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The Influence of Temperature-Induced Phase Changes on the Kinetics of Respiratory and Other Membrane-Associated Enzyme Systems

John K. Raison

Plant Physiology Unit, C.S.I.R.O. Division of Food Research, and School of Biological Sciences, Macquarie University, North Ryde, Sydney, Australia, 2113

Temperature-mediated changes in the kinetics of enzyme catalysed reactions can be due to effects on a number of different parameters. If the change in temperature does not (a) inactivate the enzyme, (b) alter the affinity of the enzyme for the substrate, an activator or an inhibitor or (c) alter the pH function of the reaction components, the velocity of enzyme catalysed reactions increases with increasing temperature. The relationship between the velocity of reaction and temperature can be expressed either as the activation energy (E) or the temperature coefficient (Q_{10}). Both expressions can be derived from the empirical Arrhenius equation relating the velocity of reaction and temperature

$$\frac{\alpha \ln k}{\alpha T} = \frac{E}{RT^2} \tag{1}$$

where k is the reaction velocity constant, R the gas constant, T the absolute temperature and E a constant, subsequently called the activation energy (also written as A or μ). Integration of equation (1) gives

$$\ln \frac{k_2}{k_1} = \frac{E}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{2}$$

from which it can be seen that the value for E can be obtained from the slope of the straight line when $\log k$ is plotted against $1/T$

$$E = 2{\cdot}303\, R \times \text{slope}$$
$$\therefore E = 4{\cdot}576 \times \text{slope (where } R = 1{\cdot}987 \text{ cal/mole/°K)}$$

The term E is not the activation energy, ΔH^*, of the activated complex formed during an enzyme reaction as defined by the transition

state theory of Eyring (cf. ref. 1) but can be related to this quantity by

$$E = \Delta H^* + RT \tag{3}$$

if the molecularity of the reaction is unity. Thus, within the physiological range of temperatures, approximately 600 cal/mole must be deduced from the calculated value of E to obtain the heat content on activation, ΔH^*. The relation between changes in heat content and other thermodynamic functions such as free energy and entropy during formation of enzyme-substrate complexes and the activation of these complexes during reactions is beyond the scope of this article. The reader is referred to the articles by Dawes;[2] Bray and White;[3] Johnson *et al.*;[1] Sizer;[4] Dixon and Webb.[5]

The other expression relating the change in reaction velocity to temperature, the temperature coefficient (Q_{10}), is defined as the factor by which the velocity increases as a result of a 10 Celsius degree rise in temperature. For most biological reactions the value of Q_{10} is between 1 and 2.

Although most enzyme catalysed reactions show a constant activation energy over a limited range of temperature, a number of reactions have been reported where a plot of the logarithm of velocity against the reciprocal of the absolute temperature (Arrhenius plot) shows two straight lines of different slope, i.e. a discontinuity or change in E with temperature.[4–7] A number of papers have also been published showing Arrhenius plots where the data is fitted to a straight line by "brute force"[8] methods. In most of these cases the realistic errors in determining the velocity have been ignored in what is apparently an attempt to make the reaction appear to conform with Arrhenius' original theory and a line of best fit applied.

The large number of examples of enzyme reactions, of both plant and animal tissues which show a distinct change in activation energy within the range of physiological temperatures, particularly reactions catalysed by enzymes associated with membrane surfaces, clearly suggests that in most cases the change is associated in some way with changes in the physical properties of the membranes. Furthermore, recent data show enzymes or multi-enzyme systems will exhibit either a varying or constant activation energy depending on the species of plant or animal from which the enzymes are derived.

This article will discuss the changes in activation energy of enzymes in terms of temperature-induced phase changes in the system, with particular reference to "crystalline" enzymes and membrane-associated enzymes of both plant and animal origin. Some of these enzymes exhibit a change in activation energy with temperature and in certain cases it is possible to relate these changes in activation energy to an alteration in the physical properties of membrane lipids. The significance of a change in activation energy is also discussed in terms

of the physiological function of plants and animals at various temperatures.

Alterations in the Activation Energy of Enzymes in Terms of Changes in Physical States

The significance of abrupt changes in activation energy of some enzyme reactions and physiological processes has been the subject of considerable controversy. The similarity between the activation energy for specific enzyme reactions and complex physiological processes such as respiration and heart beat determined by Arrhenius[9] led Crozier and co-workers (for complete discussion see ref. 1) to postulate that with each physiological process, involving a multi-enzyme series of reactions, the overall process is limited by a rate-limiting or "master reaction". The change in activation energy for these processes is considered by Crozier to represent a shift from one controlling "master reaction" to another at the critical temperature (the point of discontinuity in the Arrhenius plot). Bělehrádek,[10] on the other hand, has considered that such a process would produce a smooth catenary in the region of the temperature range over which the change occurred. Thus, according to Bělehrádek,[10] the discontinuity is the result of drawing an intercept of tangents to smooth curves and denies the existence of real discontinuities. To explain the increase in activation energy at between 26° and 32°, of fumarase acting on either fumarate or malate in alkaline medium, Massey[11] has suggested the enzyme dissociates into smaller sub-units as the temperature is increased and these smaller enzyme unit have a higher activation energy. In a more recent consideration Bělehrádek[12] states that for "a real break" to be manifest in an Arrhenius plot of a series of sequential reactions the rate-limiting processes, in the two temperature zones, must have activation energy values differing from each other by at least 16 kcal/mole. Much of the available data on discontinuities in Arrhenius plots show changes in activation energy considerably less than this value.

This problem in interpretation of discontinuities in Arrhenius plots has been discussed by Kumamoto *et al.*[13] in the light of more recent data showing that in some cases the two independent straight lines of discontinuous Arrhenius plots do not intersect at the transition temperature. Kumamoto *et al.*[13] agree with Crozier and others (cf. ref. 5) that two independent processes are required to produce a discontinuity, but further stipulate that the system must provide for the exclusive functioning of these processes in their respective temperature ranges. This proposal of the exclusive operation of the independent processes within the respective temperature ranges is justified on thermodynamic grounds by considering that the system undergoes a phase change at the critical temperature. The isothermal properties

of a phase change explain why the two independent processes operate exclusively immediately there is a shift from the temperature at which the phase change occurs. The phase change which allows for the exclusive functioning of the two independent processes could be in the ground state of one of the reactants or in the enzyme catalysing the reaction. An important consideration resulting from this explanation of discontinuities in Arrhenius plots is that the phase change in the reaction components may be determined by phase changes in structural components associated with the particular enzyme, but remote from the site of the catalysis. As Kumamoto *et al.*[13] have pointed out, under these conditions the compensation between enthalpy and entropy, predicted to maintain the rate constants of the two independent reactions at the

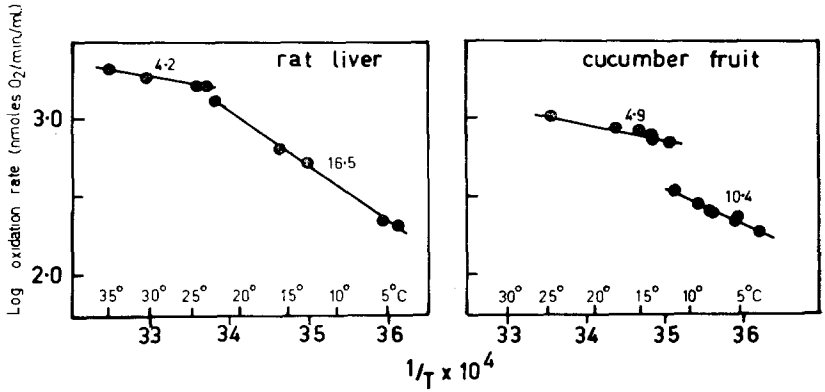

Figure 1. Arrhenius plots of state 3, succinate oxidation by rat-liver mitochondria and cucumber fruit mitochondria. The activation energies (kcal/mole) are as indicated beside each line. (Data from Kumamoto, Raison and Lyons.[13])

transition temperature constant, might not be exact. In these cases the two straight lines of an Arrhenius plot would show a non-intersecting discontinuity as typified by the examples of Arrhenius plots in Fig. 1. Many other examples of such non-intersecting discontinuities are now known (see below), but in all cases the difference in the rate constants at temperatures infinitesimally above and below the transition temperature never exceeds 0·5 log units. From thermodynamic considerations Kumamoto *et al.*[13] argue that much greater differences would be expected if the enthalpy–entropy compensation were not as exact. Their reasoning is detailed below.

By application of a simplified version of the transition state theory, neglecting partition functions, the rate constant for each of the independent reactions can be expressed as

$$k_r = \frac{Kk_\beta T}{h} e^{-\Delta F^*/RT} \qquad (4)$$

where K is the transmission coefficient, k_β is Boltzmann's constant, T is the absolute temperature, h is Planck's constant, ΔF^* the change in free energy of the activated complex and R the universal gas constant. The term

$$\frac{Kk_\beta T}{h}$$

is nearly constant over the small temperature range under consideration The ratio of the two rate constants immediately above and below the transition temperature can then be expressed as

$$\frac{k_{r_1}}{k_{r_2}} = \frac{e^{-\Delta F_1^*/RT}}{e^{-\Delta F_2^*/RT}} = e^{\Delta F_2^* - \Delta F_1^*/RT} \tag{5}$$

At a point of intersection (isokinetic point) $k_{r_1} = k_{r_2}$ and

$$\Delta F_2^* - \Delta F_1^* = 0$$

However, the two independent processes on either side of the isokinetic point have different activation energies as determined experimentally (Fig. 1). The Arrhenius activation energy (E) can be related to the enthalpy by

$$E = \Delta H^* + RT \tag{3}$$

and therefore these reactions would have different enthalpy values. Since

$$\Delta F^* = \Delta H^* - T\Delta S^* \tag{6}$$

to obtain a constant ΔF^* the change in ΔH^* for these reactions requires a corresponding change in ΔS^*.

In the case of the oxidation of succinate by mitochondria from cucumber fruit shown in Fig. 1. the values for E, above and below the transition temperature at 12°, are 4·9 and 10·4 kcal/mole, i.e., ΔH^* values of 4·3 and 9·8 kcal/mole approximately 600 cal/mole less than E.

Assuming ΔF^* is equal to ΔH^* and substituting these values in equation (5), we obtain

$$\begin{aligned} k_{r_1}/k_{r_2} &= e^{(9\cdot8\times10^3 - 4\cdot3\times10^3)/566} \qquad \text{at } 12°, \\ &= e^{(5\cdot5\times10^3)/566} \\ &= e^{9\cdot7} \\ \text{i.e. } k_{r_1}/k_{r_2} &= 10^{4\cdot2} \end{aligned}$$

i.e. the rate constants at the transition temperature should vary by $10^{4\cdot2}$ units if there were no simultaneous compensation of the entropy term. The fact that the rate constant at the isokinetic point for this reaction varies by only approximately $10^{0\cdot3}$ units clearly shows that the compensating change in the entropy is of the order of $10^{3\cdot9}$ entropy units.

In most of the reactions to be discussed the difference in activation energy, above and below the transition temperature, would predict even larger differences in the rate constants than that observed for succinate oxidation by cucumber mitochondria. Since the data show differences in rate constants of usually less than $10^{0.2}$ units it must be assumed that almost precise enthalpy–entropy compensation is the rule rather than the exception.

Discontinuities in Arrhenius Plots as a Consequence of Phase Changes

As pointed out in the preceding section, discontinuities in Arrhenius plots can be considered as a phase change in either the enzyme or the reactants and can be dominated by events removed from the active site. It is of interest therefore to consider the events occurring in some of the classical examples of discontinuities in terms of phase changes in the systems.

A. *Reactions Involving Crystalline Enzymes*

Arrhenius plots for the activity of invertase, lipase, trypsin and pepsin[15, 16] show a discontinuity or break at 0° indicative of a phase change in the water which forms one of the reactants. Dixon and Webb[5] have objected to this interpretation on the grounds that a change in activation energy at 0° was also observed with pancreatic lipase in a reaction mixture containing 36·5% glycerol.[15] However, the water involved in the hydrolysis of tributyrin by the lipase, in the region of the active centre of the enzyme molecule, is probably a separate domain which excludes glycerol and therefore reflects the phase change of pure water at 0°.[8] It is unlikely that a phase change in the lipid substrate, tributyrin, is involved since its melting point is −75°.

Massey[11] has shown discontinuities in plots of fumarase, acting in alkaline medium on either fumarate or malate, at different temperatures depending on the pH and substrate. A discontinuity was also apparent in acid medium for the hydration of fumarate by fumarase, but not for the dehydration of malate by the same enzyme. There is no simple explanation for these observations in terms of a phase change in the substrate. In alkaline conditions the activation energy changes from a larger (14·8 kcal/mole) to a smaller value (9·3 kcal/mole) as the temperature is decreased, while in slightly acid medium an increase in activation energy (6·7 to 10·6 kcal/mole) was observed. The temperature of the change also varied depending on the pH and on the direction of the reaction; hydration of fumarate or dehydration of malate. For the alkaline conditions Massey[11] suggests that the enzyme undergoes a dissociation into smaller units as the temperature increases and the smaller units have a higher activation energy. If this is indeed the case,

the two different forms of the enzyme would constitute the two phases described by Kumamoto *et al.*[13] The differences in the temperature at which the change occurs could be due to the influence of pH and substrate on the temperature-induced dissociation process. There is no satisfactory explanation of the increase in activation energy observed at 18° for the hydration of fumarate by fumarase at pH 6·35. This change in activation energy occurs at the same temperature as a change in K_m and because it occurs only in the forward reaction, was assumed to be associated with an effect on the orientation of the water involved in the reaction. In view of the observation that the affinity for inhibitors also shows a marked change in the same temperature range[17] suggests that some change occurs at this particular temperature in the structure of fumarase in the acid medium which affects the binding of substrate and inhibitors. Such changes, although inexplicable at present, obviously have the isothermal properties of the phase change discussed by Kumamto *et al.*[13] since only sharp changes are observed in the Arrhenius plots of both velocity and K_m.

The similarity between the temperature at which changes in E are observed and the temperature at which changes are observed in the structure of water adjacent to an interface, led Drost-Hansen[8] to postulate that many of the thermal anomalies in enzyme kinetics can be explained by an alteration in the physical properties of vicinal water. He stresses that these thermal anomalies occur at or near the same temperature for all systems regardless of the chemical nature of the substrates involved. The temperatures mentioned were 17°, 28°, 43° and 60°. However, as stated by Drost-Hansen,[8] changes in vicinal water must be superimposed upon the changes in the biological macromolecules, and these will undoubtedly be influenced by environmental changes. Thus for enzyme proteins an alteration in configuration reflecting structural changes in vicinal water might occur at any temperature depending upon factors such as pH and ionic strength of the medium. It is possible that changes in the structure of vicinal water might influence the conformation of the active site of an enzyme and consequently the rate constant of the reaction catalysed. This might explain the discontinuity in the Arrhenius plots of invertase, lipase, trypsin and pepsin at 0°. However, in none of the examples of discontinuities in Arrhenius plots quoted by Drost-Hansen,[8] in support of this hypothesis, has a change in vicinal water been detected.

B. *Enzymes and Multi-enzyme Systems Associated with Membranes*

(i) *Respiratory enzymes associated with mitochondrial membranes.* A major difference between the respiratory enzyme system of mitochondria derived from chilling-sensitive plants and homeothermic animals on the one hand and chilling-resistant plants and poikilothermic animals on the other is the effect of temperature on these enzyme systems.[6, 14]

Arrhenius plots of the succinate oxidase system of mitochondria from chilling-sensitive plants and homeothermic animals show a sharp discontinuity in the region of 10° to 12° and 23° respectively. A discontinuity at 17° has also been reported for succinate oxidase activity of rat-liver mitochondria[18] and at 19° for the same enzyme in a Keilin and Hartree, heart-muscle preparation from pig.[19] The same enzyme system from chilling-resistant plants[6] and poikilothermic animals[14] shows a constant activation energy over the temperature range of 0° to 25° and 0° to 37° respectively. This difference in response to temperature of the same enzyme system from the two different types of tissues is shown in Figs. 2 and 3.

Earlier studies of the effect of temperature on the kinetics of enzymes indicate that the activation energy of a particular enzyme is the same

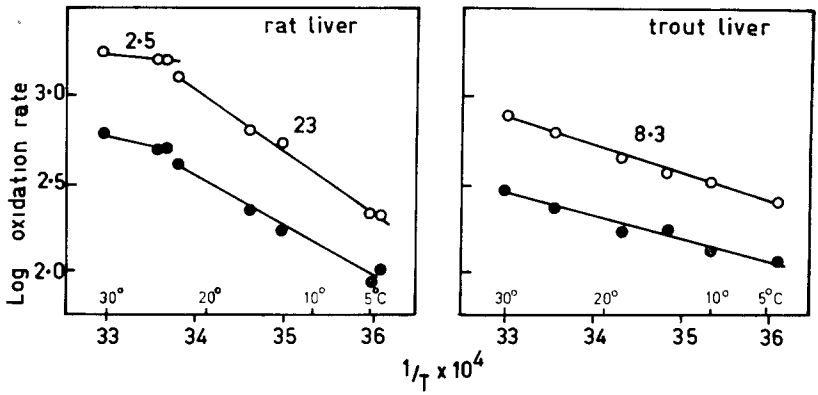

Figure 2. Arrhenius plots of state 3 (○) and state 4 (●) succinate oxidation of mitochondria from rat and trout liver. (From Lyons and Raison.[14])

regardless of the source of the enzyme and is essentially independent of environmental changes unless these factors alter the catalytic surface of the enzyme.[4] Much of the recent kinetic data on membrane-associated enzymes does, in part, confirm the earlier observations except for the significant differences between the succinate oxidase system of the two groups of the plants (chilling-resistant and chilling-sensitive) and animals (homeothermic and poikilothermic) mentioned previously.

Changes in the value of activation energy with temperature for membrane-associated systems are not confined to the succinate oxidase system. A similar discontinuity has been observed in the Arrhenius plots for the oxidation of reduced NAD (plant mitochondria only), β-hydroxybutyrate and α-oxoglutarate, the succinate:phenazine methosulphate oxidoreductase and the cytochrome *c* oxidase system measured with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate.[7] The discontinuity in the Arrhenius plots of the oxidative enzymes from the respective sensitive tissues is also apparent for both

state 3 and state 4 respiration based on succinate oxidation (see Figs. 2 and 3), and with mitochondria disrupted by hypotonic swelling and by sonic disintegration.[7] Treatment of mitochondria with low concentrations of any of several different detergents abolishes the "break" or discontinuity in the Arrhenius plots resulting in an increase in the activation energy in the higher temperature range and a decrease in the low temperature range.[7]

It is of interest to note differences in the kinetic behaviour of the succinate oxidase system of liver mitochondria from the hibernating ground squirrel, *Citellus lateralis*. Mitochondria isolated from an active

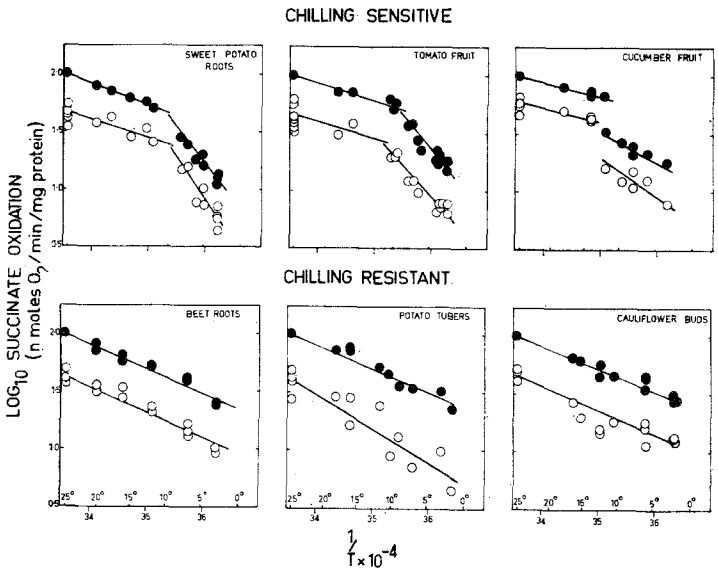

Figure 3. Arrhenius plots of state 3 (●) and state 4 (○) succinate oxidation of isolated plant mitochondria. (From Lyons and Raison.[6])

squirrel show a discontinuous Arrhenius plot for succinate oxidase activity at between 25° and 21°. However, when the mitochondria were isolated from a hibernating animal (body temperature 4°) no discontinuity is observed in the Arrhenius plot.[20] Thus the same enzyme system in the same species of animal can exhibit different temperature-dependent kinetics depending on the physiological state of the tissue. In one experiment involving one hibernating squirrel, the straight line Arrhenius plot observed with mitochondria 30 min after isolation, showed a slight discontinuity when assayed again 90 min later and a distinct discontinuity after a further 120 min.[20] This reversion from a continuous Arrhenius plot to a discontinuous plot involves a decrease in the activation energy above 25° and an increase in activation energy below this temperature. These changes in activation energy

above and below the transition temperature are in the opposite direction to the changes observed in the activation energies of succinate oxidase when rat-liver mitochondria were treated with detergent.[7] However, since these changes observed with the mitochondria from the hibernating squirrel occur above and below the transition temperature it is considered to be the result of a change in the physical properties of the mitochondrial membrane; a change to a more rigid state in the low temperature range and a more flexible state in the higher temperature range.[20]

In terms of a temperature-induced phase change it is unlikely that each of the enzymes of the respiratory system of mitochondria from sensitive tissues would undergo a conformational change at the same temperature in contrast to the same enzymes of mitochondria from resistant tissues. Since mild treatment with detergent abolishes the discontinuity in the Arrhenius plots of enzymes from sensitive tissues, but does not alter the activation energy of the same enzymes from resistant tissues, the conformational change in the enzyme protein is considered to be mediated through a phase change in the membrane lipids.[7] It should be noted, however, that a soluble succinate dehydrogenase from pig heart, activated by incubation with succinate, shows a discontinuity in an Arrhenius plot at about 18° the same temperature at which a discontinuity is observed for the succinate oxidase activity of the particular heart-muscle preparation.[19] The non-activated, soluble succinate dehydrogenase shows two points of discontinuities, at 27° and 18°. The changes in activation energy are considered to be due to conformational changes in the enzyme which alter the "catalytic-centre activity" rather than the activation enthalpy.[19] In the absence of any analytical data indicating the possibility of lipid associated with the soluble dehydrogenase it is not possible to relate these changes in activation energy to a phase change in a lipid component. However, as outlined later, some soluble preparations of succinate dehydrogenase do contain associated lipid and if present in the succinate dehydrogenase from pig heart, would provide an alternative explanation for the changes in activation energy.

Phase transitions between the so-called lamella and hexagonal phases of brain phospholipid dispersed in water have been demonstrated to occur at 23°.[21] In addition, Chapman *et al.*[22] have shown that the temperature at which a particular phase can exist for a given phospholipid will depend on the melting temperature of the hydrocarbon chains. Furthermore Steim *et al.*[19, 22] have demonstrated by differential scanning calorimetry that the temperature of the phase transitions in the membranes of *Mycoplasma laidlawii*, and the lipids extracted from these membranes, is dependent on the proportions of saturated and unsaturated fatty acids present. In relating chilling sensitivity in plants to lipid composition of mitochondrial membranes, Lyons *et al.*[24]

showed a higher proportion of saturated fatty acids in the sensitive plants than in resistant plants, and also showed that a change of as little as 5% in the amount of unsaturated fatty acids in artificial mixtures of fatty acids, which approximated the composition of plant mitochondrial membranes, would change the temperature of solidification of the lipid mixtures by up to 15°.[25] A higher proportion of unsaturated fatty acids has also been reported in the mitochondria of poikilothermic animals compared to homeothermic animals.[26,27] There is thus a well defined correlation between the maintenance of a fluid state in the hydrocarbon chains in membrane lipids by increasing the degree of unsaturation, and the environmental temperature at which the mitochondria of the particular plant or animal species functions. A more direct correlation between the physical changes of membrane lipids and the change in activation energy of enzymes associated with the membrane is obtained by direct measurement of the molecular motion of spin-labelled analogues of fatty acids associated with the membrane lipids. These data clearly show a lipid phase transition at approximately 12° for mitochondria from sweet potato (chilling-sensitive) and at 23° for rat-liver mitochondria.[28] These phase transitions coincide precisely with the temperatures at which the change in activation energy of succinate oxidase is observed in the respective tissue. No phase change is observed in the membrane lipids of potato mitochondria (chilling-resistant) or trout-liver mitochondria (poikilothermic).[28] The phospholipids extracted from the membranes of the temperature sensitive mitochondria also exhibit a phase change at the same temperature as the intact mitochondria.[28]

In physical terms the phase change inferred from the abrupt change in motion of a spin-labelled fatty acid represents a sharp transition of the phospholipids of the membrane, from what Luzzati *et al.*[21] term a liquid-crystalline structure to a cogel, as the temperature is decreased below the critical temperature. Since the enzymes associated with the mitochondrial membranes undergo a phase change (as indicated by the change in activation energy) at precisely the same temperature, the perturbation associated with the liquid-crystalline to cogel transition is assumed to induce a conformational change in the active centre of the enzyme proteins. This conformational change would thus represent the phase change described by Kumamoto *et al.*[13] and allow for the exclusive functioning of the two forms of the enzyme above and below the transition temperature.

The Mg^{2+}-activated ATPase of the inner membrane of rat-liver mitochondria also shows a discontinuity in an Arrhenius plot at approximately 23°, as shown in Fig. 4. Kemp *et al.*[18] have observed a discontinuity for the same enzyme, assayed in the presence or the absence of uncouplers, at 17°, the same temperature at which these workers observed a discontinuity for succinate oxidase activity. This

difference in the temperature of the discontinuity of both the ATPase and succinate oxidase activity in the two studies probably reflects slight differences in either the fatty acid composition or the degree of unsaturation of the fatty acids of the mitochondrial membrane lipids; both vary depending on the diet of the animal.[30] It is significant that the discontinuity for ATPase and for succinate oxidase occurs at the same temperature in the two independent studies. Further investigation of the ATPase system in this laboratory has shown that the discontinuity is only apparent for the oligomycin-sensitive reaction (see Fig. 4) and can be completely abolished by treating mitochondria with low

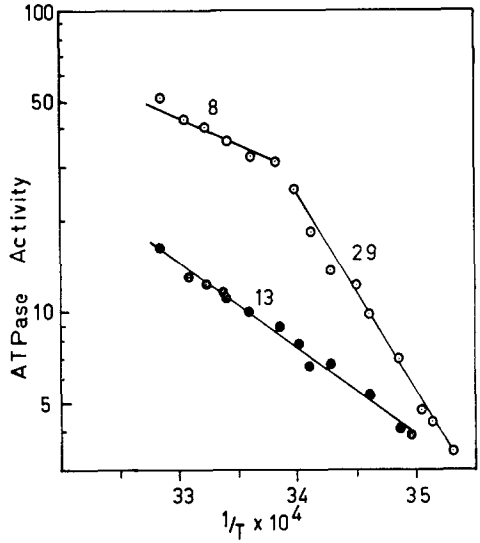

Figure 4. Arrhenius plot of the Mg^{2+}-activated ATPase of rat liver mitochondria assayed in the presence (●) and absence (○) of oligomycin. (Data of McMurchie, Raison and Cairncross.[29])

concentrations of detergent,[29] similar to the abolition of the discontinuity for succinate oxidase activity after treating mitochondria with detergent.[7] Both these observations are explicable in terms of the known structural organization of ATPase involving phospholipid and protein.[31] Oligomycin sensitivity can be abolished by even partial hydrolysis of the phospholipid with phospholipase A.[32] The coincidence of the loss of phospholipid, as a structural component of the ATPase, the loss of oligomycin sensitivity and the abolition of the discontinuity in the Arrhenius plot of activity, further enhances the concept of the induction of a conformational change in the enzyme protein through a temperature-induced phase change in the associated lipid components.

Data available regarding the temperature-dependent kinetics of the adenine nucleotide translocase of the inner membrane of rat-liver

mitochondria suggest that changes in the conformation of some membrane-associated enzymes occur at temperatures differing from the temperature of the bulk lipid phase change. The temperature response of this enzyme measured over a range of 0°–20° shows a discontinuity in the Arrhenius plot at 9°, when nucleotide exchange was measured with ATP, and at 8° with ADP.[33] The respiratory enzymes,[14] the oligomycin-sensitive ATPase[29] and the lipids of the inner membrane of mitochondria[28] all exhibit major changes at 22° to 23°. It is not at present possible to relate the change in kinetics of the translocase at 8° or 9° to temperature-induced phase changes in membrane lipids as no abrupt changes in the mobility of spin-labelled fatty acids have been detected at this temperature.[28] If a phase change in a lipid component is involved in the change in activation energy of the translocase, this lipid component is apparently not influenced by changes in the physical properties of the bulk of the membrane lipids. The change in activation energy of the translocase at 8° to 9° might be an example of a temperature-induced conformational change in the enzyme protein, independent of a change in the physical properties of membrane lipids, similar to the changes observed for myosin acting on ITP or on ADP in the presence of 2,4-dinitrophenol (DNP) or *p*-chloromercuribenzoate (PCMB)[34] (see later). The slight difference in the temperature of the discontinuity in the Arrhenius plot of translocase activity, depending on the substrate, further demonstrates a similarity with the myosin system. Further studies of the physical properties of the translocase, particularly those influenced by temperature, and its association with lipids are required before a more definite explanation can be advanced to account for the discontinuity at 8° to 9°.

The nucleotide translocase is considered by some workers to be the rate limiting step in coupled-oxidation reactions by mitochondria.[18, 35] If this were so, the activation energy of state 3 respiration could never be less than the activation energy of the translocase since in any multi-enzyme process the activation energy observed must be that of the rate-limiting event. The activation energy of the nucleotide translocase of rat-liver mitochondria between 8° and 20° is 21 kcal/mole.[34] The activation energy for succinate-dependent, state 3 respiration of rat-liver mitochondria, in approximately the same temperature range, is 18·8[14] to 23[18] kcal/mole, which is not significantly different from the activation energy of the translocase. However, an Arrhenius plot of state 3 respiration of rat-liver mitochondria exhibits a sharp discontinuity at 23° (cf. Figs. 1 and 2) as determined by Lyons and Raison[14] or at 17° as determined by Kemp *et al.*[18] Above these temperatures the activation energy of succinate oxidation is considerably less, 2·5 to 8·9 kcal/mole.[13, 14, 18] Comparison of the activation energies of the translocase and succinate oxidation in state 3 would, therefore, confirm that the translocase could be the rate limiting reaction in coupled

respiration in the temperature range of 0° to 17° or 0° to 23°. However, above these temperatures, that is in the physiological temperature range, the translocase must also decrease its activation energy if it is the rate-limiting step, since the activation energies reported for state 3 respiration, which involves the translocase, are considerably less in this temperature range. Because of the difficulties in estimating translocase activity of rat-liver mitochondria at elevated temperatures, clarification of a possible change in activation of the translocase activity at the temperature of the phase change in the membrane lipid could best be obtained from an investigation of this enzyme in mitochondria from chilling-sensitive plants. Mitochondria from these tissues show a lipid phase change at 10° to 12°[28] and a similar decrease in the activation energy of state 3 respiration above the transition temperatures as shown by the succinate-dependent respiration of rat-liver mitochondria.[6] In addition a comparison of the temperature dependence of the translocase with mitochondria from chilling-sensitive and resistant plants would also confirm whether the temperature-induced phase change in the lipids of the membrane influences the activation energy of the translocase.

(ii) *Enzymes associated with the endoplasmic reticulum and plasma membrane.* Similar conclusions regarding the dependence of the discontinuity in Arrhenius plots on temperature-induced changes in the physical properties of lipids associated with the enzyme can be inferred from kinetic studies of the Na^+ plus K^+-activated and the Mg^{2+}-activated ATPases of plasma and endoplasmic reticulum membranes of homeothermic animals.[18,29,36–38] Each of these enzymes exhibits a discontinuity in the Arrhenius plot between 18° and 20°, depending on the source and type of ATPase. For the Mg^{2+}-ATPase of the microsomal fraction of rat brain, an additional discontinuity is found at 6°.[36] For the ouabain-sensitive Na^+ plus K^+-activated ATPase the discontinuity is apparent for both the sensitive and insensitive reactions[38] although the sensitivity to ouabain diminishes as the temperature is reduced and there is no detectable ouabain-sensitive activity below about 5°.[38]

The association of these ATPases with membranes suggests that the configurational changes in the enzyme protein, which are assumed to occur at the transition temperature, are also induced by a phase change in the membrane lipids. This view is supported by the observation of a change in the molecular mobility of a spin-labelled fatty acid in the lipid region of a preparation of rat-liver endoplasmic reticulum at 22° to 23°, as shown in Fig. 5. The abolition of the break in the Arrhenius plot of the Na^+ plus K^+-activated ATPase by treatment with detergent[29] also suggests that changes in the physical properties of the membrane lipids are involved in the temperature-induced configurational changes of the enzyme protein.

These observations do not exclude the possibility of a direct, temperature-mediated configurational change in the enzyme protein since there is evidence which suggests such changes may occur with purified preparations of myosin[34] and the nucleotide translocase discussed above. The kinetics of hydrolysis of nucleotide triphosphates by myosin exhibits a sharp discontinuity in an Arrhenius plot at approximately 15° when the substrate is ITP or ATP plus either DNP or PCMB; ATP alone showed a constant activation energy in the same temperature range. Levy *et al.*[34] concluded from this study that portions of the myosin molecule, including the active site, can undergo a temperature-

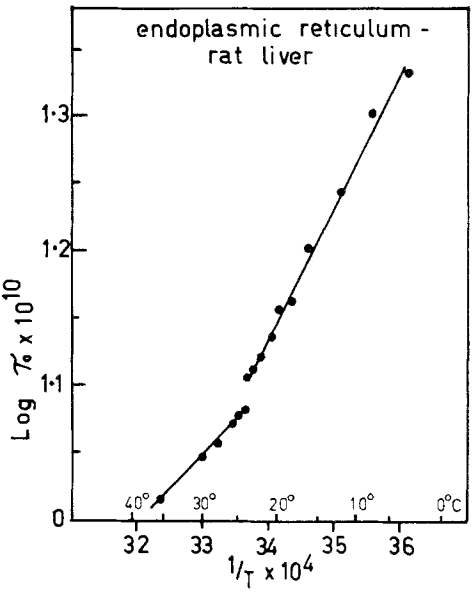

Figure 5. Arrhenius plot of the change in rotational correlation time (T_0) of the spin-label, 12 nitroxide stearic acid, in a preparation of rat-liver endoplasmic reticulum. (Data of McMurchie, Raison and Cairncross.[29])

induced conformational change, but these changes can be modified by interaction of specific groups on the protein with particular substrates or agents such as ATP, DNP or PCMB. In the binding of enzyme and substrate, the 6-amino group of the purine ring of ATP has a greater affinity for the active site than the 6-hydroxy group of ITP and evidently stabilizes the enzyme to changes in temperature since no discontinuity is observed in Arrhenius plots of activity when ATP is the substrate.[34] Cooperative transformations of enzyme protein by temperature are well known processes and the effect of the electrostatic interaction of charged groups on substrates and of compounds which either oxidize or reduce specific groups involved in cross-linking have been well documented.[1] It is of interest to note the difference between the activation energy for

the reaction of myosin involving ATP alone and the reactions with ATP plus DNP or PCMB. Above the transition temperature of 15° the activation energy for hydrolysis of ATP with or without DNP or PCMB is 12 kcal/mole. Below 15° there is no change in activation energy with ATP as substrate, but with ATP plus either DNP or PCMB the activation energy increases to 25 kcal/mole.[34] This indicates that the binding of DNP and PCMB alters the tertiary structure of the ATPase below the transition temperature, but has no effect above this temperature. This is not usually the case with enzymes associated with membranes. If the activation energy of succinate oxidase or Na^+ plus K^+, activated ATPase is altered by modifying the lipid components of the membrane with detergent, the activation energy above the transition increases while the activation energy below the transition temperature decreases.[7]

A water-soluble, ouabain-sensitive, Na^+ plus K^+-dependent ATPase,[39] prepared from NaI-treated microsomal fraction by extraction with a detergent (Lubrol W) might provide an ideal system for studying the influence of membrane association on temperature-induced conformational changes of protein. The water-soluble enzyme consists of twelve subunits arranged as three tetramers. Incubation at 37° rapidly inactivates the ouabain-sensitive reaction and causes dissociation of subunits. The resulting monomers show ouabain-insensitive activity.[39] The water-soluble enzyme can also be incorporated into ATPase depleted membranes with enhancement of activity, particularly when incorporated in the presence of phosphatidyl-L-serine. It would be of interest to determine if the water soluble tetrameric form of the enzyme exhibits a discontinuity in an Arrhenius plot.

The Ca^{2+}-dependent ATPase of sarcoplasmic reticulum also shows a discontinuity in an Arrhenius plot at 10° when assayed with a relatively high concentration (5 mM) of ATP.[40] Previous studies of the temperature dependence of this enzyme, using lower concentrations of substrate (1 mM) have shown a constant activation energy over a temperature range of 5° to 20°[41] and 0° to 37°.[42] Isolated preparations of sarcoplasmic reticulum form closed vesicles, which in the presence of an energy source, such as ATP, will accumulate Ca^{2+} with a concurrent hydrolysis of ATP by the Ca^{2+}-dependent ATPase,[43] thus indicating that Ca^{2+} transport by sarcoplasmic reticulum is linked to Ca^{2+}-activated ATPase. Additional studies with pharmacological agents have shown that while both processes can be inhibited to the same extent by mersalyic acid (Salyngan), quinidine and procaine both selectively inhibit Ca^{2+} uptake to a greater extent than the Ca^{2+}-ATPase.[40] Evidence for the view that Ca^{2+} uptake and the Ca^{2+}-dependent hydrolysis of ATP are both catalysed by the one enzyme was thus contradictory. A reassessment of the effect of temperature on

the activation energy of the two processes has shown significant differences which led Charnock and Frankel[40] to suggest that the sites of Ca^{2+} uptake and Ca^{2+}-ATPase are not identical and the uptake process does not necessarily utilize energy from the hydrolysis of ATP by the Ca^{2+}-dependent ATPase. Their data clearly show the Ca^{2+} uptake process to have a constant activation energy of 18·2 kcal/mole over a temperature range of 0° to 37° in contrast with the increase in activation energy shown by the Ca^{2+}-ATPase at 10°. In this reaction the activation energy above 10° was 16·3 kcal/mole, not significantly different from the Ca^{2+} uptake process, but below 10° the activation energy increased to 33·4 kcal/mole.[40] Whilst the interpretation of this data in terms of evidence in support of two separate sites of Ca^{2+} binding is open to question, in view of the fact that the activation energies of both processes, in the range of physiological temperatures, are not significantly different, if does indicate how a study of the effect of temperature may be utilized to differentiate between two apparently interdependent processes.

It is also of interest to note the different transition temperatures observed for the Ca^{2+}-dependent ATPase (10°) compared to the Na^+ plus K^+-activated ATPase (20° for rabbit sarcoplasmic reticulum[38] and 22° for rabbit heart and endoplasmic reticulum fraction[29]). If the discontinuity shown by Ca^{2+}-dependent ATPase[30] at 10°, is related to a phase change in a lipid component of the membrane, as is evident for the Na^+ plus K^+-ATPase, then the Ca^{2+}-ATPase and associated lipid must occupy a discreet region of the membrane where the lipid component is not influenced by changes in the physical properties of the bulk of membrane lipids. An alternative explanation for the change at 10° shown by the Ca^{2+}-dependent ATPase is that the protein is susceptible to a temperature-induced conformational change at 10°.

Physiological Processes Related to Enzymes Associated with Endoplasmic Reticulum and Plasma Membranes

Arrhenius plots of a variety of physiological processes involving a number of enzymes,[1] show the processes have a constant activation energy. In multicomponent processes such as these the activation energy observed from the Arrhenius plot represents the activation energy of the rate limiting process. Thus, in view of the recent data a discontinuity would be expected if a membrane-associated enzyme is the rate limiting step and this enzyme exhibits anomalous temperature behaviour. In a study of the effect of temperature on the beat rate of isolated rat heart,[29] a change in the slope of the Arrhenius plot is observed at approximately 19°. Heart beat ceases at 10°, but is detected again when the temperature is increased, thus demonstrating the reversibility

of the process. The change at 19° probably corresponds with the change in activation energy of Na^+ plus K^+-activated ATPase of heart endoplasmic reticulum and plasma membrane,[29] a reaction which regulates the ion balance and thus controls the propagation of an action potential on which beat rate is dependent. The complete cessation of heart beat at 10°, which is reversible, could be a reflection of the change in activation energy of Ca^{2+}-activated ATPase reported by Charnock *et al.*[40] since muscle contraction and relaxation is dependent upon the activity of this enzyme. Although a large number of enzyme reactions are implicated in determining the rate of heart beat the large apparent activation energy of the overall process (60 to 70 kcal/mole) below 19° is the result of changes in the activation energy of the rate limiting processes. However, it is conceivable that changes in the permeability of ions due to a phase change in the membrane lipids and/or the decrease in energy production as a result of the increase in activation energy of state 3 respiration of heart mitochondria[14] could impose additional restrictions on the rate limiting step and thus increase the apparent activation energy of the overall process. Regardless of which particular enzyme reaction becomes rate-limiting below the transition temperature, it appears that the most significant event which would restrict the rate of muscular contraction of homeothermic animals is the temperature-induced phase change in the membrane lipids at about 22°. In poikilothermic animals there is no phase change in the membrane lipids, no change in the activation energy of the membrane ATPases[29] or oxidative reactions on which energy production depends[14] and heart beat in these animals show a constant apparent activation energy over a temperature range of 2° to 30°.[44] The hibernating ground squirrel, *Citellus mohavensis*, provides an interesting contrast to other homeothermic animals. Although the squirrel appears to have a number of characteristics of a homeothermic animal during the active summer months, its heart beat shows the typical behaviour of a poikilothermic animal; the heart beat rate decreases with decreasing temperature, but the apparent activation energy is constant.[45]

From the earlier observations of an association of ribosomes with membranes of the endoplasmic reticulum by Palade and Siekevitz[46] protein synthesis has been considered as a membrane-associated process. Since the lipids of the endoplasmic reticulum of liver cells of homeothermic animals have been shown to undergo a phase change at approximately 22°, it is considered likely that the activity of the associated protein synthetic system might reflect this change in the physical properties of the membrane. Preliminary data has shown that the kinetics of incorporation of amino acids by a microsomal fraction from rat liver change dramatically at 23° resulting in a substantial increase in the activation energy of the process, as shown in Fig. 6. The fact that the change in activation energy occurs at 23° is strong evidence

for implying that the change in kinetic properties of the enzymes is induced by a phase change in the membrane lipids. This view is further substantiated by the observation that the incorporation of amino acids catalysed by a membrane-free preparation of ribosomes, from the cytoplasm of rat liver cells does not show a change in activation energy within the temperature range of 2° to 30°.[45] A similar increase in activation energy for the incorporation of amino acids, and hence the protein synthetic system, probably occurs in the cells of chilling-sensitive plants, at 10° to 12°, since similar phase changes have been observed in membranes of chilling-sensitive plant tissue at these temperatures. The different effects of temperature on the membrane-bound

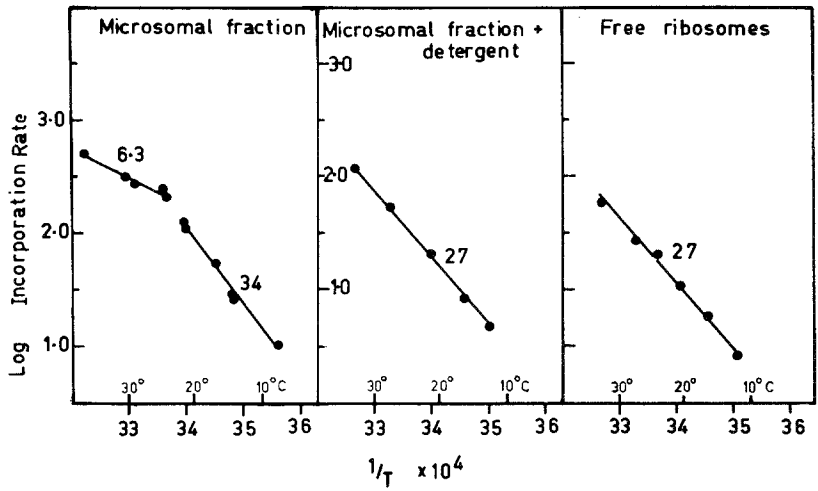

Figure 6. The effect of temperature on the activation energy of ^{14}C-leucine incorporation by a microsomal fraction and free ribosomes of rat-liver cells. (Data from Towers, Raison, Kellerman and Linnane.[47])

and free ribosome incorporating systems could be a useful method of determining whether the ribosomes of a particular incorporating system are associated with membranes, for example in organelles. The amino acid incorporation activity of rat-liver mitochondria has been found to exhibit an increase in activation energy as the temperature is decreased below 23° suggesting that the majority of the mitochondrial ribosomes are functionally associated with the mitochondrial inner membrane system.[47]

Recent evidence has shown that DNA-like RNA (D-RNA) nuclear particles, which contain messenger RNA (m-RNA), move from the nucleus to the cytoplasm of Krebs tumour cells, and become attached to cytoplasmic membranes.[48] Ribosomes attach to the bound m-RNA to form the translation complex.[48] The change in activation energy of the protein synthetic system might therefore be a reflection of a change

in the conformation of m-RNA induced by changes in the surface properties of the membrane.

Enzymes Associated with the Photosynthetic-Electron Transfer System of Chloroplast Membranes

The photosynthetic capacity of some species of plants, classified by Chen *et al.*[49] as "efficient", show a sudden decrease in activity when the temperature is reduced below about 12°. The "efficient" plants were so characterized because of their high photosynthetic capacity, low CO_2 compensation concentration and the presence of the C_4-dicarboxylic acid cycle and are mostly chilling-sensitive type plants of tropical

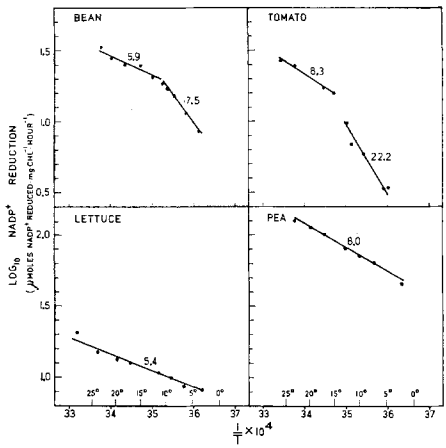

Figure 7. Arrhenius plots of the rate of NADP reduction from water for chloroplasts from chilling-sensitive (bean and tomato) and chilling-resistant (lettuce and pea) plants. The numbers beside each line are the activation energy in kcal/mole. (Data from Shneyour, Raison and Smillie.[50])

origin. Recent studies in this laboratory have shown that the decrease in the photosynthetic capacity of chilling-sensitive plants at temperatures below approximately 12° can be directly related to a change in activation energy of membrane-associated enzymes of the electron transfer system.[48]

Arrhenius plots of the rate of photoreduction of NADP from water for chloroplasts from chilling-sensitive plants (bean and tomato, Fig. 7) show a discontinuity at between 10° to 12°, similar to the discontinuity in Arrhenius plots of respiratory activity of mitochondria from some chilling-sensitive plants.[6] The increase in activation energy for the process below the transition temperature is usually in the order of three fold and is observed with chloroplasts from all the chilling-sensitive plants examined. No change in activation energy is observed with chloroplasts of chilling-resistant plants, e.g. lettuce and pea (cf.

Fig. 7). The electron transport systems of both mitochondria and chloroplasts of chilling-sensitive plants thus show similar temperature-dependent changes at the same temperature and contrast with the absence of these changes in either organelle of chilling-resistant plants.

As described previously, the distinction between the temperature response of mitochondria from chilling-sensitive and chilling-resistant plants can be directly related to the proportion of saturated and unsaturated fatty acids in the lipids of the mitochondrial membranes.[6] The same relationship, however, does not hold for chloroplast lipids of chilling-sensitive and chilling-resistant plants. Chloroplasts contain relatively high proportions of galactosyl dilinolenins (80% of total lamella lipid), and about 10% each of phosphatidy glycerol and sulphoquinovosyl diglyceride, both of which contain a linolenic or linoleic

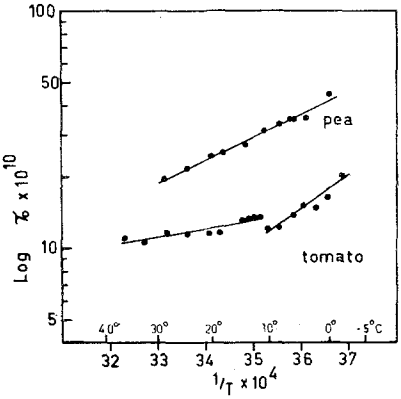

Figure 8. Arrhenius plot of the change in rotational correlation time (T_0) of the spin-label 12-nitroxide stearic acid in preparations of chloroplasts from pea and tomato leaves, (J. Raison, unpublished data.)

acid residue.[51] Based on the high content of linolenic acid in chloroplasts, the freezing point of the bulk lipids would probably be well below 10°.[25] However, physical measurements of the mobility of lipids in the chloroplast membranes by e.s.r. spectroscopy of spin-labelled fatty acids show that a phase transition occurs at approximately 10° for chloroplasts isolated from chilling-sensitive tissues (see Fig. 8). No phase change is observed in chloroplasts of chilling-resistant plants at this temperature. Thus, although a phase change in the lipids of chloroplast membranes of chilling-sensitive plants would not be predicted, considering the physical properties of the constituent fatty acids, the e.s.r. data clearly show a phase transition occurs at the same temperature as the change in activation energy of the photosynthetic electron transport enzymes and the same temperature as the phase changes in mitochondrial membrane lipids. There is little doubt that the change in activation energy of the electron transport system is a

consequence of a phase change in a lipid component of the membrane, but there is insufficient data at present to predict what proportion of the total lipids is involved in the phase change and what other enzymes are affected. The increase in the activation energy of photochemical activity at the same temperature as the increase in activation energy of respiratory enzymes, provides a biochemical basis for understanding the changes in carbon metabolism,[52] photosynthetic activity[53] and morphology of chloroplasts[54] when chilling-sensitive plants are exposed to temperatures below the transition point.

Structure and Function of Membranes in Relation to Temperature-Induced Phase Changes

Various structure-function relationships have been demonstrated with membrane systems, particularly the cristae membrane of mitochondria.[55–57] The coincidence of the phase transition in the membrane lipids and the conformational change in the enzyme protein at the same temperature in a number of different membranes of plant and animal tissues provide an excellent example of this relationship, and raises important questions concerning the molecular structure of these membranes. The available data establishes membrane lipids as the locus of temperature sensitivity, but does not provide any direct evidence to indicate whether all of the lipids of the membrane undergo the phase transition or only particular or specific lipids associated with enzymes.

The correlation between the degree of unsaturation of the fatty acids of the bulk membrane lipids and the temperature at which the organisms normally function has been well established for plant and animal mitochondria,[6,14] *Mycoplasma laidlawii*,[23] bacteria[58,59] and fungi.[60] This would suggest that the fatty acid composition of membrane lipids is regulated to maintain the bulk membrane lipids in a similar physical state at the various growth temperatures and is understandable in view of the large increase in the activation energy of membrane-associated enzymes below the temperature of the phase change. Furthermore, both the calorimetric and spin-label method of detecting the phase change have demonstrated similar temperature-dependent, physical properties for the lipids in particulate membrane preparations and in lipid micelles formed from the lipids extracted from the membranes.[23,28] In the case of the spin-label method, the temperature of the phase change coincides precisely with the temperature at which all of the oxidative enzymes of the mitochondrial, inner membrane show a change in activation energy. The available evidence would therefore favour the view that the kinetic properties of the membrane-associated enzymes are regulated by the physical state of the bulk membrane lipids. In view of the results obtained it can be assumed that specific phospho-

lipids which are known to be associated with succinate dehydrogenase,[61] cytochrome oxidase[62] and mitochondrial ATPase,[32] for example form an integral part of the bulk membrane lipids and are indistinguishable from the bulk lipids by the physical methods used to detect phase changes.

The structural proteins of membranes do not appear to influence the temperature at which the membrane lipids undergo a phase change which would suggest only a small proportion of the membrane lipids are involved in hydrophobic interactions with protein. This view is supported by the fact that the temperature of the phase change in mitochondrial membranes is the same regardless of whether the protein is heat denatured or in a native state and coincides with the temperature of the phase change in extracted lipids.[28] Furthermore, a comparison of the heat of transition for the lipids of *M. laidlawii* membranes with that for the extracted lipids indicates that 90% of the membrane lipids are in a bilayer configuration and only 10% are involved in hydrophobic interactions, presumably with protein.[63]

Since the change in the physical state of the membrane lipids must be transmitted to the membrane associated enzymes, to bring about the conformational change associated with the change in activation energy, there must be some structural association between the enzyme proteins and the bulk lipids. The specific phospholipids known to be associated with some membrane-associated enzymes might provide this link.

Another interesting question to arise from the relationship between the composition of membrane lipids and the minimum temperature for normal physiological functioning of the organism, is whether this temperature can be reduced by lowering the "freezing point" of the membrane lipids. The implications of success in being able to manipulate the membrane composition and reduce the minimum functional temperature would be of immense value to agriculture and medicine.

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