in the heterothermal season could be predicted and has been observed in the succinate oxidase system of C. lateralis (Raison & Lyons, 1971).

High energy phosphates (ATP and creatine phosphate) are maintained at near normal levels during hibernation despite reduced temperatures, whereas these compounds were rapidly decreased at low T_b in the rat (South & House, 1967; Zimny & Moreland, 1968). To explain the maintenance of ATP levels during hibernation, the structural integrity of the enzymes responsible for its production must be maintained at low temperatures. The net result, therefore, is an effective enzyme activity in the hibernant which allows maintenance of overall metabolic homeostasis in the cell at these temperatures.

One of the most commonly used indices of the efficiency of enzyme catalysis is the activation energy (E_a) which is calculated under conditions giving the maximal

velocity (V_{max}) for the enzyme (Hochachka & Somero, 1973). In many studies correlations between E_a and T_b have been observed such that organisms experiencing wide fluctuations in T_b or low absolute T_b values (i.e., ectotherms) generally have enzymes which exhibit lower E_a-values compared to the homologous enzymes of organisms with a relatively constant or high absolute T_b (i.e., endotherms) (Hochachka & Somero, 1971; 1973; Low et al., 1973). An advantage of a low E_a is a decreased sensitivity of enzyme catalysis to the temperature fluctuations encountered by the ectotherm which are less serious for endotherms (Vroman & Brown, 1963). In hibernants, no correlation was found between the temperature sensitivity of tissue lactate dehydrogenase (LDH) (Olsson, 1975; Moon, 1976) or malate dehydrogenase (MDH) (Olsson, 1975) as estimated by E_a-values, and the physiological state.

The temperature dependence of V_{max} may be a misleading index of what is happening in the cell (Somero, 1972) for substrate concentrations rarely reach saturating levels in vivo (Somero, 1972; Hochachka & Somero, 1973); in other words, under steady-state conditions most enzymes operate well below their maximal capacity. Another index of the efficiency of an enzyme is the apparent enzyme-substrate affinity (E-S affinity), which is equated with the reciprocal of the Michaelis constant, or K_m (Somero & Hochachka, 1971; Hochachka & Somero, 1973). Since K_m is the substrate concentration required to reach half-maximal saturation of the enzyme, lower K_m-values (or higher E-S affinities) will increase the reaction rate at substrate concentrations below saturation.

In fact, most enzyme modulators seem to affect E-S affinity more than the maximal velocities (Atkinson, 1968; Hochachka & Somero, 1971; Somero, 1972). At very low substrate concentrations the temperature dependence of the first order rate constant, V_{max}/K_m, can also be used as an index of adaptation in enzymes of ectothermic and endothermic animals (Borgmann, 1975; Borgmann & Moon, 1975).

Assuming that bats in the heterothermal state are essentially ectothermic (i.e., T_b determined to a large extent by T_a), it could be postulated that their regulatory functions might be affected by environmental parameters such as T_a. For example, as has been described for certain ectotherms, "positive thermal modulation" (lower K_m-values, greater apparent E-S affinity at low temperatures) of enzyme catalysis might be an important mechanism for stabilizing reaction rates in the face of short-term T_a-fluctuations (Hochachka & Somero, 1973). As pointed out by Hazel & Prosser (1974), the apparent enhanced affinity of the enzymes for their substrates may partially or even totally counteract the decrease in kinetic energy at low temperatures. Positive thermal modulation has been observed in numerous enzymes of ectotherms: brain acetylcholinesterase of mullet and ladyfish (Baldwin, 1971) and rainbow trout (Baldwin & Hochachka, 1970); muscle phosphofructokinase (PFK) in Alaskan King Crabs (Freed, 1971); muscle glyceraldehyde-3-phosphate dehydrogenase of the Antarctic fish, Dissostichus mawsoni (Greene & Feeney, 1970); liver LDH of brook trout and muscle LDH of tuna (Hochachka & Lewis, 1971); liver isocitrate dehydrogenase of

rainbow trout (Moon & Hochachka, 1971); and muscle pyruvate kinase (PK) of the Antarctic fish, Trematomus bernacchii (Somero & Hochachka, 1968). With thermal acclimation in ectotherms, enzyme variants (isozymes) have been observed for a number of enzymes [e.g., acetylcholinesterase (Baldwin & Hochachka, 1970), LDH (Hochachka & Lewis, 1971) and isocitrate dehydrogenase (Moon & Hochachka, 1971)]. Each variant has a minimum in K_m at or below the acclimation temperature. Thus, the minimum in a plot of K_m-versus-temperature is observed at a higher temperature in enzymes of warm-acclimated organisms than those of cold-acclimated ones.

Since hibernation appears to be more than just a short term compensation to fluctuations in T_a (i.e., a period of acclimation is required; Menaker, 1962; Kayser, 1965), it is conceivable that new enzyme variants are produced during the acclimation period such that lower K_m-values would be found in hibernants in the heterothermal (versus homeothermal) season. Lactate dehydrogenase isozyme distribution has been studied in the hibernants Eptesicus fuscus (Brush, 1968), Citellus tridecemlineatus (Burlington & Sampson, 1968), Myotis lucifugus (Moon, 1976), Erinaceus europaeus and Nyctalis noctula (Olsson, 1975). Shifts in isozyme patterns towards LDH-5 (anaerobic form) were observed in certain tissues of the hibernating animals which may be related to increased anaerobic glycolysis (Burlington & Wiebers, 1966; 1967). Also, two isozymes of liver PK which are present alternatively depending on the physiological state of Citellus

undulatus, have been described (Behrisch, 1974). Further, ground squirrels preparing to come out of hibernation appear to have both PK forms, as determined from electrofocusing experiments, with the activity of the normothermic form of PK equal to one third the activity of the hibernating form (Behrisch, 1974).

Affinity parameters as estimated by K_m-values have been studied in hibernant enzymes. As predicted, the enzyme variants of LDH in heart, pectoral muscle and liver of Myotis lucifugus exhibit lower K_m-values in the heterothermal season as compared to the homeothermal season (Moon, 1976). Positive thermal modulation was noted for normothermic liver LDH, but the trend of the hibernating tissue LDHs appeared to be one of thermal insensitivity (K_m-temperature-independence), an adaptive strategy permitting enzyme activity to increase as the temperature increases during arousal (Behrisch, 1974; Moon, 1976). Behrisch (1974) has reported similar results for Citellus undulatus liver PK; i.e., the K_m(PEP) is lowest at 5°C for the hibernator enzyme and essentially temperature independent above 5°C. Furthermore, this heterothermal variant demonstrated temperature-independent regulatory control and a lowered apparent affinity for inhibitory modulators, as compared to the homeothermic isozyme. Hence, biochemical adaptation to temperature is observed in the enzymes of hibernants where they have been investigated.

D. Pyruvate Kinase

Pyruvate kinase (PK; ATP:pyruvate phosphotransferase,

EC 2.7.1.40) catalyses an essentially unidirectional step in glycolysis (see Fig. 2), which would make it a potential control site for the regulation of carbon flux through this pathway (Hochachka et al., 1971; Hochachka & Storey, 1975). Phosphofructokinase (PFK) catalyses an earlier step in glycolysis and is perhaps the most important control site in glycolysis (Mansour, 1972). However, the product of the reaction catalysed by PK, pyruvate (Fig. 2), feeds into a number of metabolic pathways placing this enzyme at a primary metabolic intersection (Kayne, 1973). Furthermore, it has become apparent that control of any metabolic pathway never resides at one key site but is broken up into various segments, in each there appears to be a key control reaction (Hochachka & Storey, 1975). Since the simultaneous action of the key unidirectional enzymes of glycolysis and gluconeogenesis (see Fig. 2) would permit "futile cycles" and the hydrolysis of energy-rich phosphates (an energy-wasting process), strict regulation would be expected of these enzymes in tissues capable of carrying out both glycolysis and gluconeogenesis (e.g., liver). Such antagonistic control would prevent "cycling" and assure optimal metabolic flux (Seubert & Schoner, 1971).

It has been established that three major electrophoretically distinct and non-interconvertible forms of PK exist: type L, in liver, erythrocytes and kidney; type M_1 in muscle and brain; type M_2 in kidney, adipose tissue, leukocytes and liver (see reviews by Schloen et al., 1969; Seubert & Schoner, 1971). Alternatively these forms of PK

have been labelled (corresponding in order to types L, M_1, M_2): isozyme I, III, II (Bigley et al., 1974); types L, M, K (Cardenas et al., 1975); PK-B_4, PK-A_4, PK-C_4, plus an additional isozyme, erythrocyte PK (Susor & Rutter, 1971; Whittell et al., 1973). Taking PK tetrameric structure (Kayne, 1973) and mnemonics into consideration, probably the best nomenclature would be PK-L_4, PK-M_4, PK-K_4 since it would be a system permitting easy nomenclature of hybrids, and the initials would represent the major tissue in which the forms are found. However, this paper will adhere to the more common nomenclature (i.e., types L, M_1, and M_2).

Mammalian L-type PK displays sigmoidal kinetics with its substrate, phosphoenolpyruvate (PEP) (Schloen et al., 1969). It is sensitive to the allosteric inhibitors ATP, alanine and phenylalanine, and the heterotropic activators, fructose-1, 6-diphosphate (FDP) and H ions (Seubert & Schoner, 1971; Kayne, 1973). The classical M_1-type PK displays Michaelis-Menten (hyperbolic kinetics; Schloen et al., 1969) and is usually not sensitive to most modifiers in mammals with the exception of phenylalanine (Kayne, 1973) and alanine (Seubert & Schoner, 1971; Osterman & Fritz, 1973). However, diving mammal muscle PK has retained (or regained) the strong feedback inhibition by ATP, alanine and probably citrate and the strong feed-forward activation by FDP. (Hochachka & Storey, 1975). Similar findings have been reported for muscle PKs of lower vertebrates (Somero & Hochachka, 1968; Mustafa et al., 1971; Johnston, 1975) and some invertebrates (Somero, 1969b; Mustafa & Hochachka, 1971).

The third type of PK, type M_2 is widely distributed in the different tissues of the rat (Imamura & Tanaka, 1972) and at physiological concentrations of alanine, Mg^{++}, ADP, and PEP the activity is mainly dependent upon FDP concentrations in rat liver (Van Berkel, 1974). In mammalian liver, then, there are at least two types of PK: type L and type M_2, each with distinctive physical and kinetic properties (Susor & Rutter, 1971; Imamura & Tanaka, 1972). In contrast, only a single liver enzyme has been reported for Citellus undulatus which may combine the regulatory properties of both the L-and M_2-type PKs (Behrisch & Johnson, 1974).

According to Seubert & Schoner (1971), decreasing temperature reduces the sensitivity of L-type PK to the allosteric effectors ATP, alanine, FDP and H ions, probably through a conformational transition to a regulatory-insensitive enzyme form. A similar transition was proposed by Somero (1969b) for the muscle PK of the king-crab, Paralithodes camtschatica. The liver PK of the normothermic hibernant C. undulatus was found to be similar to non-hibernating mammalian PK in its temperature-dependent regulatory properties (Behrisch, 1974; Behrisch & Johnson, 1974). Apparent K_i-values for ATP increased from about 1.4 mM at 37°C to 3.4 mM at 5°C (a 2.4-fold increase) (Behrisch & Johnson, 1974). Further, at 5°C, 1 µM FDP did not reverse the inhibition by ATP any better than 0.5 µM FDP at 37°, even though the affinity for the inhibitor was reduced at the lower temperatures.

The regulatory properties of the liver PK from C.

undulatus in the heterothermal state appear to be quite different in their response to low temperatures; i.e., the affinity of this PK for FDP and ATP is markedly independent of temperature (Behrisch, 1974). Also, substantially higher concentrations of ATP are required to effect dissociation of the heterothermic form of PK and twice as much FDP is required to offset this dissociation of PK in the hibernating ground squirrel as compared to the normothermic animal. Behrisch (1974) suggested that the decreased affinity of the heterothermic form of PK for ATP would "favour the maintenance of a high energy charge which in turn could support a wide variety of biosynthetic reactions, including gluconeogenesis".

E. Summary

Although the literature on the physiology of hibernation is extensive, only recently have investigators attempted to educe the possible molecular strategies involved in hibernation. This study was undertaken to further our knowledge about these strategies using the important regulatory enzyme PK from breast (pectoralis major) muscle and liver of the little brown bat, Myotis lucifugus. The rationale for investigating PK revolves around its multi-modulator nature and its central role in sparing carbohydrates during lipid catabolism while maintaining glycolytic potential for arousal. Liver and pectoral muscle were chosen since they represent tissues which have distinct forms of PK and which maintain quite different activity levels during the heterothermal

period.

A biochemical approach to this thesis was used to elucidate the properties of PK isolated from hibernating and normothermic bats. Although limited in scope, the interpretation of the results presents some clues as to the molecular mechanisms employed by hibernants.

MATERIALS AND METHODS

MATERIALS AND METHODS

A. Method of Capturing Bats

Hibernating Myotis lucifugus were collected in April, 1974 from the walls of the abandoned Renfrew mines, Renfrew, Ontario. Within 5 minutes, and prior to their arousal, they were taken from the mine, decapitated with small bone clippers, and placed in plastic bags in an insulated box containing dry ice. They were then transferred to a freezer and stored at -78°C until needed. The average weight of these bats was 7.4 ± 0.6 gm (n = 15).

The normothermic bats were captured in early August, during their nocturnal flights, in a double-frame bat trap (Tuttle, 1974) at Chaffey's Locks, Ontario. These bats were decapitated as soon as possible and immediately placed on dry ice. They were then stored at -78°C. The average weight of this group of bats was 6.8 ± 0.8 gm (n = 10). Fenton (1970) demonstrated that increased disturbance caused increased weight loss in M. lucifugus. Also, Carmody et al. (1971) observed that bats longest in transit from capture site to laboratory weighed less than those which were weighed immediately upon capture. This could account for the lower weight of the bats caught in August even though these bats should typically have been heavier since they would have been preparing for hibernation (Fenton, 1970). Also, since a trap was used, it is not known with precision how much time the animals spent in the trap prior to their removal.

Although the two areas are separated by approximately 80 kilometers, previous studies have found no variations in body weight and a number of enzyme proteins in three Ontario populations of M. lucifugus (Carmody et al., 1971), and few examples of enzyme heterogeneity between individuals (Manwell & Kerst, 1966; Carmody et al., 1971), making it unlikely that any differences reported here are related to genetic differences between two isolated groups.

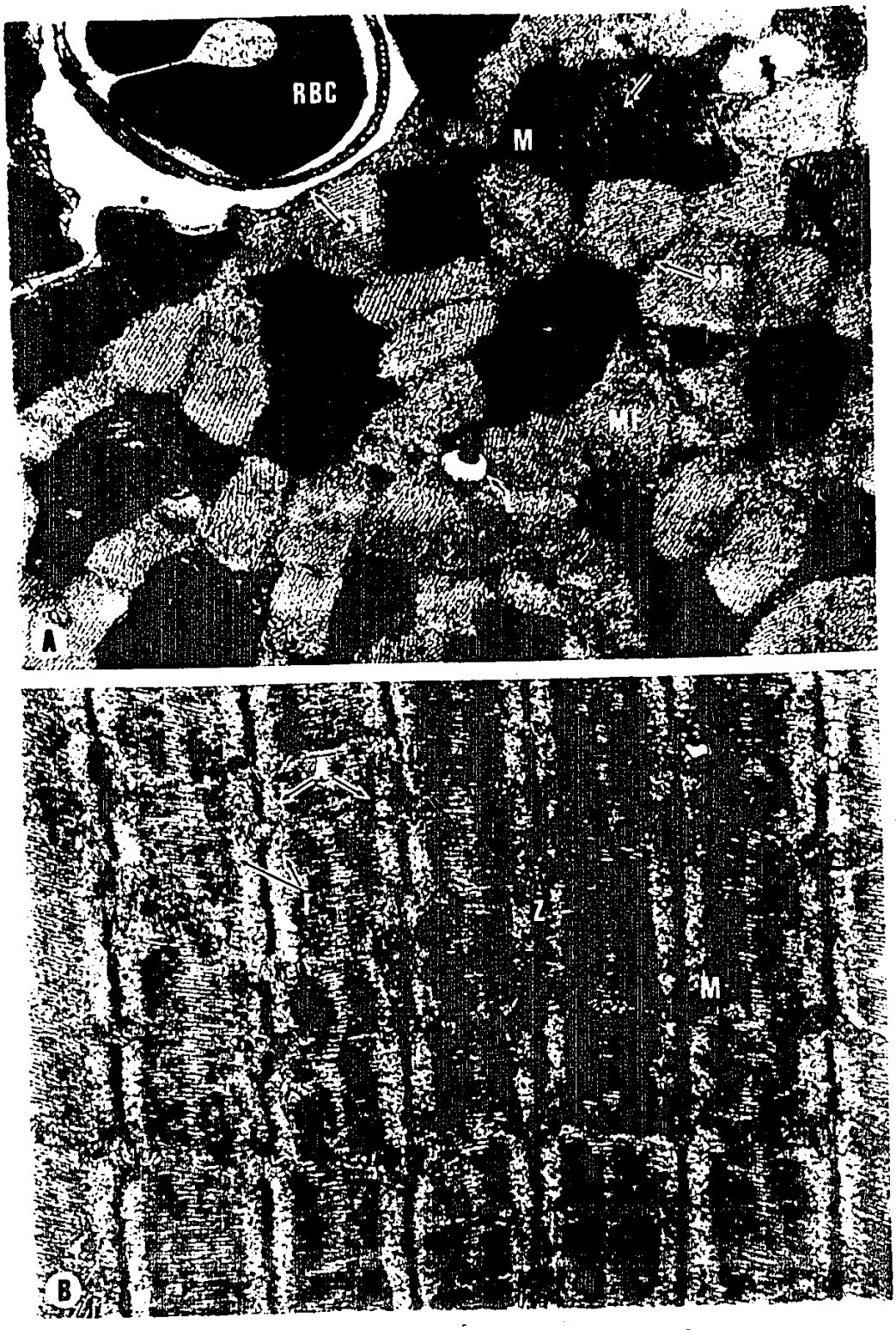
B. Excising the Tissues

Since the breast muscle (pectoralis major) of an individual bat weighed no more than 0.4 gm and the liver, 0.3 gm, the tissues from five bats were pooled for each homogenization. The tissues were excised while the bats were still partially frozen in order to maximize the amount of tissue obtained, and also to prevent loss of enzyme activity due to unnecessary warming of the tissues. Fig. 3 is an electron micrograph of bat pectoral and shoulder muscle; from the high content of densely packed mitochondria in the pectoral muscle, a high oxidative potential can be assumed for this tissue, compared to the other support muscle. The characteristics of PK isolated from such a muscle type have not been previously reported.

C. Homogenization and Centrifugation

The homogenizing buffer was 50 mM Tris-HCl, pH 7.6, containing 2 mM EDTA. Dissected tissues were placed in ice-cold homogenizing tubes (+ 5 vol buffer), puréed, and put in ice-

Fig. 3 Electron micrographs of muscle tissues from M. lucifugus: 3a. Pectoral muscle; 3b. Shoulder muscle. M = mitochondrion; SR = sarcoplasm reticulum; SL = sarcolemma; MF = myofibril; RBC = red blood cell; Z = Z-line; A = anisotrophic band; I = isotrophic band; L = lipid droplet; ⟶ = cristae. Magnification x 18,000. (From Hulbert, 1976).


RBC
M
M
A
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cold Corex centrifuge tubes. One volume of buffer was used to rinse the homogenizing vessel, and this was added to the purée and mixed thoroughly by inversion of the centrifuge tubes. The samples were then centrifuged using a Sorvall RC2-B refrigerated centrifuge at 35,000 x g (0°C) for 30 minutes. The supernatant was then pipetted into small vials (the lipid layer being carefully avoided) and ½ ml quantities were stored at -5°C until used. No loss of enzyme activity or alteration in affinity parameters were observed during storage of up to 2 months.

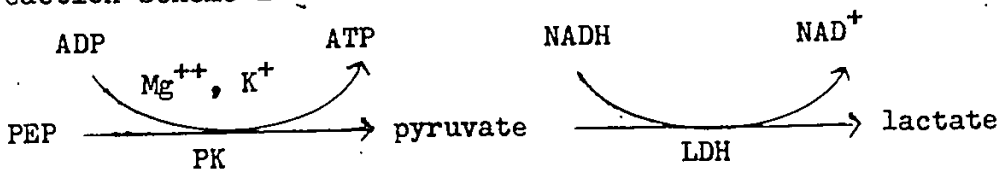
Prior to spectrophotometric analysis, the breast muscle homogenates (NM, normothermic muscle homogenate and HM, hibernating muscle homogenate) were further diluted 1:2 with homogenizing buffer, and the liver homogenates (NL, normothermic liver homogenate and HL, hibernating liver homogenate) were further diluted 1:1. Each was centrifuged at 35,000 x g (0°C) for 30 minutes to remove denatured materials and the supernatants were transferred to assay vials which were kept on ice through the analyses.

Unless stated to the contrary, all enzyme preparations followed this procedure.

D. Pyruvate Kinase (PK) Activity and Protein Assays

The activity of PK was assayed by coupling the reaction to lactate dehydrogenase (LDH) and following the oxidation of NADH by measuring the change in absorbance at 340 nm using a Unicam SP-1800 recording spectrophotometer. Assay temperatures were maintained constant by coupling the

cuvette holder to a Haake-FK refrigerated water bath. The reaction scheme is as follows:



The reaction mixture contained 50 mM Tris-maleate buffer, pH 7.0; 4 mM ADP; 10 mM $MgCl_2$; 100 mM KCl; 0.2 mM NADH; 1 U/ml LDH and 20 µl (approximately 0.1 mg protein) crude homogenate, in a final volume of 0.5 ml. The concentration of PEP used for saturation kinetics of the breast muscle PK was 2.0 mM and liver PK, 2.5 mM. This reaction mixture will be classified as the "standard assay" in all studies; modifications will be listed in the text or figure legends when necessary.

The reaction was initiated by the addition of the high-speed supernatant, after first checking for NADH oxidase activity in the absence of substrate.

In the temperature studies care was taken to maintain pH 7.0 throughout the temperature range by using the temperature coefficients reported in the Sigma technical bulletin no. 106 B.

The Michaelis-Menten constants (K_m) and theoretical maximal velocities (V_{max}) were calculated from Wilkinson plots (Wilkinson, 1961): S / v plotted against S, where S is the substrate concentration and v is the reaction velocity. The K_m is equal to the negative of the x-intercept; V_{max} is equal to the inverse of the slope (see Fig. 11 for examples of these plots).

Protein concentration was estimated spectrophotometrically using the method of Groves et al. (1968). Spectrophotometric methods for protein analysis employing this method must consider the absorbance of nucleic acid if wavelengths between 200 and 300 nm are to be used. Examination of the absorbance spectrum of the tissue nucleic acids revealed a minimum at approximately 230 nm and a maximum at approximately 260 nm, and thus to reduce absorbance interference due to nucleic acid, wavelengths near 230 nm were considered more thoroughly. Spectral measurements on protein in this latter region revealed significant absorbance which increased markedly as the wavelength decreased. The two wavelengths chosen for protein determination in this study at which the absorbance of nucleic acid is equal ("isoabsorbance wavelengths") were 224 and 239 nm. At the isoabsorbance wavelengths, the absorbance difference is due to protein alone and is proportional to its concentration. The major advantages of this method of protein determination over other methods which are more commonly used are: 1) this method is much less time consuming; 2) it allows accurate determination of protein in small samples not possible in some methods currently employed; 3) it eliminates absorbance interference due to the presence of nucleic acids; and 4) it has a variation in absorbance difference due to the content of individual proteins (amounts of aromatic amino acids) which is less than for other methods. The major disadvantage of this method is that with unpurified samples, materials other than protein and nucleic acid could conceiveably contribute

significantly to the absorbance at one of the isoabsorbance wavelengths. However, compounds containing two conjugated double bonds which would cause the greatest difficulty in this method of protein determination, such as long-chain fatty acids, carotenoids, fat-soluble vitamins, porphyrins, and steroids, which are insoluble in water, would be absent or in very low concentrations since the enzyme solution was aqueous (see previous section concerning homogenization and centrifugation).

Specific activity is defined as μmoles NADH oxidized/minute/mg protein, at the assay temperature. All enzyme activities reported in this paper are the average of at least two separate determinations with less than 10% variability between individual values.

All biochemicals were obtained through Sigma Chemical Co., St. Louis, Mo.

E. Electrophoresis

Polyacrylamide gels (8mm) were prepared essentially according to Davis (1964), with an effective gel concentration of 5.5%. Electrophoresis was continued for approximately two hours in an electrophoretic chamber similar to that described by Serman & Skreb (1972), at 200 V and 6 mA/tube, in the standard Tris-glycine, pH 8.3, buffer system.

Pyruvate kinase activity was detected by a modification of the method of Susor & Rutter (1971). The gels were removed from their tubes and incubated in the presence of

0.2 gm PEP, 0.18 gm ADP, 0.3 gm KCl, 0.16 gm $MgCl_2$, 0.016 gm NADH, 500 U LDH, and approximately 10 mg FDP in 100 ml Tris buffer (100 mM), pH 6.0. The period of incubation was closely monitored with a fluorescent light (345 nm) until the point of maximum darkening (i.e., maximum loss of fluorescence). At this point, the position and relative intensity of the bands was recorded. Gels run in the absence of PEP exhibited no banding activity.

RESULTS

RESULTS

A. Attempted Purification of Bat Pyruvate Kinase

The breast muscle and liver tissues of five hibernating bats were pooled and homogenized with 9 volumes of homogenizing buffer, 50 mM Tris-HCl, pH 7.6, containing 2 mM EDTA. Table 1 is a determination of fold-purification of the various fractions collected by adding increasing amounts of solid ammonium sulfate to the centrifuged crude homogenate. The enzyme activities of each fraction were recorded together with the protein concentrations.

The results of these experiments suggested no purification using these techniques. The greatest activities of PK for HM were found in the two fractions above 65% saturation; however, these represent only 6% of the total activity found in the crude homogenate. From these data alone, it would appear that 20 times as many individual bats would be required for the study if the enzyme were subjected to "partial purification" rather than left in its crude state. The population of bats at the Renfrew mine was, and still is, steadily declining (Dr. Fenton, personal communication); therefore, collection of great numbers of bats was not possible. Furthermore, the 65-75% fraction was found to be only 1/3 as "pure" as the crude homogenate. As for HL, the greatest PK activity was found in the very first fraction, the 0-40%-saturated-$(NH_4)_2SO_4$ fraction (32.6% of the activity in the crude homogenate), but this fraction also contained the highest amount of protein of all the

Table 1. The attempted purification of PK by ammonium sulfate fractionation of homogenates obtained from pectoralis muscle (HM) and liver (HL) of hibernating Myotis lucifugus.

tissue	fraction	Activity* (units/ml)	Volume (ml)	Tot.Act. (units)	Tot.Prot. (mg)	Spec.Act. (units/mg)	Yield (%)	Purity (_-fold)
HM	crude	9.3	16.0	149.0	83.5	1.78	100.0	1.0
	0 - 40	2.7	1.0	2.7	16.2	0.17	1.8	0.1
	40 - 50	3.8	0.7	2.6	4.9	0.53	1.7	0.3
	50 - 65	0	0.5	0	9.4	0	0	0
	65 - 75	9.6	1.0	9.6	16.9	0.57	6.4	0.3
	75+	0.5	17.0	8.5	24.5	0.35	5.7	0.2
	total			23.4	71.9		15.6	
HL	crude	2.2	17.0	37.4	185.0	0.20	100.00	1.0
	0 - 40	12.2	1.0	12.2	43.4	0.28	32.6	1.4
	40 - 50	3.2	1.4	4.5	41.2	0.11	12.0	0.5
	50 - 65	4.0	0.6	2.4	30.8	0.08	6.4	0.4
	65 - 75	0	1.0	0	16.8	0	0	0
	75+	0	17.5	0	21.4	0	0	0
	total			19.1	153.6		50.4	

*1 unit equals oxidation of 1 μmole NADH per minute at 25°C.

$$\% \text{ yield} = \frac{\text{total activity of fraction}}{\text{total activity of control}} \cdot 100$$

$$\text{Purity (purification)} = \frac{\text{specific activity of fraction}}{\text{specific activity of control}}$$

fractions. There was only a slight increase in the "purity" of this fraction as compared to the crude homogenate. These unusual results may have resulted from the small volumes which were used, but alternatively may represent an important characteristic of bat PK; this should be the subject of further studies. Therefore, for the above reasons, the HM and HL enzymes were not purified for this study and no attempt at purification of the normothermic tissues was undertaken.

B. Electrophoresis of Bat Pyruvate Kinase

There exists a very complex banding pattern in polyacrylamide electrophoresis for bat muscle and liver PK (Fig. 4). Similarities between tissues in the banding are evident. The bat muscle PK displays more isozymic forms than other mammals (see Seubert & Schoner, 1971), and with hibernation this number is reduced by about half. There are five distinct bands in NL-PK and HL-PK. The difference between these two forms of PK appears to be the positioning of these five bands.

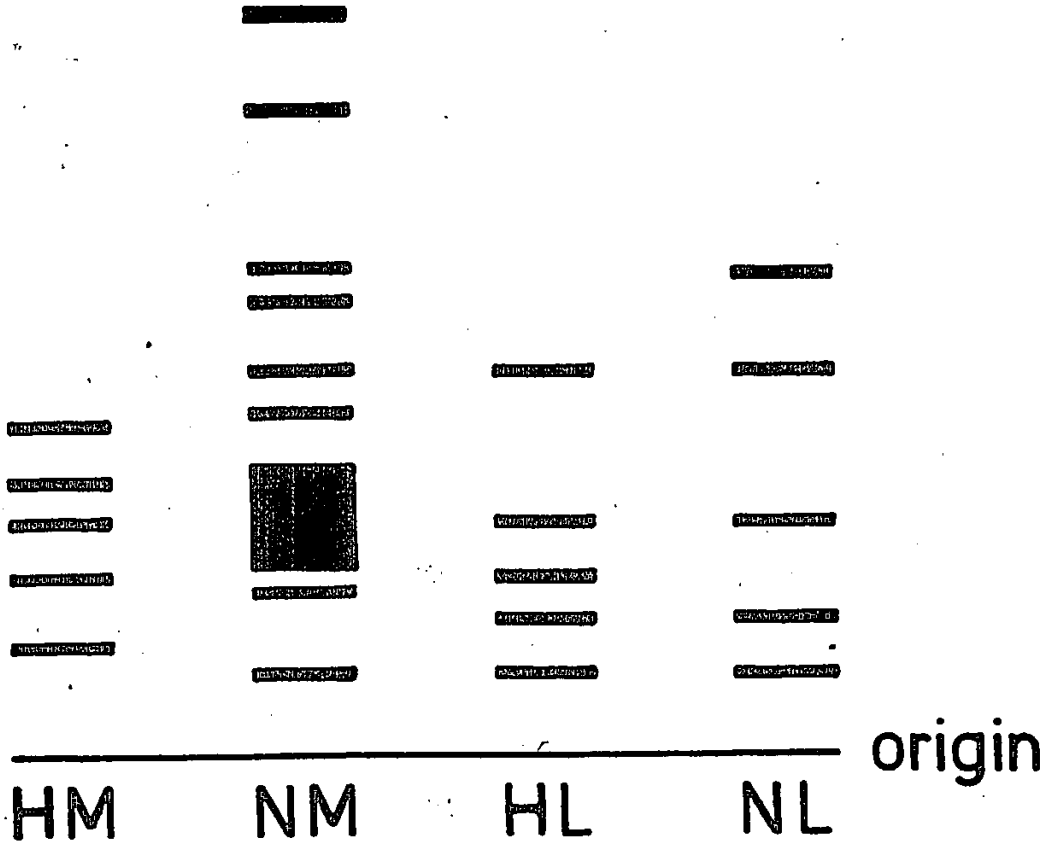
The complexity of these electrophoretic differences does not allow detailed analysis; however, such changes could possibly account for some of the kinetic differences noted in this study.

C. Effects of Ions on Bat Pyruvate Kinase

1. Potassium, magnesium, and calcium effects on hibernator PK

At very low concentrations, or without the addition

Fig. 4 Diagrammatical representation of polyacrylamide gel electrophoresis of high-speed supernatant of flight muscle and liver PK isolated from hibernating and normothermic bats. Running and staining procedures are described in the MATERIALS AND METHODS. (H = hibernating or heterothermic tissue; N = non-hibernating, homeothermic or normothermic tissue; M = breast or pectoral muscle; L = liver).


origin
HM
NM
HL
NL

of either K^+ or Mg^{++}, PK activity was either just detected, or else not detected at all. Since a crude preparation was utilized without dialysis small activities from residual ions might be expected. A relatively broad peak in specific activity of PK was found at approximately 100 mM KCl (in the presence of 10 mM $MgCl_2$; Fig. 5a) for both tissues, and at 2 to 10 mM $MgCl_2$ and 4 mM $MgCl_2$ (in the presence of 100 mM KCl) in the liver and muscle, respectively (Fig. 5b). At high concentrations, the ions inhibited PK activity: 50% inhibition occurring at about 1 M KCl and 80 mM $MgCl_2$ in both tissues.

Calcium, on the other hand, is not essential for PK activity. In the muscle, it activated PK slightly (11%) at a concentration of 0.1 mM (in the presence of 100 mM KCl and 10 mM $MgCl_2$; Fig. 5c), but greater concentrations inhibited enzyme activity. Such an effect by calcium on muscle PK has been reported for rainbow trout (Somero & Hochachka, 1968). The liver enzyme was virtually unaffected by Ca^{++} at all concentrations used, although slight inhibition is observed (Fig. 5c). In both tissues, HM and HL, 50% inhibition was found at approximately 1.0 mM Ca^{++} with complete inhibition within the concentration range of 20 to 40 mM.

As stated in the MATERIALS AND METHODS section, and as was the case for the experiments involving K^+ and Mg^{++}, the frozen muscle homogenate was further diluted in a 2:1 ratio upon thawing (i.e., before spectrophotometric analysis), and the liver, 1:1. At the end of each experiment, these diluted homogenates were discarded. However, for the

Fig. 5 The effect of ions on flight muscle and liver PK of Myotis lucifugus in the heterothermic state at 25°C. Standard assay described in MATERIALS AND METHODS. 5a. Effect of potassium ions; $[Mg^{++}]$ = 10 mM.

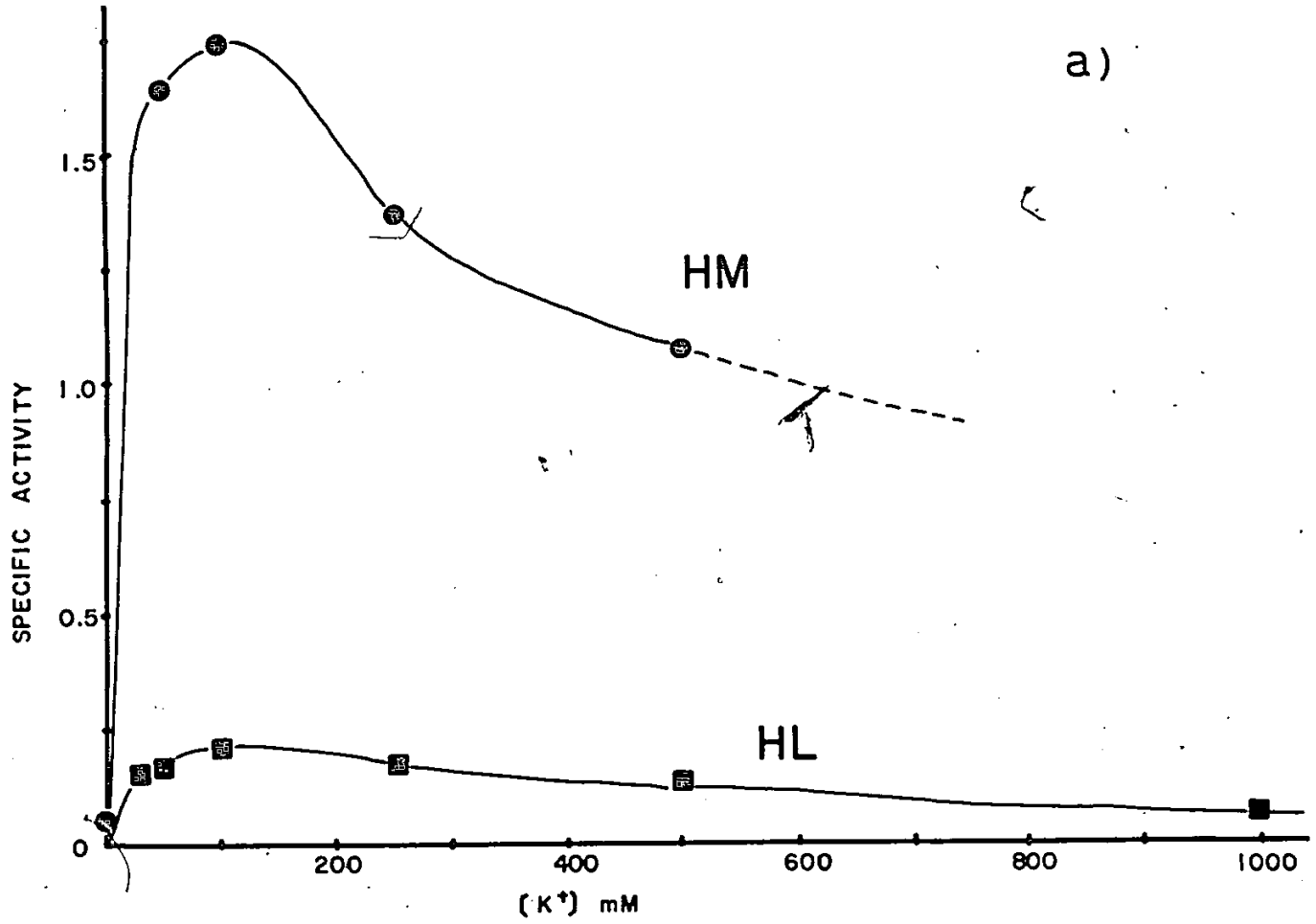

a)
HM
HL
SPECIFIC ACTIVITY
1.5
1.0
0.5
0
200
400
600
800
1000
(K+) mM

Fig. 5b. Effect of magnesium ions; $[K^+]$ = 100 mM.

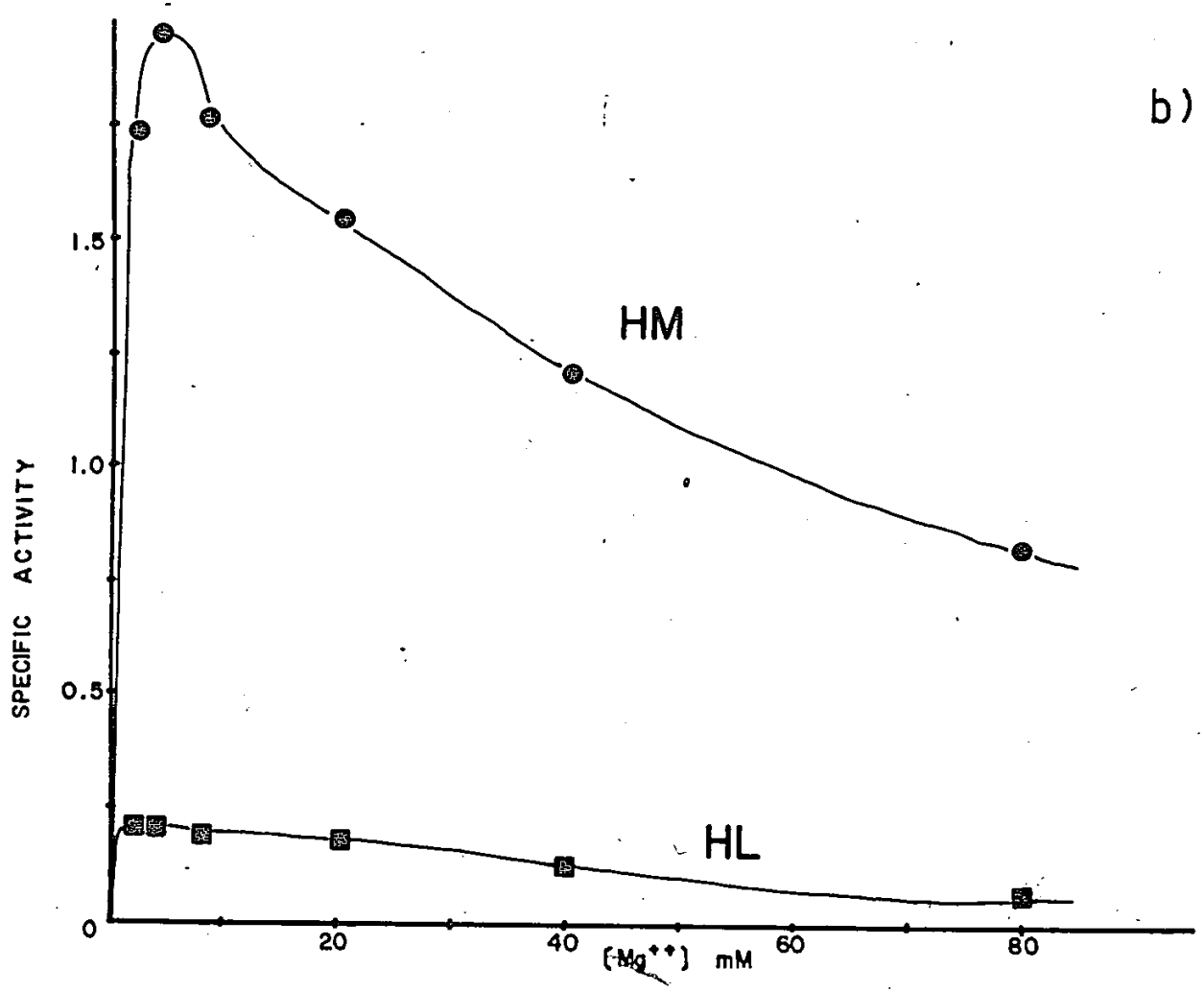
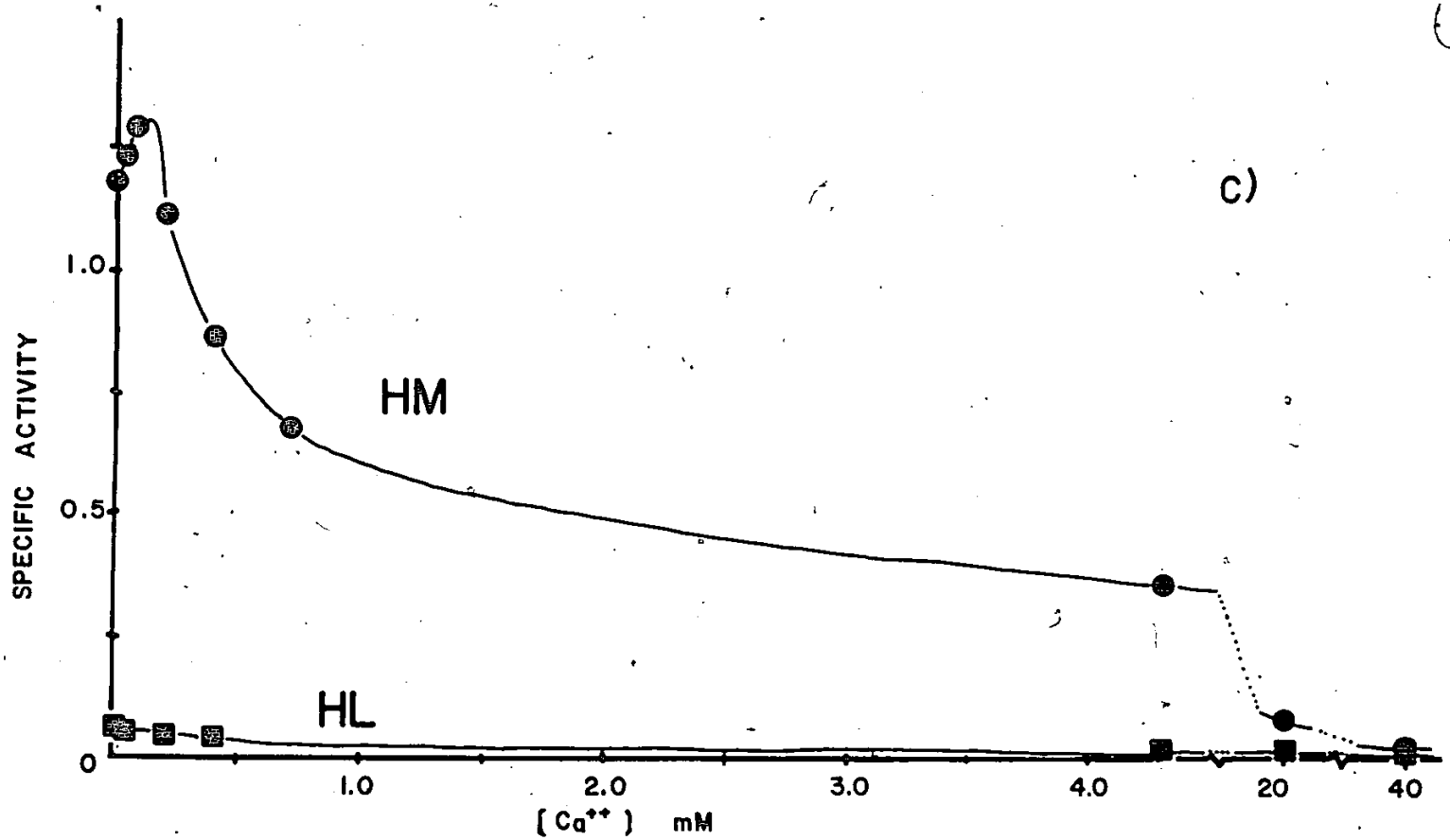

b)
SPECIFIC ACTIVITY
1.5
1.0
0.5
0
HM
HL
20
40
60
80
[Mg++] mM

Fig. 5c. Effect of calcium ions; $[Mg^{++}]$ = 10 mM; $[K^{+}]$ = 100 mM.


C)
SPECIFIC ACTIVITY
1.0
0.5
0
HM
HL
1.0
2.0
3.0
4.0
20
40
[Ca++] mM

calcium experiments, the diluted homogenates were re-frozen and used in the experiments. If Figs. 5a and 5b are compared with Fig. 5c, it will be noted that the maximum PK specific activity is about 30 - 40% lower in the muscle. In the liver, the decrease in PK specific activity approaches 75%, suggesting that bat liver PK may be more labile than muscle PK. No such loss in activity was ever noted for PK stored at high protein concentrations (approximately 4 mg protein/ml in the muscle homogenates, and 14 - 20 mg protein/ml in the liver homogenates).

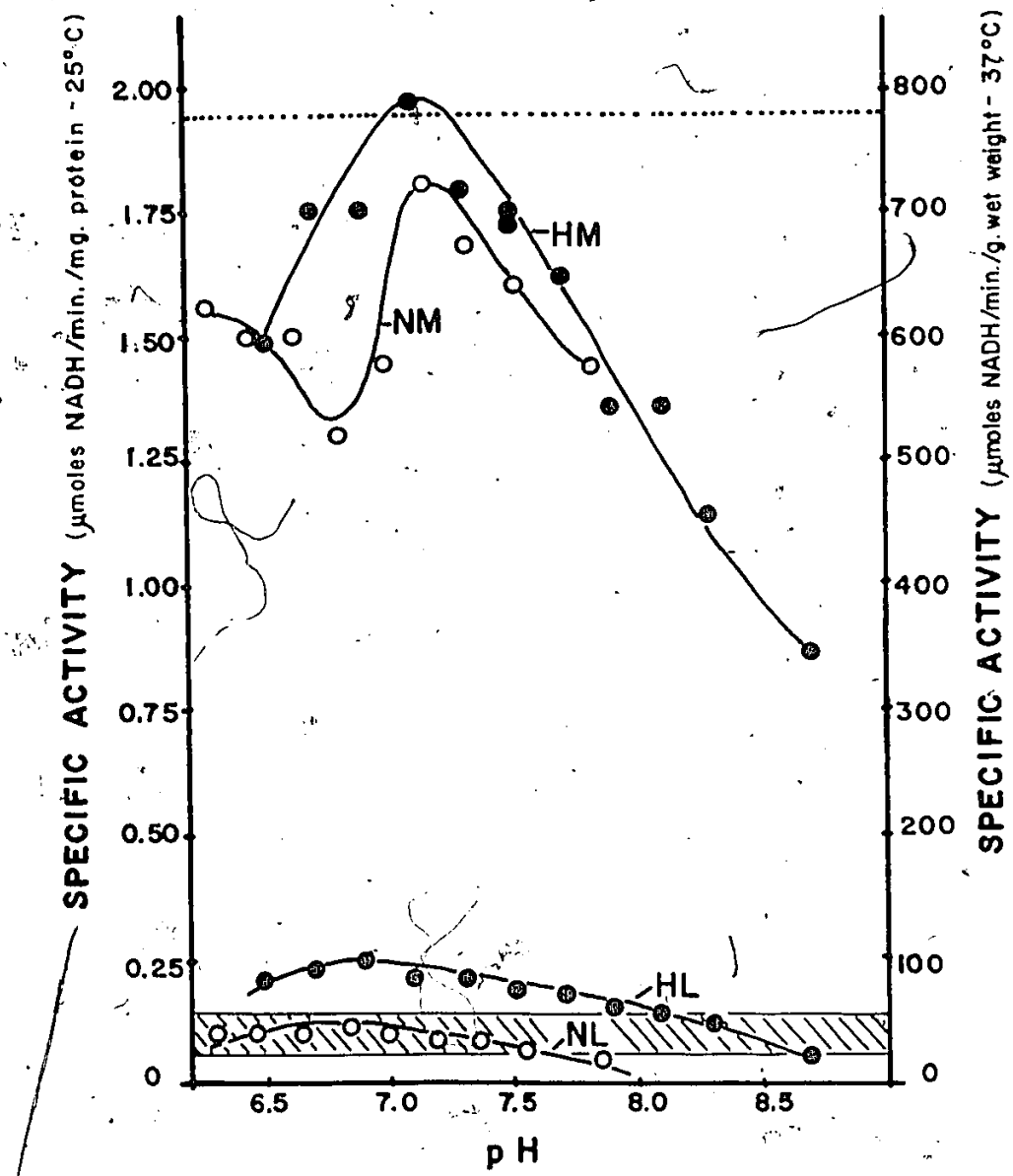
2. pH studies in hibernator and normothermic tissues

The assay conditions were as stated in the MATERIALS AND METHODS section, with the exception that the pH of the Tris-maleate buffer was varied over a range of about 2 pH units (pH 6.3 to 8.7), and 2 mM PEP was used for both tissues.

It is well known (Seubert & Schoner, 1971) that pH is a very important modulator of PK activity <u>in vivo</u>, although it has not been extensively studied here. Fig. 6 demonstrates the pH optima for NM-, HM-, NL-, and HL-PK which are, respectively, 7.1, 7.1, 6.7, and 6.8. Contrary to the muscle enzymes, the liver PKs have relatively broad pH profiles. For NM-PK there is a minimum at pH 6.7, the significance of which is unknown. A sharp rise in activity as the pH drops from 8.0 to 7.0 is observed for the muscle enzymes (not for liver PK) which may activate the enzyme during muscular activity when H ions are produced.

Also, in Fig. 6 a comparison with data from Scrutton & Utter (1968) is presented. The maximal activity for rat

Fig. 6 pH profiles of hibernating and normothermic tissue PK. The standard assay was used at a temperature of 25°C. Shaded area near bottom of graph indicates range of maximal catalytic capacities of "all species" liver PK and the dotted line near top of graph indicates the maximal catalytic capacity for rat skeletal muscle PK as reported by Scrutton & Utter (1968). The scale of specific activity as expressed in μmol/min/g wet weight at 37°C on the right hand side of graph was derived using the conversion factors presented by Scrutton & Utter (1968, p. 254).


SPECIFIC ACTIVITY (μmoles NADH/min./mg. protein - 25°C)
SPECIFIC ACTIVITY (μmoles NADH/min./g. wet weight - 37°C)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
800
700
600
500
400
300
200
100
0
HM
NM
HL
NL
6.5
7.0
7.5
8.0
8.5
pH

skeletal muscle PK is shown by a dotted line, and for liver PK in "all species" as a shaded area; values were equated using conversion factors for expressing specific activity as μmoles/minute/gm wet weight at 37°C (see Scrutton & Utter, 1968, p. 254). Although only a rough comparison, it can be seen that the PK activities of Myotis lucifugus in both physiological states are similar to those of other mammals. Note, however, the consistent difference between activity levels in muscle and liver which was also observed in Fig. 5.

The hibernating state does not result in lowered activity levels for PK (Fig. 6). In fact, throughout most of the pH range activities are 1.5- to 2.0-fold higher with hibernation. These differences in activities are not a simple function of a decrease in total protein concentration of the hibernator tissue. The trend is just the opposite (see Table 2). Therefore, this higher activity, which is also noted in the temperature experiments (see Figs. 7,8), is a real phenomenon and may indicate the importance of this enzyme to the overall metabolism of these tissues during hibernation.

D. Temperature as a Modulator of Bat Pyruvate Kinase

Arrhenius plots (log velocity vs $1/T^{\circ}K$) were determined for the four PKs studied: NM-, HM-, NL-, and HL-PK (Figs. 7,8). In each graph, the top line represents values of saturating concentrations of PEP and ADP; the

Table 2. Protein estimates in normothermic and hibernating bat pectoralis muscle and liver homogenates, using the method of Groves et al. (1968).

tissue	protein concentration	n
NM	1.9 ± 0.4 mg/ml*	8
HM	2.3 ± 0.1 mg/ml	8
NL	6.7 ± 0.8 mg/ml	15
HL	9.2 ± 0.6 mg/ml	8

*values expressed as mean ± S.E.M.

n = number of individual determinations.

Fig. 7 Arrhenius plots for the NM-(7a) and HM-(7b) PK using the standard assay with these exceptions: ○ 2 mM PEP, 4 mM ADP; ● 0.02 mM PEP, 4 mM ADP; □ 2 mM PEP, 0.4 mM ADP.

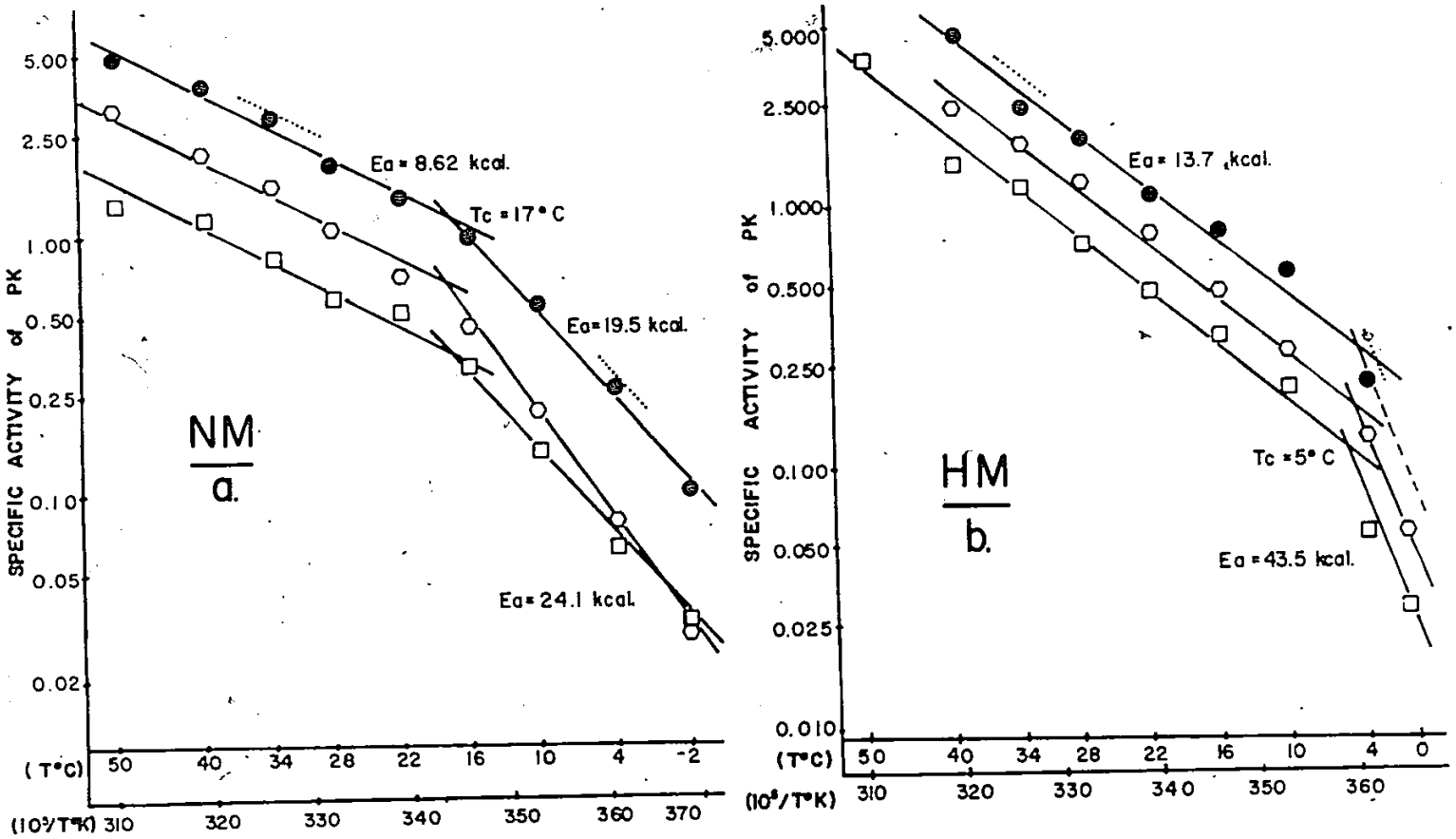
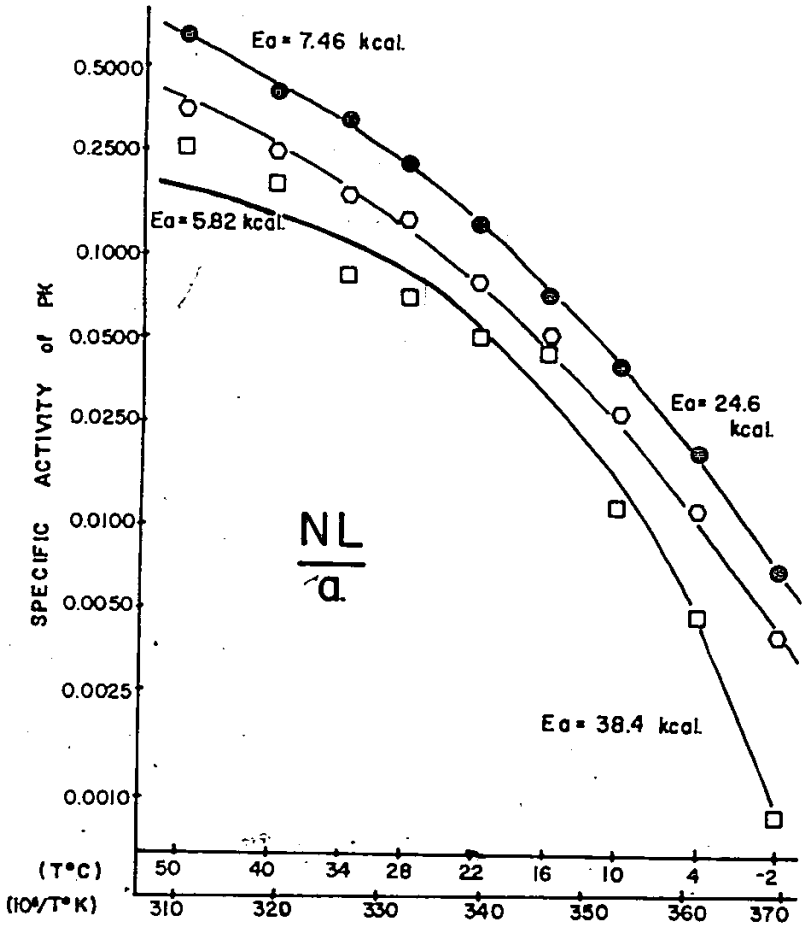
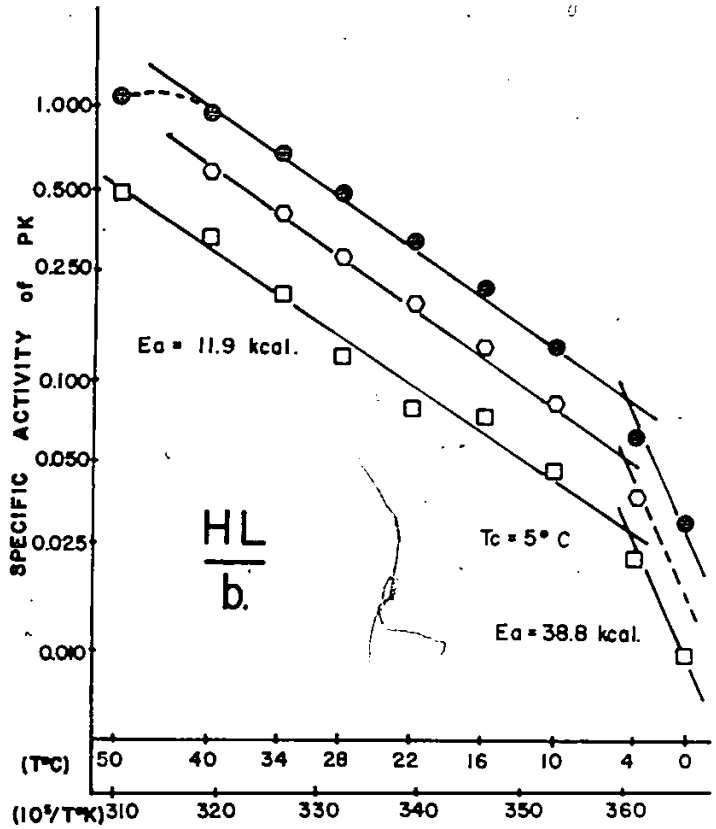

SPECIFIC ACTIVITY of PK
5.00
2.50
1.00
0.50
0.25
0.10
0.05
0.02
NM
a.
Ea = 8.62 kcal.
Tc = 17° C
Ea = 19.5 kcal.
Ea = 24.1 kcal.
(T°C) 50 40 34 28 22 16 10 4 -2
(10³/T°K) 310 320 330 340 350 360 370
SPECIFIC ACTIVITY of PK
5.000
2.500
1.000
0.500
0.250
0.100
0.050
0.025
0.010
HM
b.
Ea = 13.7 kcal.
Tc = 5° C
Ea = 43.5 kcal.
(T°C) 50 40 34 28 22 16 10 4 0
(10³/T°K) 310 320 330 340 350 360

Fig. 8 Arrhenius plots for the NL-(8a) and HL-(8b) PK using the standard assay with these exceptions: ○ 2.5 mM PEP, 4 mM ADP; ● 0.2 mM PEP, 4 mM ADP; □ 2.5 mM PEP, 0.4 mM ADP.


Ea = 7.46 kcal.
Ea = 5.82 kcal.
Ea = 24.6 kcal.
Ea = 38.4 kcal.
NL
a.
SPECIFIC ACTIVITY of PK
0.5000
0.2500
0.1000
0.0500
0.0250
0.0100
0.0050
0.0025
0.0010
(T°C)
50 40 34 28 22 16 10 4 -2
(10⁴/T°K)
310 320 330 340 350 360 370


Ea = 11.9 kcal.
Ea = 38.8 kcal.
HL
b.
Tc = 5° C
SPECIFIC ACTIVITY of PK
1.000
0.500
0.250
0.100
0.050
0.025
0.010
(T°C)
50 40 34 28 22 16 10 4 0
(10⁴/T°K)
310 320 330 340 350 360

middle line, low concentration of ADP; and the bottom line, low concentration of PEP.

1. The ADP-temperature interaction

Maximal activation of PK in HM and HL occurs at an ADP concentration of 3 to 4 mM (Fig. 9). Inhibition is more marked in the HL such that 50% inhibition occurs at approximately 20 mM as compared to more than twice this concentration in the HM. Such large concentrations of ADP are not physiological and the significance of this inhibition is not immediately apparent.

The effect of temperature on the K_m(ADP) in the NM, HM, NL, and HL can be seen in Figs. 7, 8 by comparing the top lines (saturating concentrations of ADP, i.e., 4.0 mM) with the middle lines (low concentration of ADP, i.e., 0.4 mM). The trend for the K_m(ADP) is temperature independence as indicated by the parallel lines in these figures, with one exception: for NM-PK (Fig. 7a), below 17°C the slope is much greater at the lower than at the higher concentration of ADP. The composite plot (Fig. 10) demonstrates that the K_m(ADP) for both the NM- and HM-enzyme is temperature independent above 17°C, but below there is an approximate four-fold rise in the K_m(ADP) for the NM-enzyme. (See below for discussion of correlation of K_m and parallel lines in Arrhenius plots; Section D.3.) Also, at all temperatures, NM-PK has a slightly higher K_m(ADP) compared to the HM-enzyme. As will be discussed in more detail (see DISCUSSION), at temperatures below 17°C, the decrease in available thermal energy coupled to the

Fig. 9 ADP saturation curves for heterothermic flight muscle and liver PK. The standard assay was used at the temperature of 25°C.

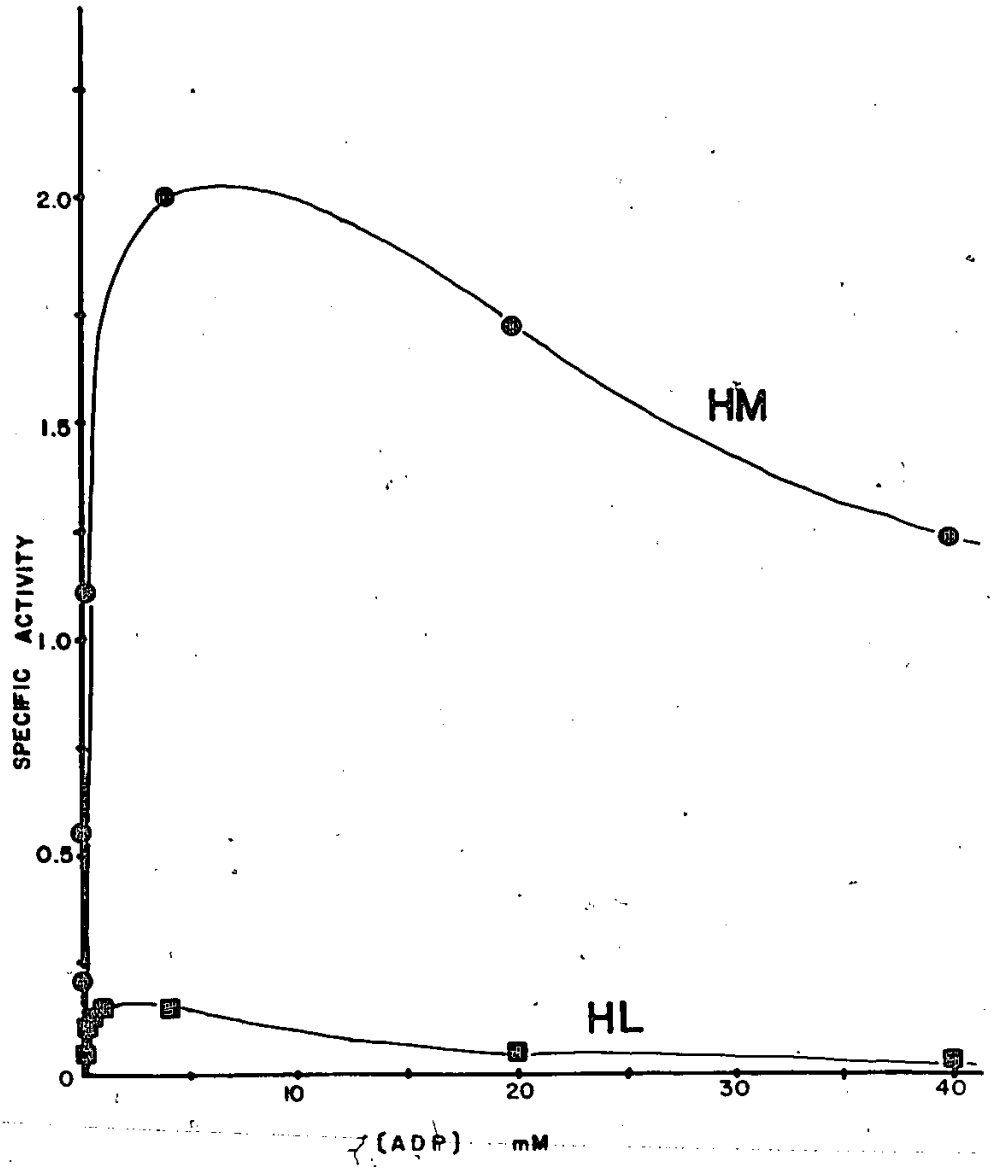
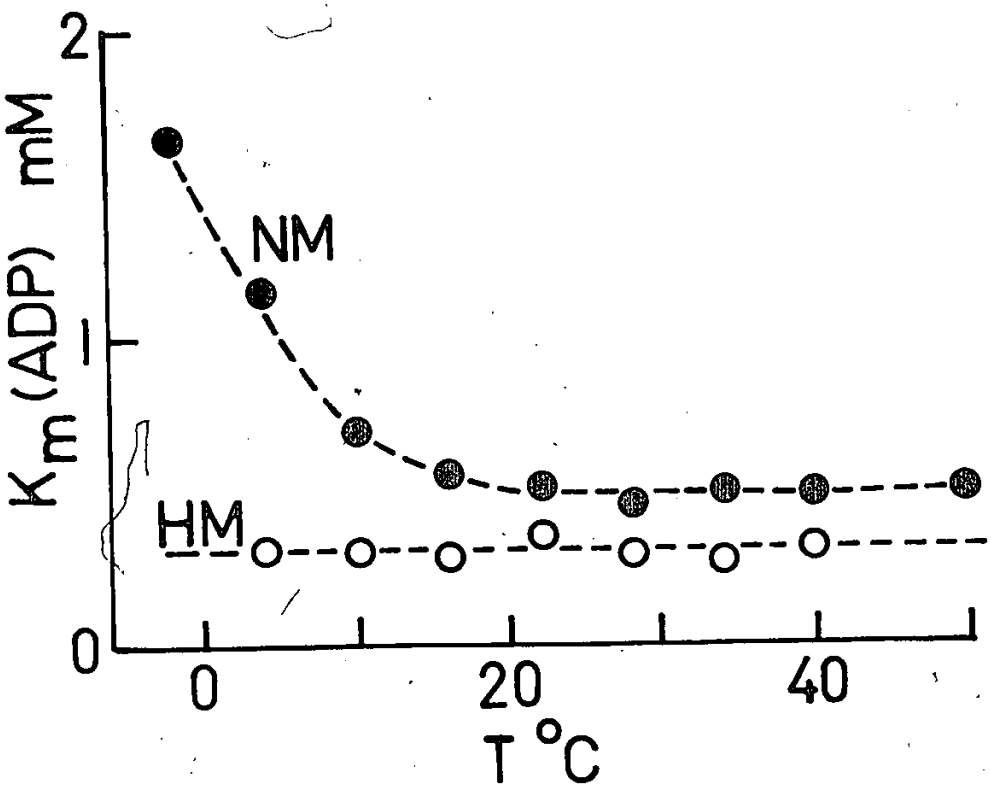

SPECIFIC ACTIVITY
2.0
1.5
1.0
0.5
0
HM
HL
10
20
30
40
(ADP) mM

Fig. 10 Estimated K_m(ADP) values as a function of temperature for the NM- and HM-PK isolated from the bat. Values were calculated from data illustrated in Fig. 7; see text.


2
1
0
K_m (ADP) mM
NM
HM
0
20
40
T °C

increase in K_m could result in very large reaction rate decreases, and possible inactivation of the enzyme. Such enzyme kinetics would be disadvantageous to an organism exposed to low temperatures, and as seen in Fig. 10, do not occur in HM-PK.

2. The PEP-temperature interaction

a. Muscle PK

In Fig. 7a, b, a parallel relationship exists between activities at high and low concentrations of PEP throughout the temperature range. This result indicates that the K_m(PEP) is temperature-independent. The association of parallel Arrhenius plots to constant K_m-values was arrived at from the following reasoning. Taking the Michaelis-Menten equation,

$$\text{velocity} = \frac{V_{max} \cdot [PEP]}{K_m + [PEP]},$$

where V_{max} is the maximal rate of the reaction, and K_m is the apparent Michaelis constant. If both the K_m(PEP) and the concentration of PEP are held constant at all assay temperatures, the above equation can be simplified:

$$\frac{[PEP]}{K_m + [PEP]} = \text{a constant, e.g., C,}$$

and C will always be less than 1. This can be illustrated by using the values shown in Fig. 7 and holding K_m at 0.05 mM.

Case 1, where $[PEP] = 2.0$ mM (saturating)

$$\text{velocity} = V_{max} \cdot C$$

$$= V_{max} \cdot \frac{2.0}{0.05 + 2.0}$$

$$= V_{max} \cdot \frac{2.0}{2.05}$$

$$= V_{max} \cdot 0.96$$

Case 2, where $[PEP] = 0.02$ mM (near K_m)

$$\text{velocity} = V_{max} \cdot C$$

$$= V_{max} \cdot \frac{0.02}{0.05 + 0.02}$$

$$= V_{max} \cdot \frac{0.02}{0.07}$$

$$= V_{max} \cdot 0.29$$

Thus, in the first case, $C = 0.96$, and in the second, $C = 0.29$; velocity is always a constant percentage of V_{max}. In this example, velocity would be equal to 96% of the maximal velocity in Case 1, and 29% of V_{max} in Case 2 if the K_m was a constant 0.05 mM throughout the temperature range. Represented on a logarithmic graph, the velocities at one concentration of PEP would be parallel to the velocities at another concentration of PEP. A similar argument has been used by Somero (1969a); if no change in the K_m of the substrate occurs with a change in temperature, the Q_{10} (or E_a) would be independent of the substrate concentration.

Arrhenius plots can also be used to give an indication of the thermal sensitivity of the maximal rate of

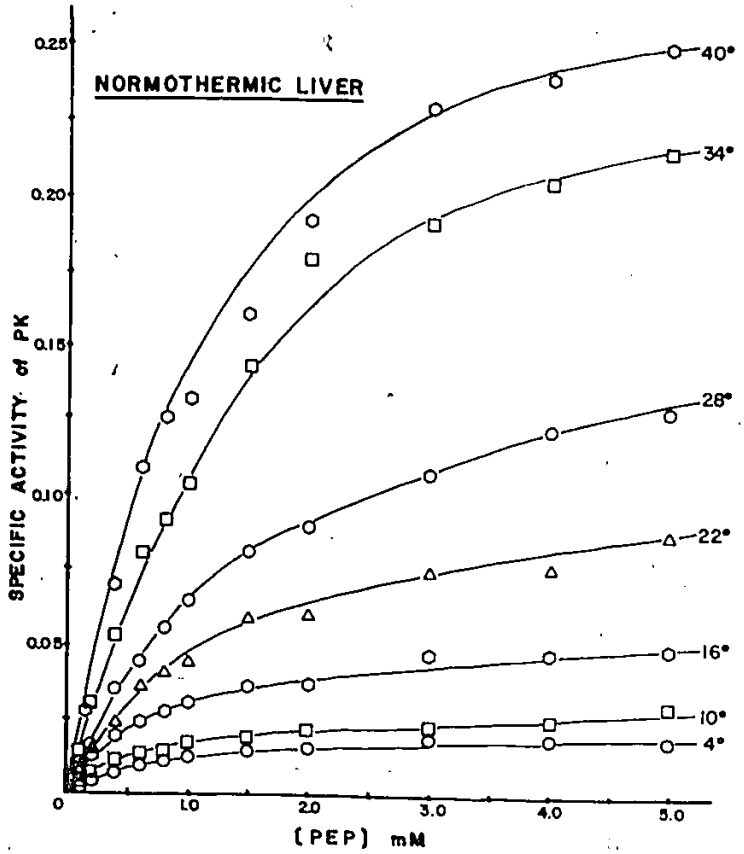
catalysis by an enzyme. For example, at temperatures above 17°C, the NM-PK is less thermally dependent compared to HM-PK, as illustrated by its lower activation energy (E_a = 8.62 kcal/mole compared to E_a = 13.7 kcal/mole). In the temperature range of approximately 17 to 50°C, and using thermal insensitivity as a criteria (i.e., a low E_a), the NM-PK would be considered the better catalytic species compared to HM-PK. In the range of 5 to 17°C, however, the temperature dependence doubles for NM-PK and it is the HM-PK which has the lower E_a (13.7 vs 19.5 kcal/mole). Thus, at the low temperatures the HM-PK would seem to be the most catalytic efficient species. Below 5°C there is a three-fold increase in E_a for the hibernator tissue enzyme.

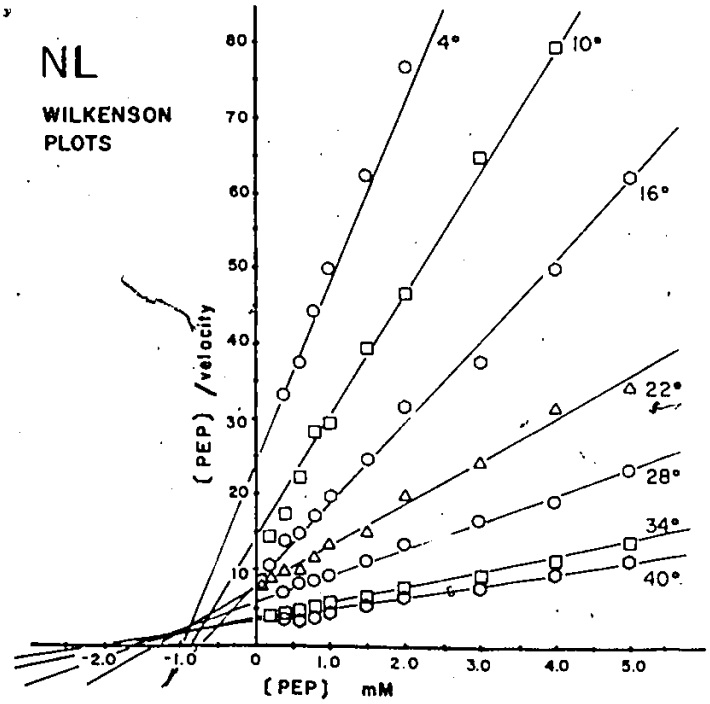
b. Liver PK

Basically, the same pattern of activation energies found above for muscle PK was also found for the liver enzyme. Here, the K_m(PEP) was found to be temperature-independent for HL-PK (Fig. 8b), and the Arrhenius plot demonstrated a break at approximately 5°C. However, the lines describing NL-PK activities at saturating and low-concentrations of PEP (Fig. 8a) were not parallel, especially at the thermal extremes (as was consistently found for the other three PKs), and may be curvilinear (see Section D.3). This suggested that the K_m(PEP) for NL-PK was temperature-dependent, and further investigations were carried out to validate this hypothesis.

Fig. 11 (top) has a plot of the PEP saturation curves at various assay temperatures (4° to 40°C) for NL-PK.

Fig. 11 PEP saturation curves (top) and Wilkinson plots (bottom) for NL-PK. The standard assay was used at the various temperatures.


NORMOTHERMIC LIVER
SPECIFIC ACTIVITY of PK
0.25
0.20
0.15
0.10
0.05
0
1.0
2.0
3.0
4.0
5.0
(PEP) mM
40°
34°
28°
22°
16°
10°
4°


NL
WILKENSON
PLOTS
(PEP) /velocity
80
70
60
50
40
30
20
10
-2.0
-1.0
0
1.0
2.0
3.0
4.0
5.0
(PEP) mM
4°
10°
16°
22°
28°
34°
40°

These data were analyzed using Wilkinson plots (Fig. 11, bottom) to calculate the K_m at each temperature. A similar set of experiments (Fig. 12) and calculations were carried out for HL-PK. The following graph (Fig. 13) combines the data obtained from the two series of experiments, except for the one value recorded at -2°C connected by a dashed line which was obtained from the Arrhenius plots in Fig. 8a. Since only two points were used, this calculated value is less reliable, but it does indicate the trend towards negative thermal modulation at low assay temperatures.

The K_m(PEP) for HL-PK (Fig. 13) demonstrates thermal-insensitivity whereas the NL-PK response is one of complex thermal-sensitivity. Similar results have been reported by Hochachka & Somero (1973) for a number of ectothermic enzymes (see DISCUSSION). Also, it should be noted that at all temperatures the K_m(PEP) of the HL-PK is lower than that of NL-PK, this being especially obvious at the thermal extremes (Fig. 13).

The V_{max}/K_m data (Fig. 14) further emphasizes these adaptive strategies. Between 34° - 40°C, which coincides with the T_b range of active bats, the E_a for NL-PK is threefold lower than the E_a for HL-PK. (3.0 vs 11.9 kcal/mole), suggesting rates at low substrate concentrations (see Borgmann & Moon, 1975) are essentially temperature-independent. Therefore, as will be discussed later, within the thermal range of 15 - 40°C, which corresponds with environmental temperatures usually experienced during diurnal torpor, NL-PK can function relatively independent of temperature. Under

Fig. 12 PEP saturation curves as a function of temperature for HL-PK. Conditions were similar to those for Fig. 11.

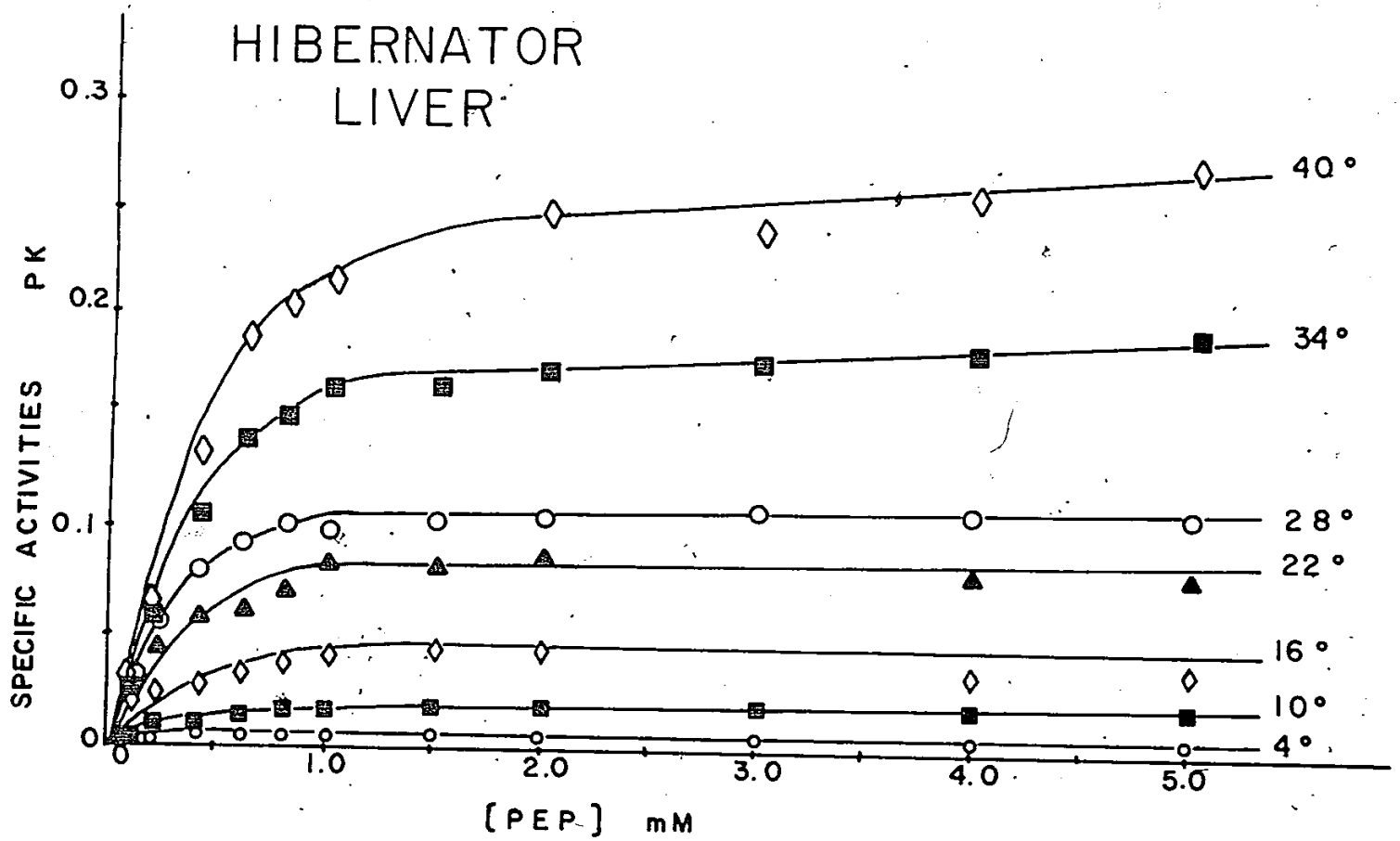

HIBERNATOR
LIVER
SPECIFIC ACTIVITIES PK
0.3
0.2
0.1
0
0
1.0
2.0
3.0
4.0
5.0
[PEP] mM
40°
34°
28°
22°
16°
10°
4°

Fig. 13 K_m(PEP) as a function of temperature for NL- and HL-PK from the bat. Values were determined from Wilkinson plots of Figs. 11a and 12 using the standard assay and varying PEP concentrations. The $-2^{\circ}C$ values (NL-PK) were estimated from Fig. 8a.

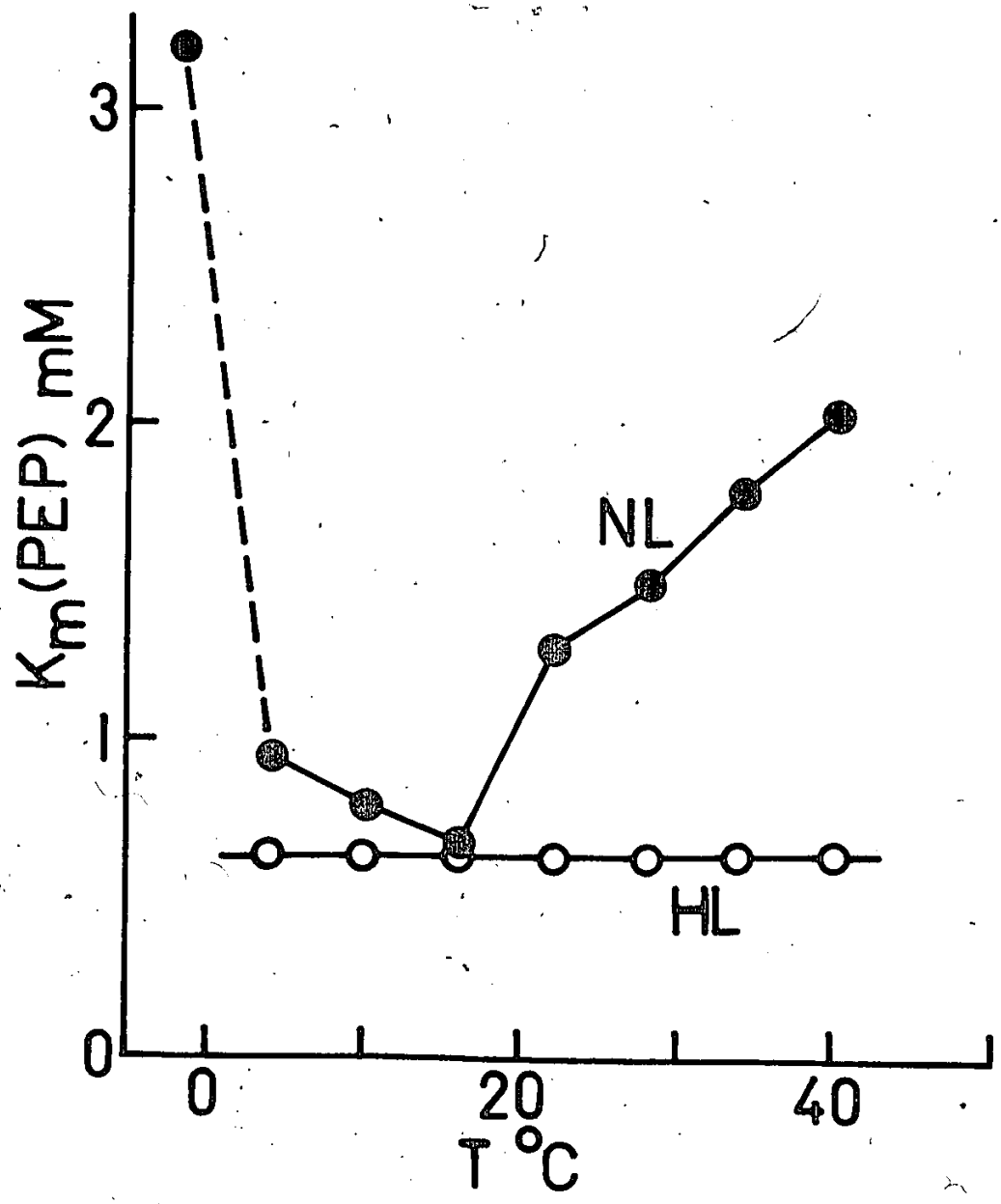
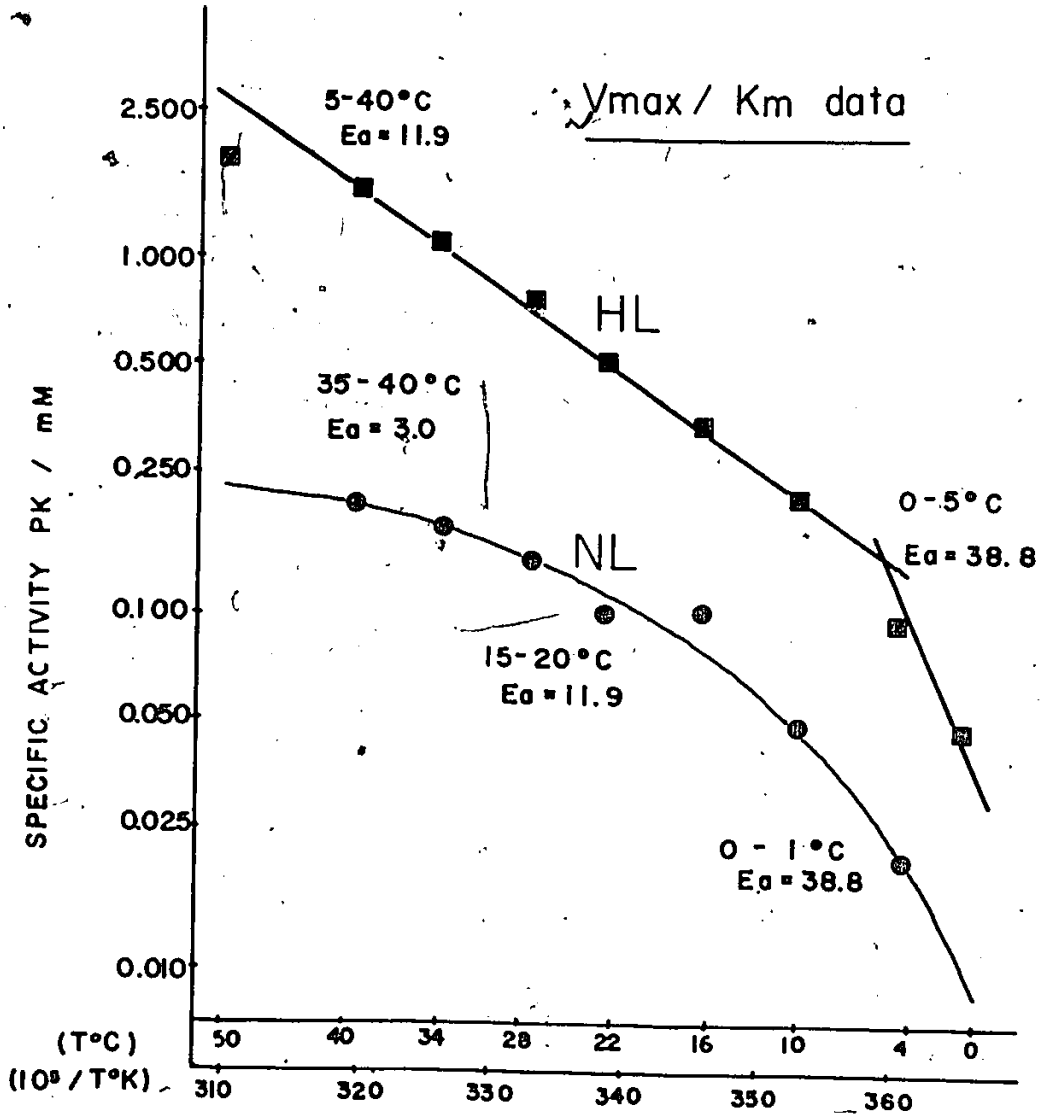

3
2
1
0
K_m(PEP) mM
NL
HL
0
20
40
T °C

Fig. 14 V_{max}/K_m values as a function of temperature for the NL- and HL-PK. Values of V_{max} and K_m were calculated from Wilkinson plots of data in Figs. 11, 12.


Vmax / Km data
5-40°C
Ea = 11.9
HL
35-40°C
Ea = 3.0
0-5°C
Ea = 38.8
NL
15-20°C
Ea = 11.9
0-1°C
Ea = 38.8
SPECIFIC ACTIVITY PK / mM
2.500
1.000
0.500
0.250
0.100
0.050
0.025
0.010
(T°C)
50
40
34
28
22
16
10
4
0
(10³ / T°K)
310
320
330
340
350
360

these conditions, metabolite control of the enzyme is probably of prime importance (see below). Similarily, between 5 and 15°C, which coincides with hibernating T_b for M. lucifugus, the lowest thermal sensitivity is observed for the hibernator enzyme.

As is seen in Fig. 7a, b, the difference between specific activities of NM- and HM-PKs at all temperatures is small. However, from Fig. 8a, b, it is obvious that HL-PK is at least 2.5-fold greater in activity than the corresponding NL-PK; examination of Fig. 14 demonstrates that these differences are exaggerated at low PEP concentrations. These differences result from the marked temperature dependence of the K_m(PEP) of NL-PK, which are not observed in the HL enzyme. Such large differences may indicate an altered function of liver PK during the two physiological states.

3. Correlation Analysis

A computer programme, polynomial curve fitting, using the standard polynomial, $y = ax + b$ (Poly 1, straight line), to compare with the polynomial, $y = ax^2 + bx + c$ (Poly 2, curved line), was employed to weigh the discontinuous against the curvilinear form for the Arrhenius plots of Figs. 7, 8. The statistical correlation of the data points (r^2) for the two equations are recorded in Table 3. In every case, the second polynomial explains more of the random variation than the straight line polynomial, based on the r^2 values. However, upon examining the differences in the correlation of the two polynomials (last column), the greatest difference

Table 3. Correlation analysis of Arrhenius plot data (Figs. 7, 8).

tissue	condition	r^2(Poly 1)*	r^2(Poly 2)	r^2(Poly 2) − r^2(Poly 1)
NM	saturated	0.952	0.996	0.044
	low [PEP]	0.937	0.993	0.056
	low [ADP]	0.952	0.997	0.045
HM	saturated	0.976	0.981	0.005
	low [PEP]	0.961	0.979	0.018
	low [ADP]	0.969	0.994	0.025
NL	saturated	0.968	0.998	0.030
	low [PEP]	0.907	0.973	0.066
	low [ADP]	0.950	0.996	0.046
HL	saturated	0.951	0.996	0.045
	low [PEP]	0.967	0.976	0.009
	low [ADP]	0.986	0.996	0.010

*Poly 1 is the standard polynomial, $y = ax + b$, and describes a straight line; Poly 2 is the curved-line polynomial, $7 = ax^2 + bx + c$; r^2 is the statistical correlation of data as described by one of the equations.

encountered is 6.6%. Perhaps the normothermic tissue Arrhenius plots are better expressed as curved lines. The hibernator tissue Arrhenius plots, on the other hand, are not significantly improved using the second polynomial instead of the first, as indicated by the lower r^2-difference. Because the same conclusions would result whether using polynomials #1 or #2, it was decided to maintain the original lines and emphasize the gross differences between the lines and not the specific form of the individual line.

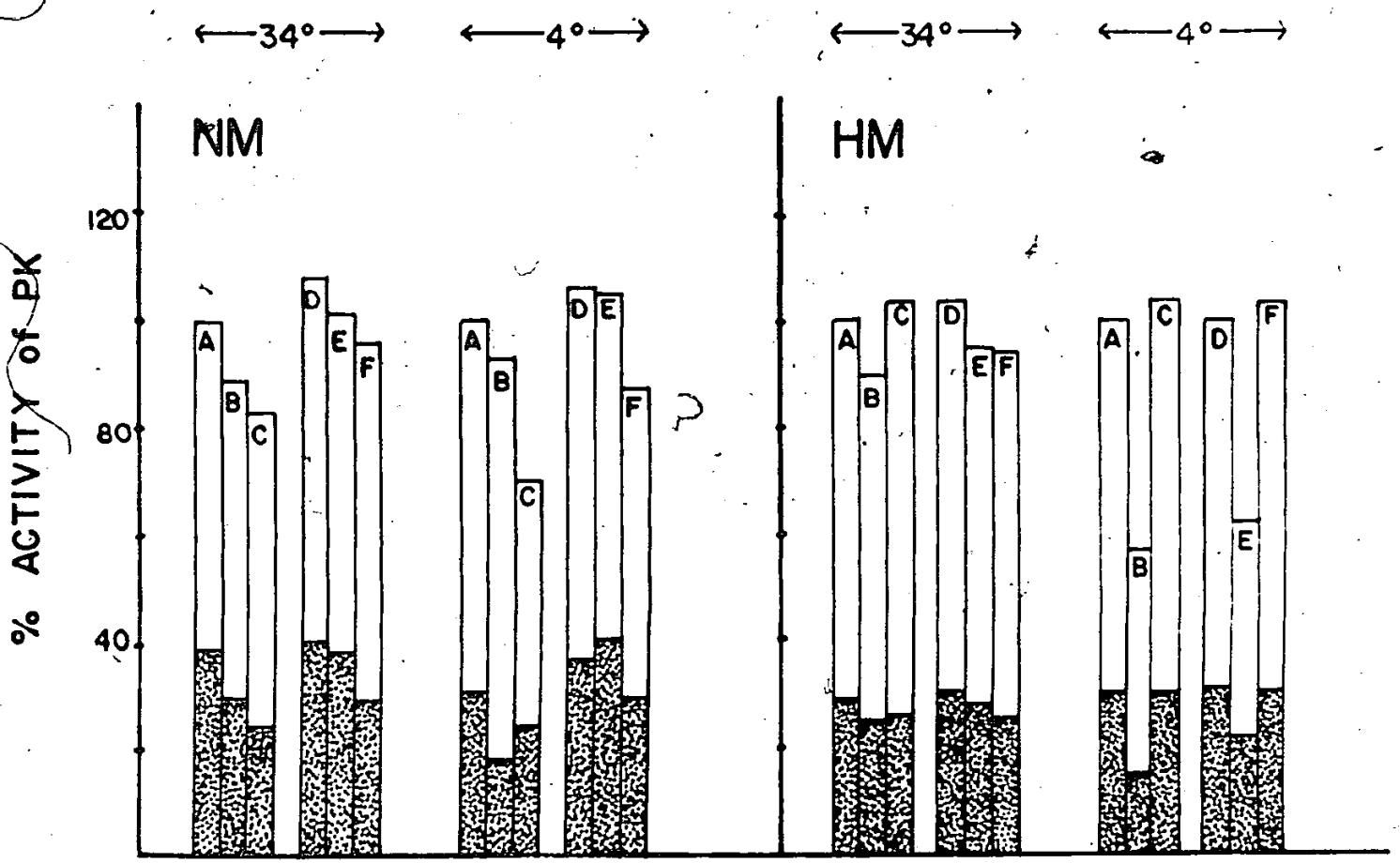
E. Metabolites as Modulators of Bat Pyruvate Kinase

1. Bat muscle PK

Preliminary metabolite studies are reported as a histogram in Fig. 15. As is apparent from these studies, bat muscle PK is much like muscle PK from other mammals, in not demonstrating any significant regulatory characteristics. A few exceptions to this trend can be noted from this histogram. Alanine, at 2 mM, caused a 17% decrease in the NM-PK at 34°C, but not at 4°C, or in the HM-PK at either temperature. The inhibitor ATP appears to have an effect, particularlity at 4°C in the HM enzyme, but little effect elsewhere. At high concentrations of PEP, there was an approximate 10% inhibition at 34°C for both tissues whereas the inhibition increased to approximately 80% at 4°C for HM-PK. These effects were further studied as a function of PEP saturation.

As expected from the above data, the greatest

Fig. 15 Histogram illustrating relative effects of various metabolites under saturating (unprimed letters) and below-K_m (primed letters) concentrations of PEP for NM- and HM-PK. A = control (standard assay); B = influence of 6 mM ATP; C = 2 mM alanine; D = 0.1 mM FDP; E = 6 mM ATP plus 0.1 mM FDP; F = 2 mM alanine plus 0.1 mM FDP.


←34°→
←4°→
←34°→
←4°→
NM
HM
% ACTIVITY of PK
120
80
40
A
B
C
D
E
F

effect of ATP can be seen for the HM-PK at 4°C (Fig. 16). In general, K_m(PEP) is increased without major effects on V_{max}. This is best illustrated in Table 4, expressed as V_{max}/K_m. At 4°C, the activity of HM-PK is only 14% of the control value. There was apparently no differential effect between the two tissues at 34°C when ATP was present, although rates at low PEP concentrations were reduced by 50% in both cases. Since ATP has its greatest effect at 4°C and preferentially in HM-PK, a simple chelation phenomenon, as reported in the literature (Van Berkel, 1974) may not totally explain this activity change. Again, it is important to note that the affinity between the hibernator enzyme and PEP is higher than that for the normothermic enzyme, even with ATP present (Table 4).

At low, and presumably near-physiological, concentrations of PEP (0.2 mM), variations in ATP concentrations resulted in the typical inhibition curves of Fig. 17. This inhibitor appeared to be more effective at low temperatures for both muscle enzymes. At 34°C, the K_i(ATP) values were found to be six times higher than those found at 4°C (Table 5). Furthermore, the lower K_i-values were consistently found in the HM-enzyme, suggesting higher affinities between the inhibitor and the enzyme.

Various FDP concentrations other than 0.1 mM were also tried (data not shown) with no effect except that greater than 1 mM FDP caused inhibition of both NM- and HM-PK activities; this result is consistent with homologous muscle

Fig. 16 The effect of concentration of ATP on NM- and HM-PK at 34° and 4°C. The standard assay was employed except for varying the concentrations of PEP. Symbols: □ HM control; ■ HM + ATP (6 mM); ○ NM control; ● NM + ATP (6 mM).

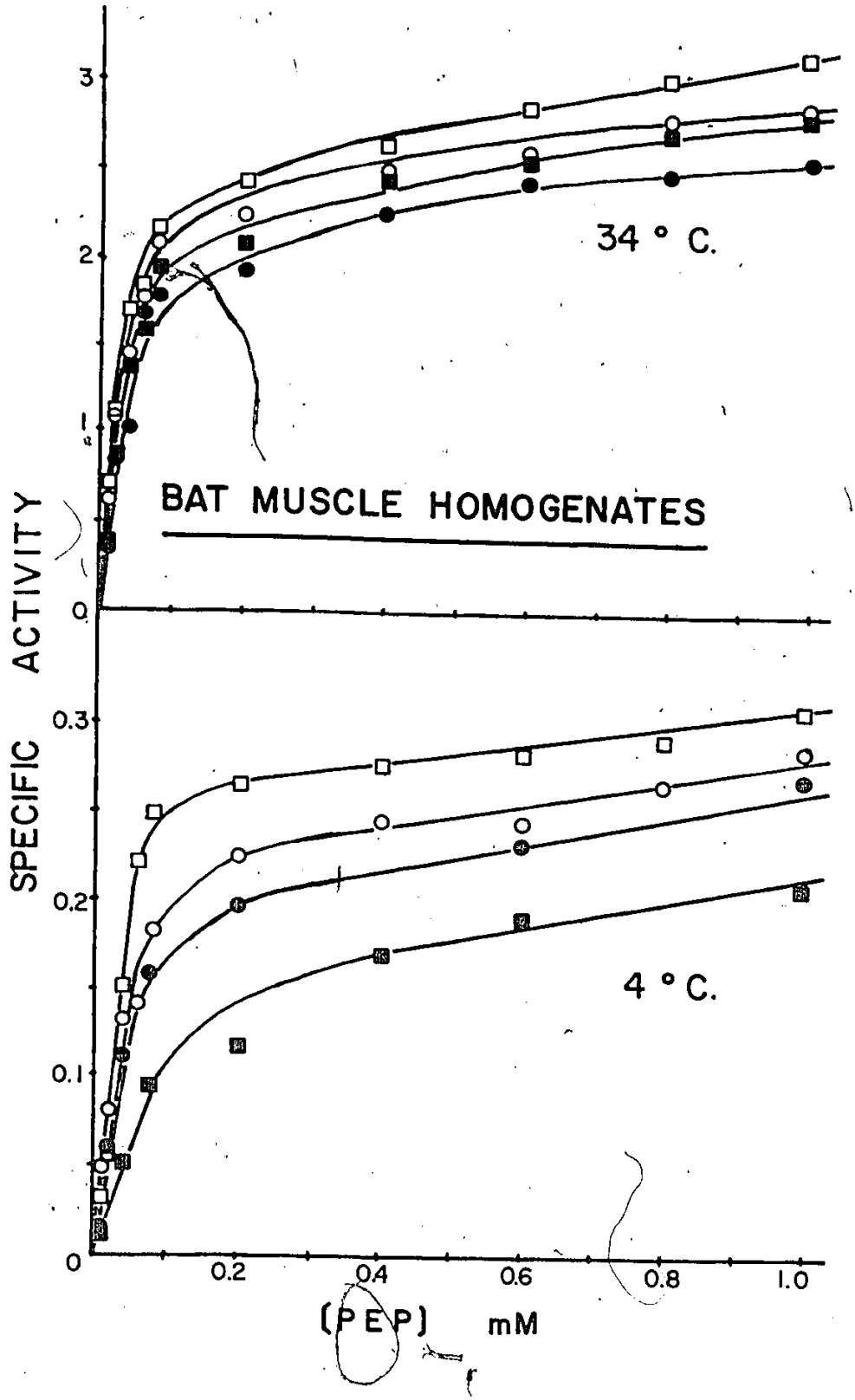

BAT MUSCLE HOMOGENATES
34 ° C.
4 ° C.
SPECIFIC ACTIVITY
(PEP) mM
0
0.1
0.2
0.3
1
2
3
0.2
0.4
0.6
0.8
1.0

Table 4. The effects of 6 mM ATP on the binding of PEP to PK from breast muscle of the bat at 34° and 4°C. K_m(PEP) and V_{max} values estimated from Wilkinson plots.

T(°C)	tissue	condition	K_m(PEP) (mM)	V_{max}	V_{max}/K_m	$\frac{V_{max}/K_m}{control}$
34	NM	control	0.042	3.0	71.5	-
		ATP	0.075	2.7	36.0	0.50
	HM	control	0.030	3.2	106.7	-
		ATP	0.055	3.0	54.5	0.50
4	NM	control	0.052	0.28	5.38	-
		ATP	0.060	0.25	4.20	0.80
	HM	control	0.025	0.28	11.20	-
		ATP	0.145	0.23	1.60	0.14

Fig. 17 ATP inhibition curves at 0.2 mM PEP, 34°C and 4°C, using the standard assay for PK isolated from tissues of M. lucifugus.

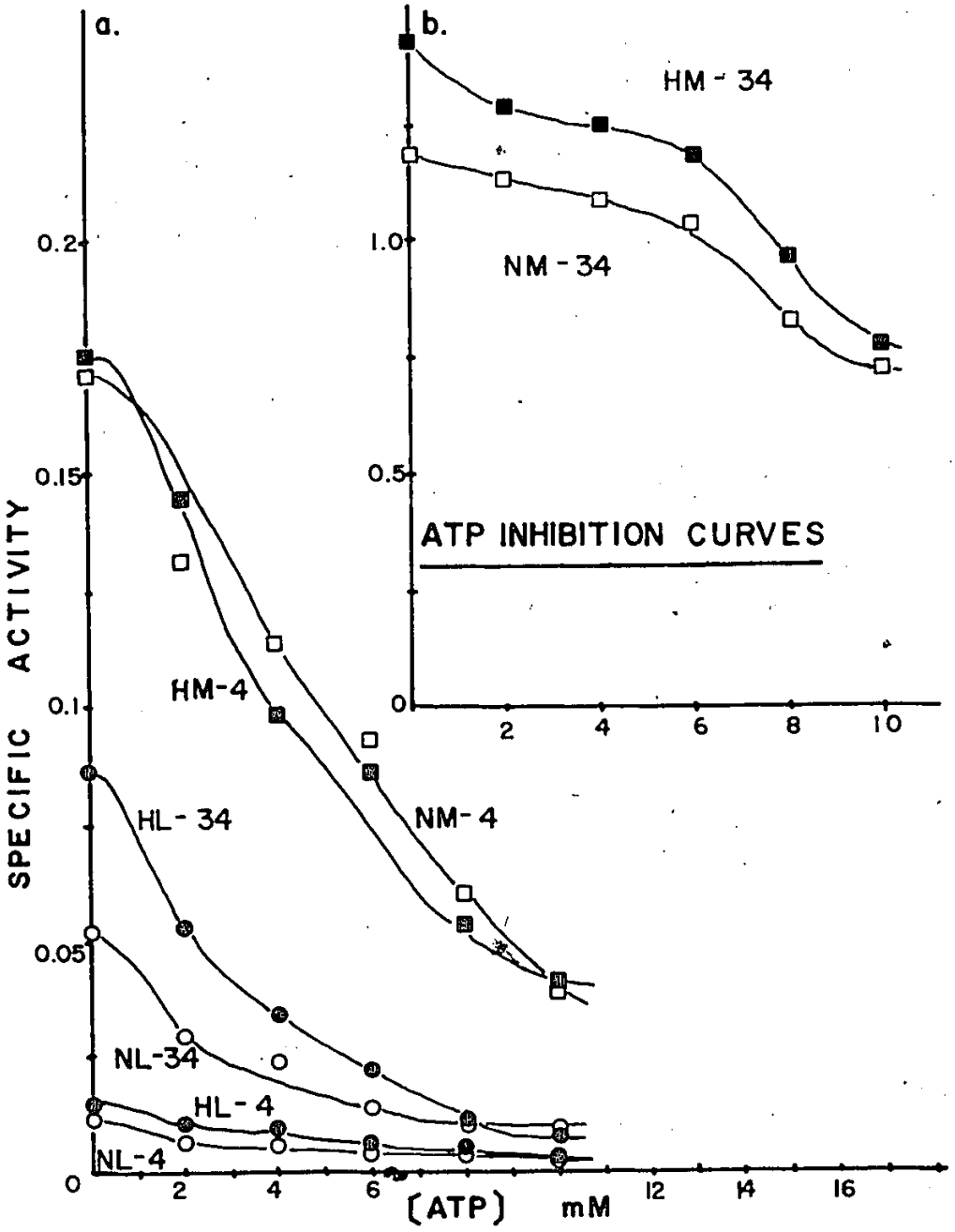

a.
b.
HM - 34
NM - 34
ATP INHIBITION CURVES
SPECIFIC ACTIVITY
0.2
0.15
0.1
0.05
0
1.0
0.5
0
2
4
6
8
10
HM-4
NM-4
HL-34
NL-34
HL-4
NL-4
2
4
6
12
14
16
(ATP) mM

Table 5. Binding affinities of ala, ATP, and FDP to *M. lucifugus* tissue PKs at 34°, 4°C and 0.2 mM PEP. Values estimated from Wilkinson plots.

tissue	K_i(ala) mM	K_i(ATP) mM	K_a(FDP) μM
NM-34	-	33.6	-
HM-34	-	29.4	-
NL-34	1.18	2.2	0.6
HL-34	0.72	2.6	0.2
NM-4	-	6.8	-
HM-4	-	4.2	-
NL-4	0.54	4.3	7.7
HL-4	0.20	4.4	2.4

PKs from other mammals (Seubert & Schoner, 1971).

The effect of alanine can be seen in Fig. 18; even at the very high concentration of 10 mM there was no observable alanine effect.

Therefore, in terms of metabolite interaction, bat muscle PK is very similar to other mammalian muscle homologs. However, the differential effects of ATP at 4°C and in the HM-enzyme may indicate some regulatory control over this enzyme, at least in vitro.

2. Bat liver PK

At low concentrations of PEP (0.2 mM), 0.1 mM FDP had a marked effect, causing greater than a three-fold increase in both NL- and HL-PK activities (Fig. 19). Note also from this figure that FDP exerted a stronger influence in the HL- than the NL-enzyme at 4°C and at both high (2 mM) and low (0.2 mM) concentrations of PEP. At a saturating concentration of PEP and 34°C, the HL- and NL-PK responded similarily to FDP, although not at low (0.2 mM) PEP concentrations.

When FDP was added in the presence of inhibitors (ATP or alanine), in all cases the inhibition was reversed, and at low PEP concentrations the inhibition was overcome sufficiently to allow strong activation. Once again, especially in the latter case (i.e., alanine inhibition), the HL-PK appeared to be most strongly modulated by FDP in the presence of either inhibitor and at both temperatures. As an example, at 4°C, there is a difference of 83% between the

Fig. 18 Alanine inhibition curves. Conditions similar to those for Fig. 17.

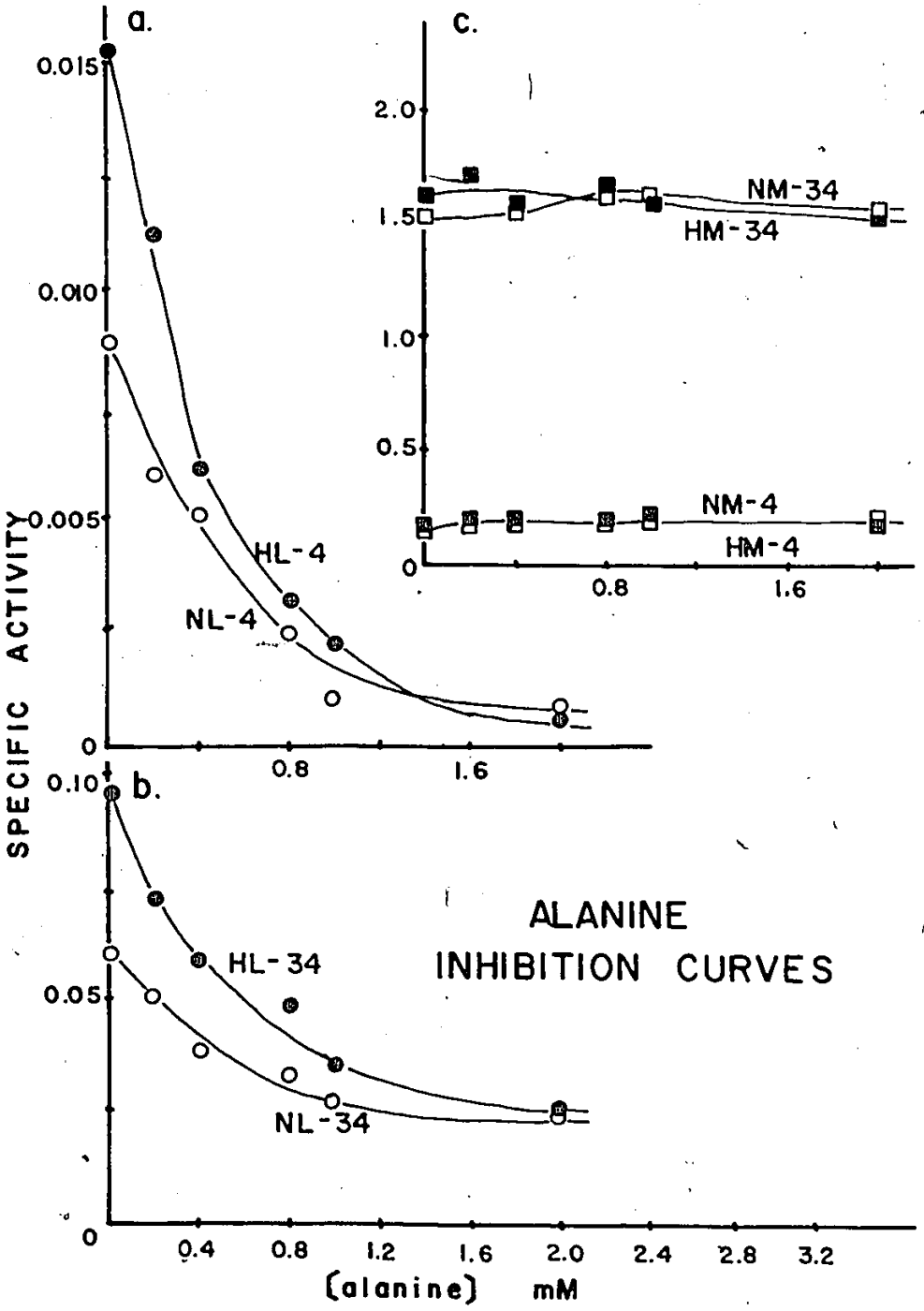
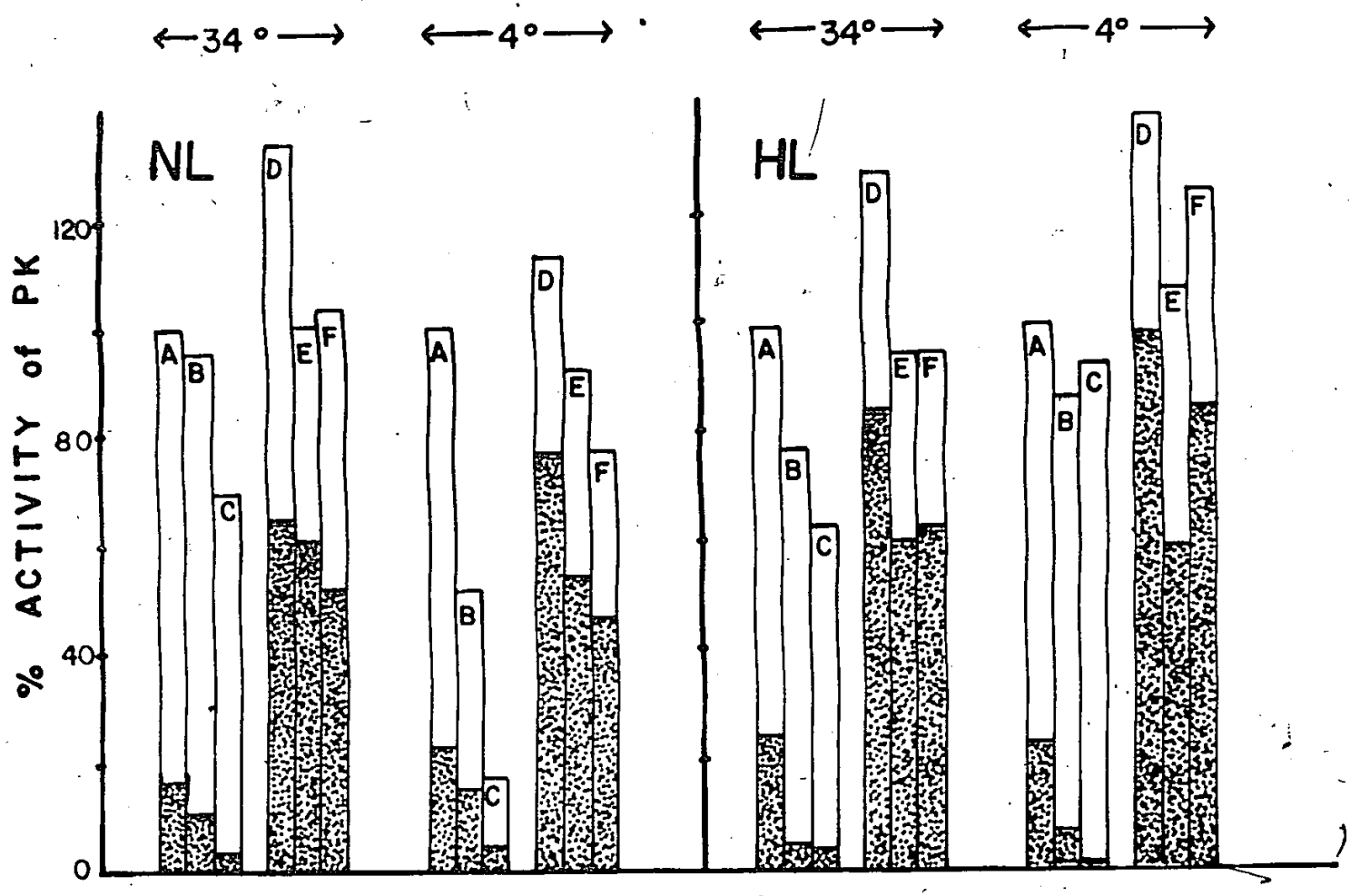

a.
0.015
0.010
0.005
0
HL-4
NL-4
0.8
1.6
c.
2.0
1.5
1.0
0.5
0
NM-34
HM-34
NM-4
HM-4
0.8
1.6
SPECIFIC ACTIVITY
b.
0.10
0.05
0
HL-34
NL-34
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
(alanine) mM
ALANINE
INHIBITION CURVES

Fig. 19 Histogram illustrating relative effects of various metabolites under saturating-(unprimed letters) and below-K_m (primed letters) concentrations of PEP for NL- and HL-PK. A = control (standard assay); B = standard assay plus 4 mM ATP; C = 2 mM alanine; D = 0.1 mM FDP; E = 4 mM ATP plus 0.1 mM FDP; F = 2 mM alanine plus 0.1 mM FDP.


←34°→
←4°→
←34°→
←4°→
NL
HL
% ACTIVITY of PK
120
80
40
0
A
B
C
D
E
F

alanine and alanine-plus-FDP activities for HL-PK whereas for NL-PK, the difference is 41%

ATP exerted its greatest effect at low concentrations of PEP as well, with a 1.5-fold decrease in NL-PK activity at a 4- to 5-fold decrease in HL-PK activity, regardless of the temperature (Fig. 19). An exception to this trend is at high concentrations of PEP for the NL-PK at 4°C; activities are reduced by approximately 50%, which might indicate a strong modulatory action by ATP. For alanine, a five-fold decrease was noted at high concentrations of PEP and 4°C for NL-PK (versus no significant decrease in HL-PK under similar conditions) and at both temperatures when a low concentration of PEP was employed. These differences are marked and may indicate a strong differential control over the two enzyme types. In the latter case (low PEP concentrations), this inhibitor has its greatest effect on the HL-PK; i.e., a seven-fold decrease at 34°C and a 19-fold decrease at 4°C.

As is obvious from the histogram (Fig. 19), the modulation by FDP, ATP and alanine are more significant in the control of liver PK than the muscle enzyme. All three metabolites modulate the enzyme activity in liver very strongly. A better understanding of these modulator functions were obtained through experiments involving: a. variation in substrate concentrations at constant modulator concentrations: $[FDP] = 0.1$ mM, $[ATP] = 4$ mM, and $[alanine] = 2$ mM (Figs. 20, 21), and b. variation in modulator concentrations at a constant substrate concentration: $[PEP] = 0.5$ mM (Figs. 17, 18, 22).

a. PEP saturation kinetics in the presence of modulators

Table 6 gives a list of K_m(PEP) or $S_{0.5}$(PEP) (i.e., the inverse of the apparent PK-PEP affinity), V_{max}, and n_H (Hill coefficients) values as calculated from Figs. 20, 21, using both Wilkinson and Hill plots. A plot of log $[v/(V_{max} - v)]$ against log [substrate] has a slope of n_H and such a plot is commonly known as a Hill plot, having first been used by A.V. Hill in his studies of the binding of O_2 to hemoglobin (Laidler & Bunting, 1973, p. 356). If $n_H = 1$, the curve is hyperbolic and obeys Michaelis-Menten kinetics; the greater the value of n_H, the more sigmoidal the curve (White et al., 1968, p. 762).

PEP saturation kinetics are complex, in that hyperbolic kinetics (reflected by FDP curves) and sigmoidal kinetics (reflected by alanine or ATP curves) are apparent depending upon the modulator present. In the absence of a modulator (control), the curves were somewhat sigmoidal (n_H = 1.1 to 1.2, Table 6). FDP noticeably increased the apparent PK-PEP affinity in the presence and absence of inhibitors (Figs. 20, 21 and Table 6), and at the same time abolished the sigmoidal nature of the PEP saturation curves as reflected by n_H-values which approached unity in the presence of this allosteric activator (Table 6). As an example of the FDP effect on the E-S affinity, in NL at 34°C (NL-34), the addition of FDP caused a 5-fold drop in $S_{0.5}$(PEP) from the control value (1.20 to 0.25 mM), without any change in V_{max}. The effect of an increased apparent PK-PEP affinity and no change in V_{max} resulted in more than a five-

Table 6. The effects of the heterotropic agents, ATP, ala and FDP, on the homotropic binding of PEP to bat liver PK. K_m- and V_{max}-values were estimated from Wilkinson plots, and n_H- and $S_{0.5}$-values from Hill plots. Concentrations: alanine, 2 mM; ATP, 4 mM; FDP, 0.1 mM.

tissue	condition	n_H	K_m(PEP)*	V_{max}	V_{max}/K_m
NL-34	control	1.1	1.20	0.23	0.19
	alanine	2.4	1.50	0.16	(0.11)**
	ATP	1.8	1.30	0.20	(0.15)
	FDP	1.0	0.25	0.24	0.95
	ala + FDP	1.0	0.35	0.18	0.50
	ATP + FDP	1.0	0.31	0.20	0.65
NL-4	control	1.2	0.64	0.019	0.03
	alanine	1.4	0.80	0.004	(0.01)
	ATP	1.8	0.75	0.012	(0.02)
	FDP	1.0	0.12	0.020	0.17
	ala + FDP	1.0	0.16	0.013	0.08
	ATP + FDP	1.0	0.15	0.015	0.10
HL-34	control	1.2	0.63	0.40	0.64
	alanine	3.9	1.25	0.25	(0.20)
	ATP	2.1	0.92	0.31	(0.34)
	FDP	1.0	0.11	0.43	3.86
	ala + FDP	1.0	0.13	0.33	2.56
	ATP + FDP	1.0	0.13	0.31	2.40
HL-4	control	1.2	0.63	0.021	0.03
	alanine	2.3	1.31	0.019	(0.02)
	ATP	2.1	0.92	0.019	(0.02)
	FDP	1.0	0.09	0.025	0.28
	ala + FDP	1.0	0.10	0.022	0.22
	ATP + FDP	1.0	0.11	0.019	0.18

*K_m(PEP) = $S_{0.5}$(PEP) when $n_H > 1.0$.

**Values in parentheses represent an upper limit and are used only as relative comparisons. The higher the n_H-value, the greater the difference between the rate at low substrate concentrations and V_{max}/K_m.

Fig. 20 Effects of metabolites on NL-PK at 34°C (20A, 20B), 4°C (20C, 20D) and various concentrations of PEP. Concentrations of metabolites: 0.1 mM FDP; 4 mM ATP; 2 mM alanine.

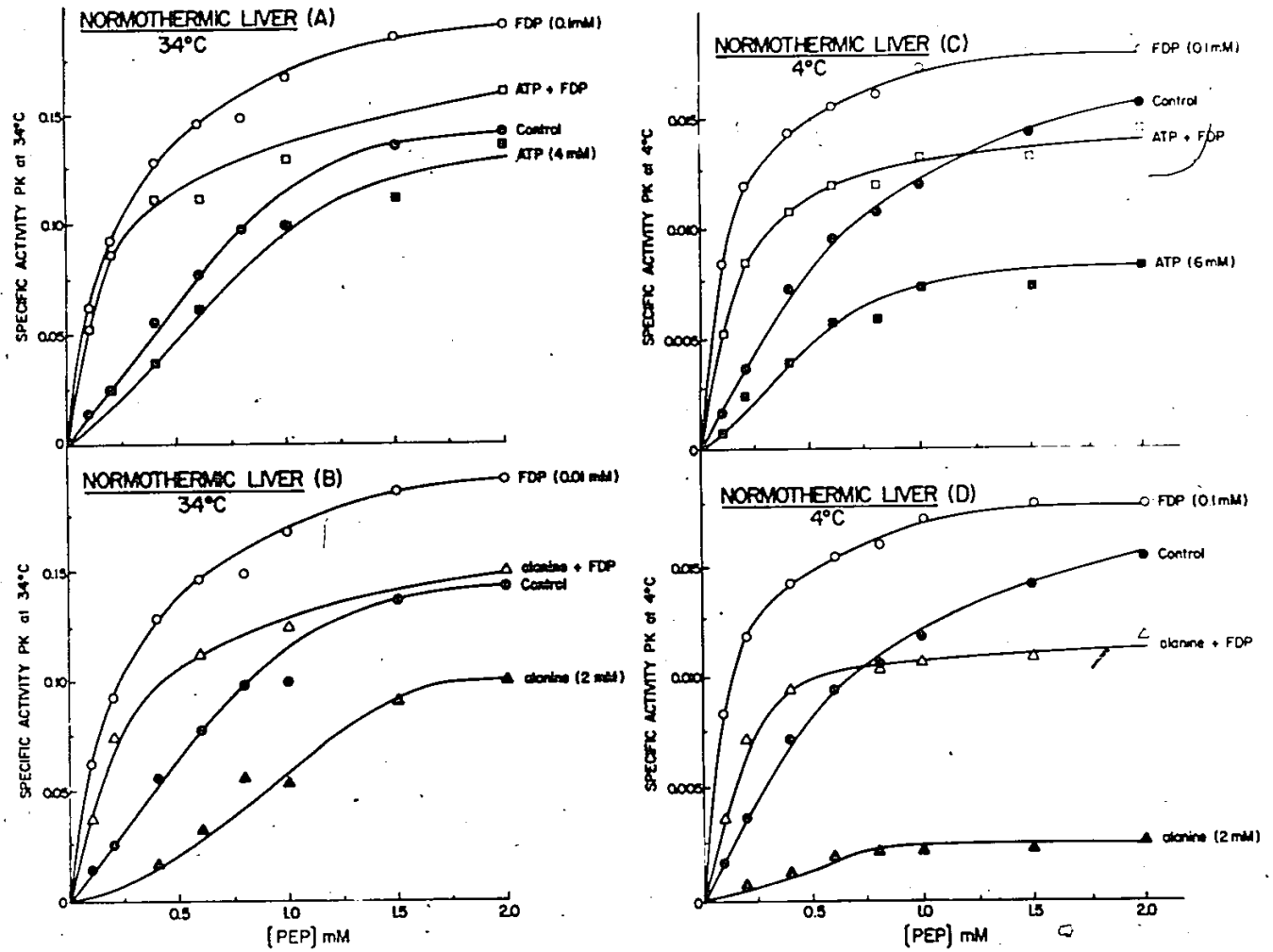
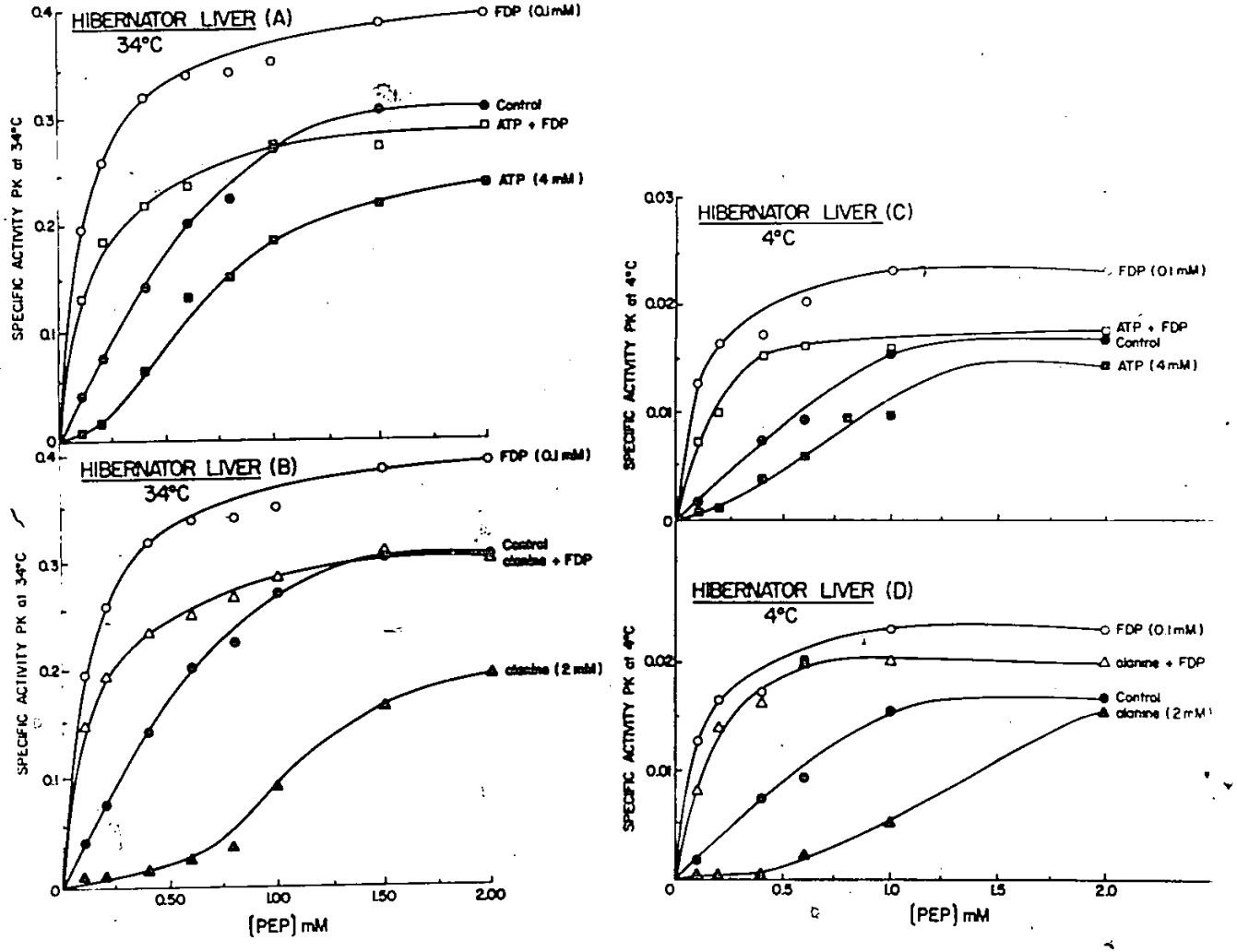

NORMOTHERMIC LIVER (A)
34°C
FDP (0.1mM)
ATP + FDP
Control
ATP (4 mM)
SPECIFIC ACTIVITY PK at 34°C
NORMOTHERMIC LIVER (B)
34°C
FDP (0.01 mM)
alanine + FDP
Control
alanine (2 mM)
SPECIFIC ACTIVITY PK at 34°C
[PEP] mM
NORMOTHERMIC LIVER (C)
4°C
FDP (0.1mM)
Control
ATP + FDP
ATP (6 mM)
SPECIFIC ACTIVITY PK at 4°C
NORMOTHERMIC LIVER (D)
4°C
FDP (0.1mM)
Control
alanine + FDP
alanine (2 mM)
SPECIFIC ACTIVITY PK at 4°C
[PEP] mM

Fig. 21 Effects of metabolites on HL-PK at 34°C (21A, 21B), 4°C (21C, 21D) and various concentrations of PEP. Concentrations of metabolites: 0.1 mM FDP; 4 mM ATP; 2 mM alanine.


HIBERNATOR LIVER (A)
34°C
FDP (0.1 mM)
Control
ATP + FDP
ATP (4 mM)
SPECIFIC ACTIVITY PK at 34°C
HIBERNATOR LIVER (B)
34°C
FDP (0.1 mM)
Control
alanine + FDP
alanine (2 mM)
SPECIFIC ACTIVITY PK at 34°C
0.50
1.00
1.50
2.00
[PEP] mM
HIBERNATOR LIVER (C)
4°C
FDP (0.1 mM)
ATP + FDP
Control
ATP (4 mM)
SPECIFIC ACTIVITY PK at 4°C
HIBERNATOR LIVER (D)
4°C
FDP (0.1 mM)
alanine + FDP
Control
alanine (2 mM)
SPECIFIC ACTIVITY PK at 4°C
0.5
1.0
1.5
2.0
[PEP] mM

fold increase in V_{max}/K_m as compared to the control (>0.19 vs 0.95) or greater than a five-fold increase in activities at low concentrations of PEP. In the HL-34, the allosteric activation was greater; FDP increased the apparent PK-PEP affinity of the control from 0.63 to 0.11 with a concomittent greater than 6-fold rise in V_{max}/K_m. The lower temperature acted synergistically with this activator such that the HL-PK showed the greatest response of all, a greater than 9-fold increase in V_{max}/K_m over the control. In no case is there a significant effect of FDP on V_{max}.

Inhibition by ATP affected the apparent PK-PEP affinity more so than the V_{max} (Table 6 and Figs. 20, 21). At 34°C, ATP caused a slight increase in $S_{0.5}$(PEP) from 1.2 to 1.3 mM in NL (Table 6) while causing a 1½-fold increase from the control in HL (0.63 to 0.92 mM). At the lower temperature, the effect of ATP was slightly increased in the NL-enzyme, but the HL-PK did not differ from the values reported at 34°C.

Both V_{max} and the apparent PK-PEP affinity were affected more by alanine than ATP (Table 6 and Figs. 20, 21). At both temperatures, alanine decreased the apparent PK-PEP affinity in the normothermic liver, and n_H-values (see Table 6) decreased as temperature decreased, suggesting a complex allosteric effect of this metabolite. The greatest effect of alanine was observed for HL-PK where at both temperatures the $S_{0.5}$-values of the control were doubled (0.63 to 1.25 mM; 0.63 to 1.31 mM, Table 6). The n_H-value decreased with decreasing temperature as above.

Therefore, as in other mammalian liver PKs, the bat liver enzyme is a regulatory enzyme, its activity being modified by ATP, alanine and FDP. In most cases, the HL-PK was under tighter allosteric control, yet at the same time demonstrating a higher apparent PK-PEP affinity than seen for the NL-enzyme. In all cases, FDP reversed the inhibitory nature of ATP and alanine, but did not significantly activate at V_{max}.

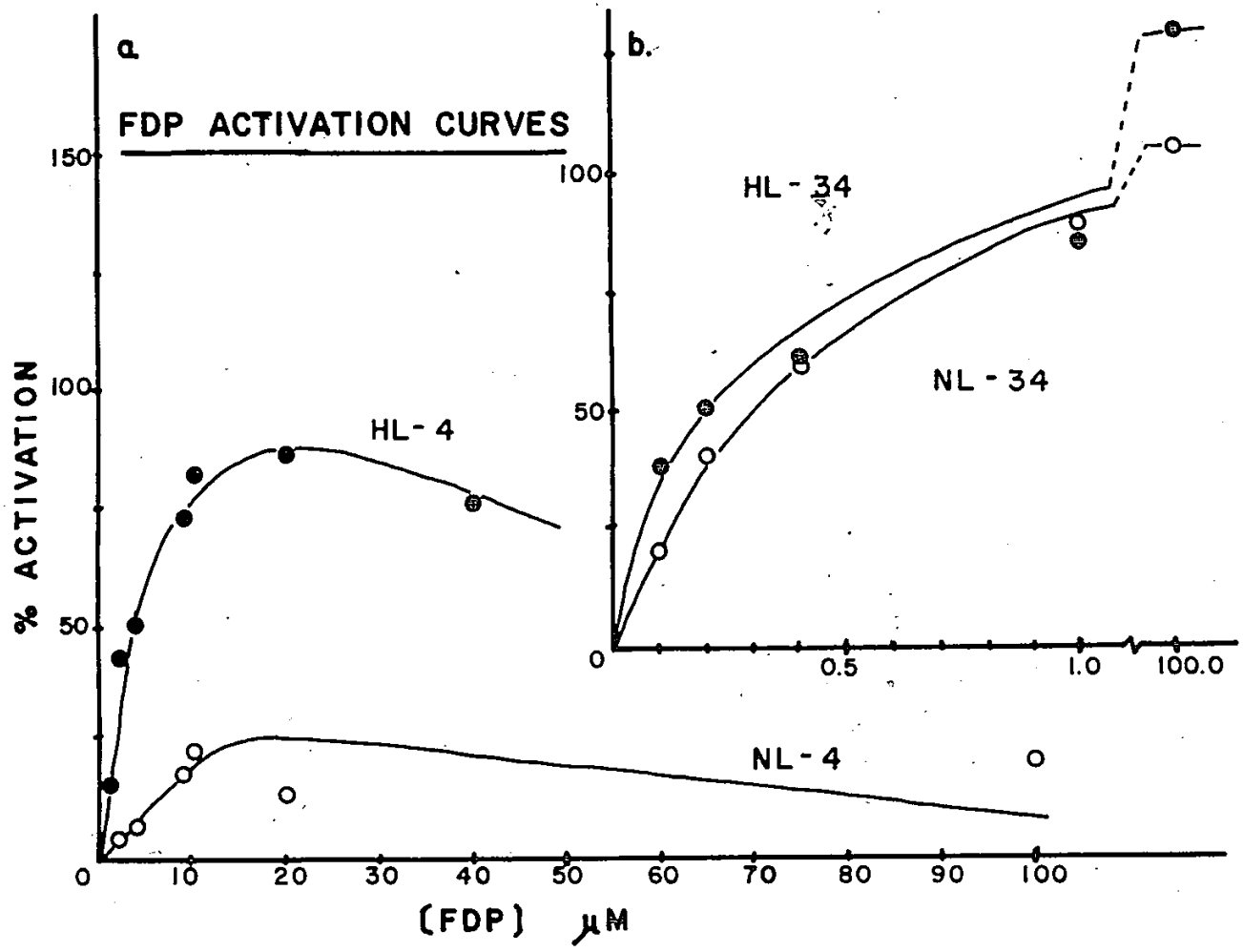
b. K_a and K_i values for the liver enzymes

At 34°C, 0.1 mM FDP appeared to cause maximal activation of liver PK (Fig. 22). The higher temperature increased the sensitivity of PK to this modulator as can be seen in Table 5, and Fig. 22, with K_a(FDP) values being 10-fold lower at 4°C compared to 34°C. Also the K_a(FDP) values are three times lower for the HL-enzyme at both temperatures. As noted in Fig. 22, maximal PK activity at 4°C occurred at about 20 μM FDP with subsequent inhibition at higher FDP concentrations; note that at this concentration, the HL-enzyme is enhanced approximately four times greater than the NL-enzyme.

As with FDP, ATP exerted its strongest influence at the higher temperature (Fig. 17); although this inhibitor bound to both forms of PK equally at both assay temperatures (Table 5).

In the case of alanine, binding to PK was enhanced at the lower temperature in contrast to the other two modulators (Fig. 18 and Table 5). Again, as with ATP, the HL-PK showed the strongest binding with the K_i(ala) values about

Fig. 22 FDP enhancement curves for liver PK of normothermic and hibernating bats as a function of temperature (34°C and 4°C). Standard assay was used.


a
FDP ACTIVATION CURVES
% ACTIVATION
150
100
50
0
HL-4
NL-4
10
20
30
40
50
60
70
80
90
100
(FDP) μM
b.
100
50
0
HL-34
NL-34
0.5
1.0
100.0

half those found for NL-PK (0.20 versus 0.54 mM and 0.72 versus 1.18 mM, Table 5).

Therefore, bat liver PK is a regulatory enzyme of the same type demonstrated for other mammalian species. Here, however, the HL-enzyme is more sensitive to both positive and negative modulators compared to NL-PK. These characteristics are undoubtedly of some significance to the functioning of the enzyme under each of these physiological conditions.

DISCUSSION

DISCUSSION

In an attempt to understand the molecular strategies involved in hibernation, the effects of temperature and modulators on breast muscle and liver PK of Myotis lucifugus have been investigated. Definite differences have been found between PK from bats in the hibernating and normothermic states in terms of A. isozymic patterns and specific activity, B. E_a or Arrhenius values, T_c-values, and K_m-values, and C. FDP activation, and ATP and alanine inhibition.

A. Isozymic Patterns and Specific Activity

1. Isozymic patterns

At least six mammalian (Seubert & Schoner, 1971, Whittell et al., 1973) and five lower vertebrate (Schloen et al., 1974) PK isozymes have been reported. In the absence of quantitative studies and because isozymic patterns change as a function of dietary and hormonal state (Seubert & Schoner, 1971) and developmental state (Schloen et al., 1974) of the organism, it is impossible to classify the five (in liver) to ten (in muscle) electrophoretically distinct forms of bat PK observed in this study (Fig. 4), or assign cause-and-effect to the changes which are a function of the physiological state of the bat. What may be gleaned from these qualitative changes is perhaps a partial explanation for the observed kinetic differences between bat PKs. The possible importance of multiple molecular forms of enzymes

in hibernation has been discussed for LDH in bats (Brush, 1968; Moon, 1976) and ground squirrels (Burlington & Sampson, 1968), and PK in Arctic ground squirrels (Behrisch, 1974).

2. Specific activity

The specific activities of some enzymes have been found to be lower in the heterothermal state compared to the homeothermal state of a hibernant (Chaffee et al., 1961; Daudova & Stepanova, 1966; Zimny & Moreland, 1968; Brabec & McColloch, 1970; Moon, 1976); some have been found to be similar in the two states, particularily in heart homogenates (Burlington & Klain, 1967; Brabec & McColloch, 1970; Moon, 1976); while still others have been found to be higher (Burlington & Klain, 1967; Burlington & Sampson, 1968; Atherton & Zimmerman, 1974; Fang & Willis, 1974). One possible explanation for differences in enzyme activities between hibernator and normothermic tissues could be related to tissue activities, per se (Moon, 1976). Essentially, a tissue which maintains a high activity during the hibernation period (e.g., heart) would also have a high activity during normothermia, whereas the tissue with a lower activity (e.g., liver) would have an even greater reduction in its hibernation activity pattern. Tissues such as pectoral muscle used for locomotion would be totally inactive with hibernation. Another explanation for higher specific activities during hibernation relative to normothermia could be seasonal induction where these enzymes are specifically synthesized prior to hibernation (Bowler & Duncan, 1969). In M. lucifugus,

PK activities are enhanced during hibernation (see Figs. 6, 7, 8). Since hibernation in the bat is essentially a state of chronic starvation, a decline in liver PK activities would have been predicted based on the 60% decline recorded by Weber (1969) in the starved rat liver.

The importance of maintaining PK activity during hibernation, therefore, might be related to at least two processes: 1. the switch from lipid oxidation to anaerobic carbohydrate utilization known to occur with arousal (Lyman, 1970; Burlington, 1972) which would necessitate the presence of an active PK, and/or 2. are involved in a futile cycle (energy-wasting process) to generate heat, a hypothesis which could explain the ability of hibernating bats to maintain their T_b slightly above the T_a. This heat would be in addition to the insulative effect of tissues and pelage. Such pyruvate recycling has been reported by a number of researchers (Friedmann et al., 1971; Rognstad & Katz, 1972; Friedrichs & Schoner, 1974) in the tissues of rats but it is not known if cycling also occurs in bat tissues or any tissue of hibernants. This cycling is indirectly referred to in Behrisch's (1974) discussion of the possible importance of the decrease in the affinity of HL-PK in the Arctic ground squirrel to the allosteric inhibitor, ATP. He states, "the desensitization of a key enzyme ... to its major inhibitor may be an efficient way of ensuring the continued evolution of small amounts of heat during the deep hibernation state ...".

B. Temperature as a Modulator of Pyruvate Kinase

1. E_a or Arrhenius values

When the logarithm of velocity is plotted against the reciprocal of the absolute temperature, most biological reactions give a straight line, the slope of which is $-E_a/4.6$, where E_a is the activation energy and 4.6 is 2.303 (the conversion factor from natural to base 10 logarithms) multiplied by the gas constant, R (1.98 cal/deg-mole).

As noted in Figs. 7, 8, 14, the predominant form of PK found in a particular physiological state is the one with the lowest thermal sensitivity (as indicated by a reduced E_a-value) over the range of temperatures associated with that state. Catalytic efficiencies of enzymes of different thermally-adapted animals have previously been compared with the Arrhenius activation energy, E_a, as the index of the capacity of an enzyme to reduce the energy barrier of a reaction (referred to as $\Delta G^{\ddagger}$, or the free energy of activation). Due to the complex thermodynamic parameters implicit in the expression of catalytic efficiency, the equating of E_a-values with this term has been criticized (Hochachka & Somero, 1973). In this study, however, E_a-values are used as comparative indicators of temperature sensitivity; a lower E_a-value indicates a reduced slope on an Arrhenius plot which in turn suggests a smaller change in the reaction rate for a given change in temperature. In a study of rat and frog liver succinic dehydrogenases where the enzyme of the ectotherm was found to have the reduced slope, Vroman &

Brown (1963) concluded that the level of the frog enzyme activity would be maintained relatively constant in the face of the small T_a changes encountered by this ectotherm, a property not necessary for the rat succinic dehydrogenase which is always found in a constant temperature environment. However, in two recent studies, no correlation was found between E_a-values for either LDH (Olsson, 1975; Moon, 1976) or malate dehydrogenase (Olsson, 1975) and the hibernating and non-hibernating condition, as has been observed in this present study. Unlike PK, LDH and MDH are not regulatory enzymes and hence there may be less selective pressure for changes in the values of temperature characteristics as a function of the state of hibernation and normothermia.

According to Davis & Reite (1967), changes in T_a alone over a broad range do not induce arousal in bats. However, a small rise in T_a (at the lower thermal extreme) during hibernation could cause a large increase in the activity of PK if only the normothermic form were present (as indicated by the larger E_a-values at lower temperatures of NM-PK and NL-PK, Figs. 7, 8, 14), and arousal might be carried to completion (Lyman, 1970). In bats, however, the driving forces which cause arousal sometimes quite suddenly come to a halt; Hayward & Lyman (1967) recorded a 4°C rise in interscapular temperature of E. fuscus and a fall of the same amount in a matter of 6 minutes. Therefore, at least in the Chiroptero, temperature-insensitivity of key enzymes in hibernators at the lower thermal extreme would allow other

modulators to halt the unnecessary and costly process of arousal in the face of small temperature rises.

The NM-PK of M. lucifugus activation energies (Fig. 7a) compare favourably with those reported for homeothermic rat muscle PK (Somero & Hochachka, 1968) where above the critical temperature (in the rat, T_c = 25°C), E_a = 10 kcal/mole and below T_c, E_a = 20 kcal/mole. For the muscle PK of the zooarcid and the king crab, activation energies of 15 and 12 kcal/mole respectively, in the temperature range of 5 to 20°C were reported (Somero, 1969a). The breast muscle enzyme of the little brown bat during the state of hibernation (HM-PK; Fig. 7b) with an activation energy of 13.7 kcal/mole (5 to 40°C) is within the range of E_a-values reported for these two poikilotherms.

Just as a reduced E_a for V_{max} indicates a lower temperature sensitivity of the enzyme-catalysed reaction at high substrate concentrations, a smaller temperature dependence in V_{max}/K_m would also indicate less thermal sensitivity of the reaction at low substrate concentrations which can be used as further evidence for adaptation to a fluctuating or low absolute T_b (Borgmann & Moon, 1975). Although infrequently estimated in the literature, three papers pertinent to this study use V_{max}/K_m for interspecifically differentiating enzyme properties of cold and warm-blooded animals: AMP-aminohydrolase of hen and carp muscle (Zydowo & Purzycka-Preis, 1972); adenosine deaminases of chicken and bay scallop tissues (Harbison & Fisher, 1973a); LDH of beef and flounder muscle (Borgmann & Moon, 1975). In all cases, the E_a of

V_{max}/K_m was lowest in the poikilotherm, especially at temperatures within the normal habitat range. In this study, the E_a of V_{max}/K_m was found to be lowest for PK of the normothermic liver in the temperature range of 25° to 40°C and for the hibernator at temperatures optimal for hibernation (3° to 10°C) (Fig. 14). The E_a of V_{max}/K_m for both muscle and liver PKs tends to mimic those found for the E_a of the V_{max}-values (see above). In addition, it is possible that thermal sensitivity may be reduced in exchange for modulator control. For example, below 17°C, HL-PK is temperature-insensitive but appears to be more sensitive to the modulators FDP and alanine than NL-PK (see Section C.2).

White fat which represents the largest portion of stored fat energy in bats, and brown adipose tissue which is specialized for heat production (Henshaw, 1970), must be deposited in the summer and fall for their subsequent utilization during hibernation and arousal. A thermally insensitive liver PK (such as is NL-PK, more so than HL-PK) would continue to be metabolically active during periods of diurnal torpor and thus the synthesis of lipid precursors would not be curtailed at this time, a definite advantage to the bat. Further, during the homeothermal period, positive modulators other than temperature could possibly be enhancing the storage of various metabolites (even though modulation of NL-PK by FDP and alanine is not as effective as that of HL-PK, their control over NL-PK would be more specific than that by temperature). Indeed, this would be especially important for juvenile bats (weighing significantly less prior to

hibernation than adults: Ewing et al., 1970; Fenton, 1970) about to experience their first winter since they do not have as much time to accumulate fat as do adults and they may be less efficient at catching prey (Gould, 1955).

2. T_c-values

Sharp breaks in Arrhenius plots have been observed for mitochondrial enzymes in a large number of organisms, including hibernating species (Raison & Lyons, 1971), and are probably due to lipid-based changes involving liquid-to-solid phase transitions (Hochachka & Somero, 1973), or lipid cluster formation (Lee et al., 1974; Wunderlich et al., 1975). Breaks have been reported for PK of the rat, and the fish Trematomus (Somero & Hochachka, 1968), and rabbit muscle PK (Kayne & Suelter, 1968). Since PK is not known to be bound to membranes, the breaks observed here and in other PK studies could be caused by protein conformational changes (Kayne & Suelter, 1968), and/or lipid effects, but not lipid phase changes. PK is known to be inhibited by free fatty acids (Weber, 1969) which may be crucial in this study since the enzyme preparations were crude homogenates. Mammalian PK is known to undergo a temperature-induced conformational change to a modulator-insensitive form (Seubert & Schoner, 1971); examination of the data on Table 6 would suggest such a modulator-insensitive form is not produced in the NL-enzyme below T_c. Therefore, the differences in T_c-values seen here between hibernator and normothermic bat enzymes (Figs. 7, 8) may represent the influence of pH and

substrates on this conformational transition (Raison, 1973), as well as temperature.

Whatever mechanism is the cause for the observed changes in PK sensitivity to temperature, the differences in T_c-values between the hibernator and normothermic PKs are consistant and appear significantly different. Temperature-induced changes in activity can act as a barrier to metabolic flow (Raison & Lyons, 1971). By lowering the T_c-value, hibernating bat PK prevents a potential glycolytic blockage resulting from the temperature reduction. Also, since the T_c-value is near the average recorded T_b of Ontario cave-hibernating M. lucifugus (Fenton, 1972), it would be expected that further T_b decreases would be avoided. Such appears to be the case since a potentially dangerous drop in T_a is not followed by a similar drop in T_b; in fact, many hibernants thermoregulate (i.e., increase their T_b) with or without arousing (Reite & Davis, 1966; Lyman, 1970; Hudson, 1973). It would be of interest to examine PK from bats with lower hibernating T_b (e.g., E. fuscus and M. leibii; Fenton, 1972) to see whether the T_c may be further depressed in these species of hibernants.

3. K_m-values

The temperature-insensitivity of the apparent K_m, which is a measure of E-S affinity, for both HM- and HL-PK could be viewed as an adaptation to reduce the biochemical problems of a lowered T_b. In contrast, the increase in K_m-values at low temperatures for the normothermic enzymes (Figs. 10, 13), called negative thermal modulation by

Hochachka & Somero (1973), could biologically inactivate these enzymes in the absence of large changes in substrate concentration. Thermal insensitivity of the K_m has also been demonstrated for liver PK of hibernating Citellus undulatus (Behrisch, 1974) and for LDH of hibernating M. lucifugus (Moon, 1976).

At the higher thermal extreme, the K_m-values show positive modulation (i.e., K_m-values decrease between 34° and 16°; Fig. 13) in NL-PK. This decrease in K_m(PEP) or an increase in E-S affinity is accompanied by reduced Q_{10}-values (a drop to 1.4 at 0.1 mM PEP in NL-PK as compared to no change in HL-PK, Table 7), which again indicates decreased sensitivity of HL-PK to temperature changes over this thermal range. This data supports both V_{max} and V_{max}/K_m data for the NL-enzyme mentioned above. Liver PK in Citellus undulatus also counters the effect of a decrease in temperature from 37° to 5°C on enzyme activity by increasing the apparent affinity for the substrate PEP by two-fold and 7.5-fold for ADP, reducing Q_{10}, and thus maintaining enzyme and metabolic activity more or less constant in an environment of fluctuating temperatures (Behrisch, 1974). Such a kinetic response could be beneficial during diurnal torpor when synthetic potential is necessary to accumulate storage materials either for activity or the upcoming hibernation period; this could be directed specifically by metabolites.

Unlike normothermic PK, HL-PK does not demonstrate positive thermal modulation. During arousal, this temperature

Table 7. Temperature coefficient (Q_{10}-values) for NL- and HL-PK at saturating levels of ADP and varied concentrations of PEP. Values determined from Wilkinson plots.

	Q_{10}(16-34°C)	
[PEP] mM	NL-PK	HL-PK
2.0	2.4	2.1
0.8	2.0	2.1
0.4	1.8	2.1
0.2	1.7	1.9
0.1	1.4	1.9

independence of K_m-values could be important. Thermally insensitive K_m-values indicate relatively high Q_{10}-values are maintained (Behrisch, 1974), as was found for HL-PK (Table 7). Therefore, constant large increases in activity at low PEP concentrations for this enzyme (in contrast to NL-PK) would be expected as T_b rises during arousal. Increasingly more pyruvate would be made available for oxidative metabolism via the Krebs cycle and ATP production enhanced. Since ATP is necessary to drive heat producing reactions (Hochachka, 1974), body temperature increases would result.

Temperature, it would seem then, has a profound influence on bat PK. Two electrophoretically and kinetically distinct forms of PK in each of bat breast muscle and liver are seasonally induced and require an undetermined period of acclimation. An ingredient of seasonal induction may of course be ambient temperature. Furthermore, temperature acts as a modulator of these isozymes, modulating each for efficient functioning under the appropriate physiological conditions, and thus instantaneous adaptation to temperature exists. Further modulation of these isozymes has been investigated in this study in the form of metabolite regulation.

C. Metabolite Control of PK: ATP, Alanine and FDP

1. Bat muscle PK

Both normothermic and hibernating PKs isolated from breast muscle are affected by metabolites in a similar manner to the homologous M_1-type enzymes of other mammals (Llorente

et al., 1970; Seubert & Schoner, 1971; Kayne, 1973; Bigley et al., 1974; Flory et al., 1974; Van Berkel et al., 1974), with one exception. Bat breast muscle PK, particularily at 4°C, is sensitive to feedback inhibition by ATP such as has been reported for PK of certain invertebrate muscles (Mustafa & Hochachka, 1971), white (Somero & Hochachka, 1968; Mustafa et al., 1971) and red (Johnston, 1975; Hulbert & Moon, unpublished) skeletal muscle of fish, and diving mammal muscle (Storey & Hochachka, 1974; Hochachka & Storey, 1975). Although no specific correlation between muscle type and PK-ATP inhibition has been attempted in the literature, it is interesting to note that bat breast muscle is a highly oxidative tissue (see Fig. 3) as are some of the above muscle types.

According to several authors (e.g., see Van Berkel, 1974; Van Berkel et al., 1974), ATP inhibition may be an artifact of the chelation by ATP of free magnesium ions which are required for PK activity, causing the enzyme activity to drop to low levels. Could chelation be responsible for the inhibitory action of ATP encountered in bat PK, either the muscle or liver enzymes?

The present data is insufficient to answer this question, although the differential effects of ATP suggest it might be more than simple chelation. Similar to the findings of Van Berkel (1974), ATP inhibition in bat muscle PKs at 34°C occurs principally as a result of chelation. However, in bat liver, and perhaps bat muscle at 4°C, ATP inhibition may not be solely attributable to chelation

effects since marked inhibition is observed at low ATP concentrations. In retrospect, the problem of whether or not ATP inhibition is physiological may have been partially overcome with the use of glycerol-1-phosphate-Mg^{++} which buffers the magnesium concentration (Irving & Williams, 1973; Kayne, 1973).

What, then, would be the significance of the temperature-dependent ATP sensitivity in bat muscle PK? In the case of HM-PK, ATP could be instrumental in decreasing PK activity (acting synergistically with temperature) during entry into hibernation. The NM-PK would feature a similar ATP effect but it is assumed that this form of PK would not be subjected to such a temperature shock.

2. Bat liver PK

Qualitatively, the effects of the modulators ATP, alanine, and FDP on bat liver PK mimic those reported for other mammalian L-type PKs. Differences are noted, however, in the magnitude of the interaction coeficient (n_H-value) for the homotropic effector (substrate as an effector) seen in Table 6; in general, these values are lower than the typical L-type enzyme (Llorente et al., 1970; Flory et al., 1974; Van Berkel et al., 1974). Marked differences also exist between the hibernating and normothermic forms of the bat liver enzymes: 1. although affinity for ATP is similar in the two forms of PK (Table 5), HL-PK appears to be more sensitive than NL-PK, at both 34° and 4°C, to alanine and FDP (Table 5); and 2. the influence of the heterotropic

effectors (those effectors other than the substrate) upon the PK-PEP binding appears to be thermally independent in the HL-PK in contrast to that in the NL-PK (Table 6).

The importance of these heterotropic effectors in liver function is well documented. Alanine, one of the major gluconeogenic amino acids in mammals, is known to inhibit glycolysis only at PK (Friedrichs & Schoner, 1974). Thus, alanine may serve an important role in directing carbon flow either towards or away from glucose. Also, at physiological concentrations of PEP, ATP and alanine, PK is inactive in rat liver, and only the presence of FDP allows for significant activity (Llorente et al., 1970; Flory et al., 1974; Van Berkel et al., 1974). In the bat, the sensitivity of HL-PK to the two metabolites alanine and FDP, is greater than for NL-PK (Figs. 18, 22 and Table 5) suggesting a tighter control of the reaction catalysed by the HL-PK. This enhanced affinity of the HL-PK for alanine and FDP is consistent with the heightened heterotropic interaction of these effectors seen in Table 6. At some point during the heterothermal season, although this is not consistent in all hibernants (Galster & Morrison, 1974), gluconeogenesis which requires inhibition of PK must occur since blood glucose levels are known to be maintained at a reduced but controlled level during hibernation (Burlington, 1972). Also, PK inhibition could spare glycogen in favour of lipid and protein utilization during hibernation (Hudson, 1973). In these two cases, greater alanine control over HL-PK would be essential. The

thermal dependence of the K_i(ala) would enhance this effect (Table 5). On the other hand, control by FDP may be important in the shift from lipid to carbohydrate utilization during arousal, especially so in bats and birchmice which arouse much faster than other hibernants (Johansen & Krog, 1959; Heldmaier, 1969; Lyman, 1970). Here, FDP acts to reverse ATP and alanine inhibition, and activate glycolysis. It is interesting to note that the sensitivity of PK to FDP is temperature-dependent, decreasing as temperatures decrease (Table 5); this is also seen for L-type PK of other mammals (Seubert & Schoner, 1971) and may be a general characteristic of all mammalian PKs. Thus, it would seem that the hibernating form of liver PK in the bat is particularily well-suited for hibernation and arousal. Furthermore, should a thermogenic role be indicated in the bat liver, such as has been suggested for this tissue of chickens (Freeman, 1967) and rats (Stoner, 1973), the importance of a more sensitive HL-PK would be further enhanced.

Contrary to the findings in this study, Behrisch (1974) has reported that the HL-PK of Citellus undulatus is less sensitive than the NL-PK to ATP and FDP at all temperatures assayed. This lowered affinity of HL-PK for ATP would permit a higher energy charge within the hibernating liver cells of the Arctic ground squirrel (Behrisch, 1974). The lowered sensitivity to ATP could prevent substantial inhibition by ATP as this metabolite is rapidly produced during thermogenesis. Although no difference was observed

between the normothermic and hibernating bat PKs with regards to ATP inhibition, K_i(ATP)-values were doubled at 4°C. This change could permit a high energy charge at this low temperature as suggested by Behrisch. The lowered PK affinity for FDP may be related to the slower arousal of this mammal in contrast to bats.

There is also a marked difference in the effect of the heterotropic agents on the apparent PK-PEP affinity in hibernating versus normothermic M. lucifugus. In C. undulatus, the K_m(PEP) for HL-PK is less temperature sensitive than that for NL-PK (Behrisch, 1974). Similarily, the K_m(PEP) in bat HL-PK is temperature-insensitive compared to the thermally-dependent interaction observed for the NL-enzyme (Table 6). Furthermore, this pattern is observed whether the effectors are present or absent; i.e., the PK-PEP interaction is differentially temperature-sensitive. Therefore, hibernating bat liver PK differs markedly from the classic mammalian L-type enzyme in that temperature decreases result in the maintenance of control by the heterotropic effectors, not a desensitization (Llorente et al., 1970; Seubert & Schoner, 1971). It is apparent then, that the form of bat liver PK present during hibernation (HL-PK) not only allows maintenance of PK metabolic potential but also its regulatory potential.

CONCLUSIONS

CONCLUSIONS

This study has demonstrated that there are electrophoretically and kinetically distinct forms of bat liver and breast muscle PK which are unique to the physiological state (homeothermal or heterothermal) of this hibernant. However, no major qualitative differences have been detected between these enzymes and the homologous M_1- and L-type mammalian PKs. Certain minor changes in isozymic numbers and a possible regulatory role for ATP over breast muscle PK activities were observed for the bat enzymes, but general activity levels and regulatory properties are very similar. The only major exception to these generalizations is in the temperature sensitivity of the liver enzyme. Mammalian L-type PK has two interconvertible states, a regulator sensitive and insensitive form (Seubert & Schoner, 1971), which corresponds to the R and T states of the Monod et al. (1965) model. Unlike other mammalian forms, this equilibrium is unaffected by temperature in the bat liver PK, but may be affected by metabolites (i.e., FDP, ATP and alanine).

Although the bat PKs do not differ markedly from other mammalian PKs, differences do exist between the homeothermic and heterothermic forms of bat enzymes. For example, there is tighter control over the activity of HL-PK by FDP (which is in contrast to the findings of Behrisch (1974) for the homologous enzyme of C. undulatus), and alanine. Stronger inhibition by alanine would permit gluconeogenesis for restoration of glycogen stores during deep hibernation in

anticipation of the requirement for this energy source during arousal. At the same time, alanine inhibition could spare glycogen in favour of lipid utilization during the heterothermal period. Stronger activation of HL-PK (as compared to NL-PK) by FDP would be advantageous upon the initiation and early stages of arousal when anaerobic metabolism may be important.

The specific activities of tissue PKs in the hibernating bat were found to be higher than those of the normothermic bat; this is thought to be related to the need for glycolysis during arousal, and possibly the need for a thermogenic cycle between PK and the antagonist PEPCK during the heterothermal period. Furthermore, the form of PK found in hibernation (HL-, HM-PK) or normothermia (NL-, NM-PK) appears to be the one with the lowest thermal sensitivity, as determined by the lowest E_a-values for V_{max} and V_{max}/K_m. The advantage of a thermally-insensitive liver PK in a normothermic bat could be related to the maintenance of metabolic flux through this point independent of temperature but dependent on metabolites directing carbon flux for storage of materials during diurnal torpor. The insensitivity to temperature in bat tissue PKs during the heterothermal period might prevent energy-consuming arousal when only small T_a increases are encountered by the hibernating bat.

Thermal insensitivity of the apparent affinity of HL-PK for its substrate, PEP (i.e., the K_m(PEP)), is linked with the maintenance of high Q_{10}-values which suggests that constant increases in PK activity at low or physiological

PEP concentrations could be expected as T_b rises during arousal. Large increases in PK activity would permit production of large amounts of energy and heat. Conversely, during entrance into hibernation, rates would drop more quickly with this type of kinetic pattern. Similar kinetics have been reported for liver PK of C. undulatus (Behrisch, 1974). Furthermore, thermal insensitivity in HL-PK has been found in the presence as well as the absence of allosteric effectors (ATP, alanine, FDP). On the other hand, a decreased K_m(PEP) for NL-PK between 34° and 16°C (coinciding with reduced E_a- and Q_{10}-values) is thought to indicate a decreased sensitivity of this enzyme form to temperatures encountered in diurnal torpor at a time when synthetic potential is important.

Another property of the hibernating form of bat tissue PKs which would seem to be advantageous during the heterothermal period is lowered T_c-values since temperature-induced changes in enzyme activity (found in the normothermic forms of bat PK) can act as a barrier to metabolic flow.

Therefore, as has been found to exist when enzymes from homeotherms and poikilotherms are compared (Hochachka & Somero, 1973; Borgmann & Moon, 1975), enzymes of hibernating and normothermic bats, Myotis lucifugus, are tailored through evolutionary time to function optimally in a specific physiological state. Since few studies have reported enzyme differences in hibernants, it is impossible to suggest how general these results will be found.

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