AN ABRUPT TEMPERATURE-DEPENDENT CHANGE IN THE ENERGY OF ACTIVATION OF HORMONE-STIMULATED HEPATIC ADENYLYL CYCLASE

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1. In the stimulation of rat hepatic adenylyl cyclase by glucagon or epinephrine we observe an abrupt change in the energy of activation at 32°C (seen as an increase in the slope of the Arrhenius plot). The energy of activation for the cyclase reaction above 32°C is about 1.7 times that found below this temperature. Cyclase activity stimulated by fluoride, prostaglandin E_1, or 1-propanol, or activity in the absence of added stimulators does not show this change. The structural differences between the hormones suggest that they interact with the cyclase system at different loci. But the mechanism by which they stimulate cyclase activity appears to involve a common, temperature-dependent step.

2. In the presence of 1-propanol the change at 32°C in the energy of activation of the hormone-stimulated activity is not observed.

3. In view of the relatively large mole fraction of cholesterol present in the rat liver plasma membrane (which appears to inhibit phase transitions in bulk membrane lipids), it is suggested that this thermal sensitivity resides in protein rather than lipid components or that the cyclase is restricted to cholesterol-poor membrane regions.

4. The occurrence of anomalous Arrhenius plots of enzyme activities (with abrupt changes of slope) for both membrane-bound and soluble enzymes is reviewed.

INTRODUCTION

Significant changes in the slope of the Arrhenius plot as a function of temperature have been reported for the activities of a number of enzymes, especially for those which are bound to a membrane. In some cases the change in energy of activation appears to reflect a "phase transition" in the bulk lipid of the membrane. In other cases, especially when the enzyme in question is purified and soluble, such an explanation is not possible and the two-slope anomaly in the Arrhenius plot must reflect changes in the enzyme itself. Both upward breaks in the Arrhenius plot (increased slope and hence increased energy of activation at higher temperatures) and downward breaks (decreased energy of activation at higher temperatures) have been described. The mechanisms to account for the two types of behavior may in many cases be different. In this paper we consider only reversible effects on the enzyme activity (i.e., effects due to irreversible "thermal denaturation" are excluded).

It has been suggested that upward breaks might be explained by the presence of two

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parallel reactions catalyzed by different active centers with different energies of activation. At higher temperatures the reaction with the higher energy of activation would predominate and the Arrhenius plot would curve upward (1). Downward breaks might be explained in terms of two successive reactions with different energies of activation. Since the step with the higher energy of activation would be more rapid at higher temperatures, the step with the lower energy of activation would become rate limiting at higher temperatures and the Arrhenius plot would curve downward (2). A difficulty with both of these explanations is that unless the difference in activation energy for the two reactions is enormous, the Arrhenius plot will curve over a very wide range of temperature and the data will not be well represented by straight lines.

A downward break (or curvature) for urease was explained by Kistiakowsky and Lumry (3) in terms of reversible inactivation of the enzyme. If the heat change on converting from the active to the inactive form is ΔH, and the energy of activation for the catalytic process is E, at lower temperatures when the enzyme is mainly in the active form the slope will represent E. At higher temperatures when the enzyme is mostly in the inactive form the slope will represent $E-\Delta H$. Massey has extensively studied two enzymes, furmarase (4), and D–amino acid oxidase (5), which in solution exist in different forms, both of which have catalytic activity, but with different energies of activation. At alkaline pH, fumarase gives upward breaks with either fumarate or malate as substrates. At acid pH a downward break is seen with fumarate but no break with malate. In addition, at low pH a plot of log K_m for fumarate against 1/T shows a break.

For D–amino acid oxidase there is a conformational change at 14°C which is reflected in tryptophan fluoresence and in other physical properties as well as in the catalytic activity. With alanine as substrate a downward break is seen at 14°C; with methionine a downward break is seen at 24°C (the change in temperature apparently reflecting a difference in binding of this substrate to the two forms of the enzyme). For either of the above mechanisms the break in the Arrhenius plot will not be sharp unless the enthalpy change for converting from one form of the enzyme to the other is quite large. Massey (5) has shown that if the enthalpy change for this process is 50 Cal/mole, the data can be well represented by two straight lines connected by a region of curvature over just 2°C. For a greater enthalpy change the region of curvature will be less.

A number of membrane-bound enzymes in E. coli show downward breaks in Arrhenius plots of their activity. In most cases these breaks appear to be correlated with a phase transition in the bulk membrane lipid. An unsaturated fatty acid auxotroph utilizes whatever fatty acids are available from the medium, permitting manipulation of the lipid content of the membrane. Thus the temperature at which the membrane "melts" can be changed and breaks in Arrhenius plots of respiration (6), galactoside transport (6–8), glucoside transport (7, 8), and succinic ubiquinone reductase activity (9, 10) vary also. When lipids are extracted with acetone the activity of succinic ubiquinone reductace is reduced to about one third of its previous value and the break is abolished. Addition of appropriate lipids to the delipidated enzyme increases the activity and restores the break (with the break temperature dependent on the mixture of lipids added back). Some membrane-bound enzymes in E. coli either exhibit no break or have a break (or curvature) of the Arrhenius plot which is almost totally independent of the lipid composition of the membrane (11). For all of the membrane enzymes which show a break in the Arrhenius plot, it is downward, with a smaller energy of activation at high temperature.

Activities of a number of the enzymes of mitochondrial oxidative phosphorylation also have Arrhenius plots with breaks. Succinate dehydrogenase, cytochrome oxidase, and

β-hydroxybutyrate oxidase activities of rat-liver mitochondria shown downward breaks at 23°C. In mitochondria of chill-sensitive sweet potatoes these enzymes have breaks at 9°C (12). In mitochondria from fish liver or chill-insensitive sweet potatoes there are no breaks. In the mitochondria which show the breaks, freezing and thawing have little effect but treatment with detergents abolishes the breaks (12). When succinate dehydrogenase is solubilized from beef-heart mitochondria by butanol extraction it retains the downward break at 18°C which is seen with whole mitochondria and in addition shows a new upward break at 27°C (13). Sanadi showed that ATP-dependent reversal of electron transport and the oligomycin-sensitive ATPase activity show breaks in the Arrhenius plots in the range 27–37°C. Reversal of electron transport at coupling-site 1 and the ATPase have downward breaks while reversal of electron transport at coupling-site 2 has an upward break (14). The mole fraction of cholesterol in the mitochondrial membrane is less than 0.05 (15), so a phase transition of bulk membrane lipid is possible and may account for many or all of the biphasic Arrhenius plots seen with mitochondrial membrane-bound enzymes.

In this paper we describe a biphasic Arrhenius plot found with hormone-stimulated adenylyl cyclase (cyclase) activity of rat liver. There is an upward break at 32°C for glucagon- or epinephrine-stimulated cyclase activity but no break for fluoride- or prostaglandin-stimulated cyclase activity, or for activity in the absence of added stimulators (basal). In view of the high cholesterol content of the liver plasma membrane it is probable that the break does not reflect a phase transition in bulk membrane lipid. So our observation suggests either that cyclase is restricted to cholesterol-poor membrane domains or that the change in its energy of activation reflects properties of the cyclase apparatus itself.

MATERIALS AND METHODS

Washed membrane particles from the livers of normal and adrenalectomized weanling (50 g) female Sprague Dawley rats were prepared as previously described (16). For the cholera experiments weanling female rats (50 g) were given 50 μg of purified (17) cholera toxin intraperitoneally and killed after 4 hours. In evaluating effects of stimulators on cyclase, the compounds were routinely preincubated with the membrane particles for 15 minutes at ice temperature. Porcine crystalline glucagon and L-epinephrine bitartrate were purchased from Sigma. The prostaglandin (PGE_1) was a gift from Drs. Caldwell and Speroff of Yale University. The purified cholera toxin was kindly provided by Dr. Robert Northrup of NIAID.

Cyclase activity was assayed as previously described (18). Labeled cyclic AMP was purified by descending thin-layer chromatography (19). Assay of cyclase was carried out using a 3-minute incubation period at the temperatures indicated, in the presence of 1.6 mM [8–^{14}C] ATP (45 Ci/mole), or [8–^{3}H] ATP diluted to the same specific activity, 7 mM aminophylline, and an ATP regenerating system (38 mM phosphocreatine and 2 mg/ml creatine phosphokinase) in buffer 1 (3 mM $MgSO_4$, 0.4 mM EDTA, and 32 mM glycylglycine, pH 7.4). Assay of 3′,5′ nucleotide phosphodiesterase was carried out by incubating in the presence of 1.0×10^{-3} [8–^{3}H] cyclic AMP (diluted to 50 Ci/mole) in buffer 1, and measuring the disappearance of substrate in 1 minute. Alkaline phosphatase was assayed using p-nitrophenyl phosphate (20). Creatine phosphokinase was assayed in the presence of 38 mM phosphocreatine and 1 mM ADP in buffer 1 by following the consumption of H^+. Protein concentrations were determined by the method of Lowry

(21). The slopes for the Arrhenius plots and the existence and location of break points were determined by linear regression analysis. The fit for a single line (from 23 to 39°C) was compared to various possible two-line fits (e.g., one line from 23°C to 30°C, a second from 30°C to 39°C; one line from 23°C to 32°C, a second from 32°C to 39°C, etc.). The fit which gave the smallest value for the sum of the squares of the deviations in activity was selected. The energy (or energies) of activation were determined from the slope(s), and for a two-line fit the temperature at which the two lines intersect was determined.

RESULTS

Figure 1 shows the temperature dependence for both epinephrine- and glucagon-stimulated hepatic systems. Examination of the Arrhenius plots of these data (Fig. 2) indicates that in the presence of hormones, there is a change in the thermodynamic properties of the enzymic mechanism which is characterized by an abrupt increase in the energy of activation at 32°C. For both hormone-stimulated reactions, the energy of activation is approximately 1.7 times as great above 32°C as below. This difference in energy of energy of activation is statistically significant ($p<0.001$). The temperature at which this change is observed is very reproducible (e.g., for glucagon the mean value for seven different temperature curves is 32.2 ± 0.5°C; for epinephrine the mean value for five different curves is 31.8 ± 0.7 (Table I). The abrupt increase in energy of activation is not observed in the fluoride-, prostaglandin E_1, or 1-propanol-stimulated systems, or in

Table I. Effects of Stimulators and 1-Propanol on the Energy of Activation for the Cyclase Reaction

Stimulator (concentration)	Number of temperature curves[a]	Temperature at which energy of activation breaks (range 23°–39°C)	Energy of activation (kcal/mole) Below 32°	Above 32°
None (basal)	9	No break	– 9.8±0.5	–
Glucagon (1.6×10^{-5} M)	7	32.2±0.5°C	10.0±0.7	17.5±1.1
Epinephrine (2.5×10^{-5} M)	5	31.8±0.7°C	9.4±0.5	17.6±1.7
Prostaglandin E_1 ($2.8 \times 10-^{-5}$ M)	4	No break	– 11.1±1.3	–
Fluoride (10^{-2} M)	4	No break	– 11.0±1.0	–
Glucagon (1.6×10^{-5} M) and 1-propanol				
(0.42 M)	3	No break	– 20±1.8	–
(0.21 M)	2	No break	– 18±1.3	–
(0.08 M)	2	No break	– 16±1.9	–
(0.02 M)	2	No break	– 15.4±1.6	–
Epinephrine (2.5×10^{-5} M) and 1-propanol				
(0.42 M)	2	No break	– 14±1.6	–
1-propanol (0.42 M)	4	No break	– 12±1.6	–
Cholera toxin	2	No break	– 10..7±1.7	–

[a]Each temperature curve consists of triplicate determinations for each of 10 temperatures. Errors shown are standard deviations of the mean for the number of curves indicated.

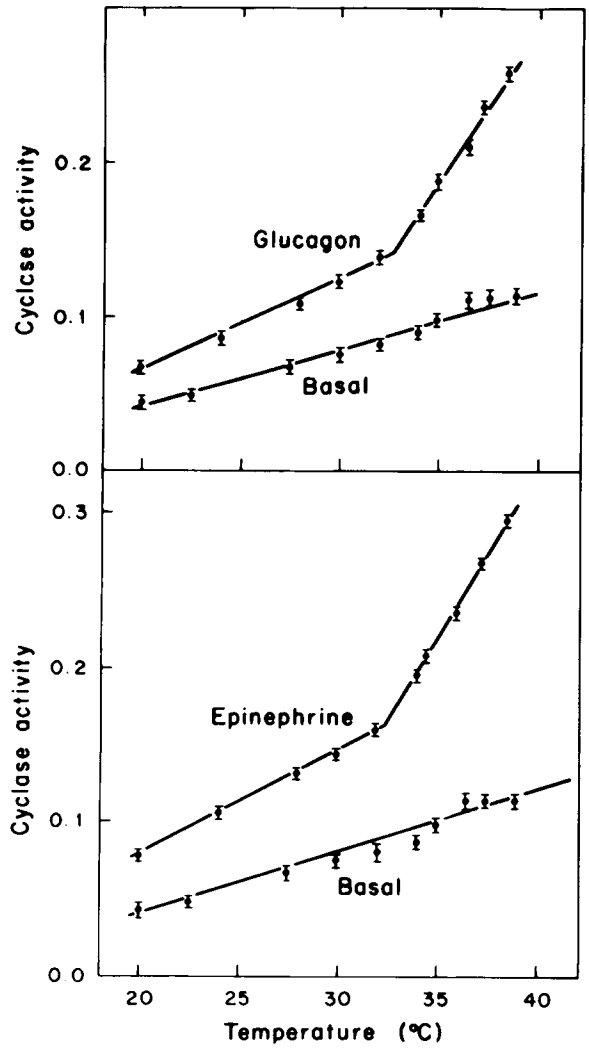

Fig. 1. Cyclase activity (nmole cAMP/min/mg. of protein) of liver washed particles. Data shown are for one of several experiments, with mean and extreme values of a triplicate determination. Top: with no added stimulator (basal) or with 1.6×10^{-5} M glucagon. Bottom: with no added stimulator or with 2.5×10^{-5} M epinephrine.

the absence of stimulators (basal cyclase activity) (Fig. 3). When the best two-line fit with a break at 32°C was found for the basal, fluoride-, 1-propanol-, or prostaglandin-stimulated systems, an insignificant change in energy of activation of the fluoride- and 1-propanol-stimulated systems was found as a function of temperature between 23° and 39°C. A small decrease in the energy of activation (in contrast to the marked increase seen with the glucagon- or epinephrine-stimulated system) was found for basal and the prostaglandin-stimulated cyclase activities. This small decrease in activation energy might reflect denaturation at higher temperatures. For data obtained with the hormone-stimulated systems a two-line model with a sharp break gave a much better fit than a one-line model (sum of the squares of the deviations at least three times greater for the one-line model).

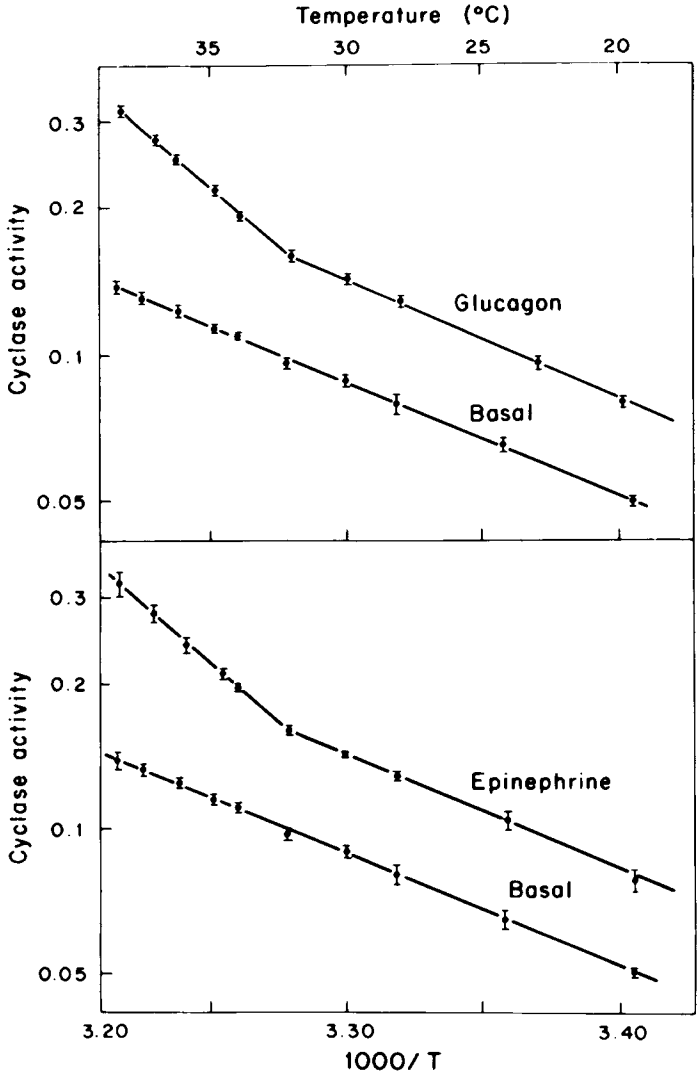

Fig. 2. Arrhenius plot of cyclase activity data from Fig. 1.

For data obtained with basal, prostaglandin-, 1-propanol-, or fluoride-stimulated systems there were no significant advantages to a two-line fit. It is interesting that the energies of activation for the basal cyclase system and for the fluoride- or prostaglandin-stimulated enzymes are similar to the low-temperature values for the hormone-stimulated systems. Above 39°C there is a fall in basal and epinephrine-stimulated cyclase activity presumably due to thermal denaturation. A fall in glucagon-stimulated activity during the assay is only apparent above 50°C. The serine protease inhibitor phenylmethylsulfonyl fluoride (10^{-4} M) does not significantly enhance the enzyme activity (in the presence or absence of hormones) at higher temperatures. Previous experiments (22) have shown that hepatic cyclase (measured in vitro) is activated by cholera toxin injected in vivo (4 hours prior to sacrifice). The temperature characteristics of cholera toxin stimulation of hepatic cyclase were studied. Cholera toxin appears to resemble fluoride and prostaglandin E_1 in produc-

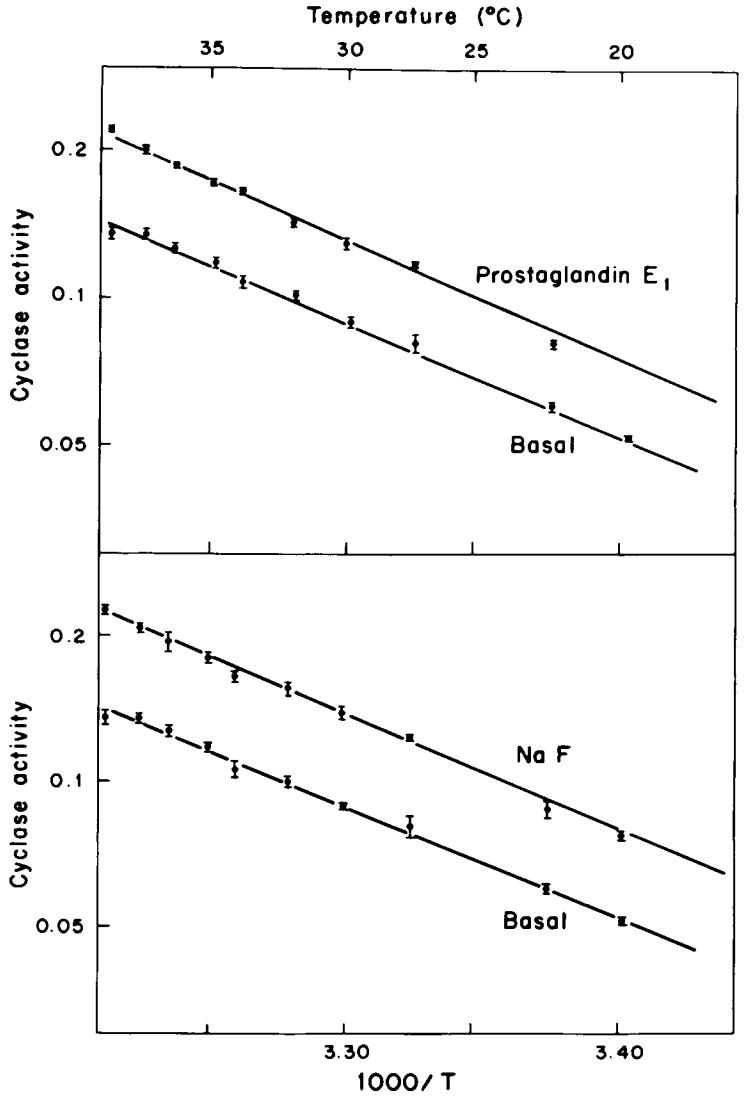

Fig. 3. Cyclase activity (nmole cAMP/min/mg. of protein). of liver washed particles. Top: with no added stimulator (basal) or with 2.8×10^{-5} M prostaglandin E_1. Bottom: with no added stimulator or with 10^{-2} M NaF.

ing stimulation without a break in energy of activation between 32° and 39°C.

The process producing the break in the Arrhenius plot appears to be reversible since membrane particles which were preincubated at different temperatures (30°C or 37°C for 5 min) or at one temperature for different times (30°C to 1 or 10 min) exhibit the same temperature-activity relationships for basal or glucagon-stimulated cyclase as membrane particles which have been kept at ice temperature. The apparent change in energy of activation with temperature is not explained by changes in the duration of a "lag phase" [which has been described in association with stimulation by hormones (23)] as a function of temperature. The reactions were linear during the entire 3 minutes of incubation at temperatures above, at, and below the break point. The increased energy of activation

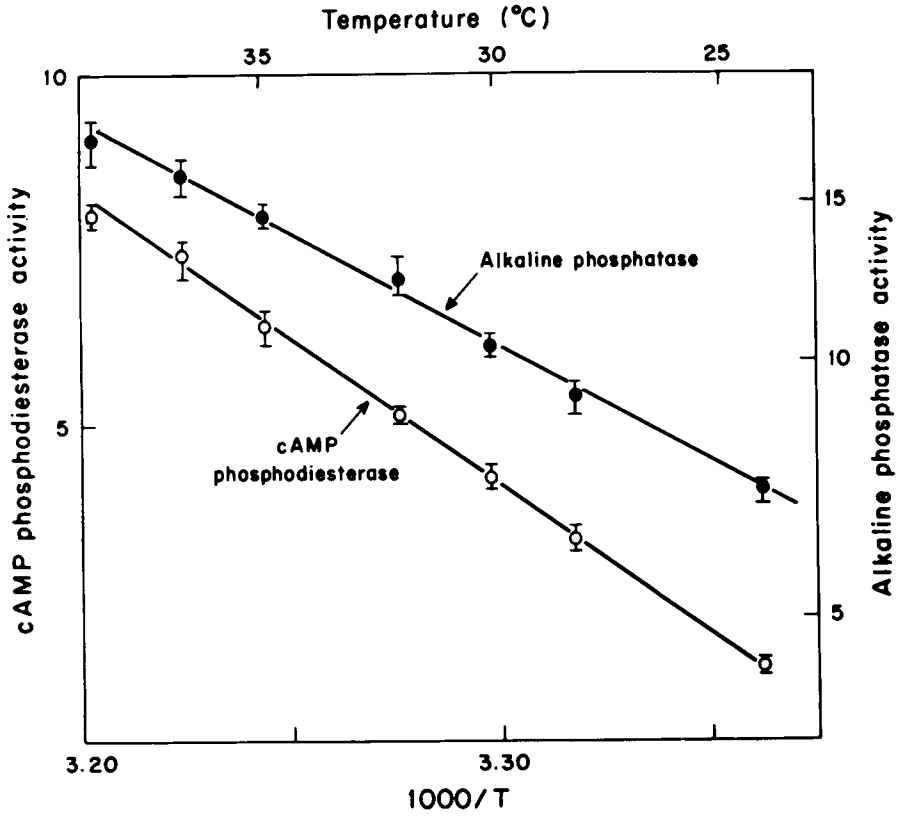

Fig. 4. Alkaline phosphatase activity (•) (nmole P_i /min/mg. of protein) and cAMP phosphodiesterase activity (○) (nmole cAMP/min/mg. of protein) of liver washed particles.

for the reaction found with hormones at higher temperatures is accounted for by an increased V_{max}, since for the glucagon-stimulated cyclase the K_m for ATP increases slightly at higher temperatures (0.66 ± 0.11 mM at 28°C and 0.90 ± 0.17 mM at 38°C). The effect of temperature on cyclase activity cannot be explained by a change in the pH of the assay mixture with temperature. There is a pH change from 7.4 at 23°C to 7.0 at 39°C, but the activity of the enzyme is almost constant in this pH range. The slight perturbation introduced by temperature-dependent changes in pH would tend to reduce rather than increase the change in energy of activation.

The anomalous Arrhenius plot with glucagon is not explained by increased glucagon solubility at higher temperatures, since it is observed at a variety of glucagon concentrations below the solubility limits for this peptide. In addition, the effect was observed in the epinephrine-stimulated system, where saturating concentrations of hormone are well below its solubility limit. A change in the energy of activation (at 32°C) was not found for the membrane-associated fraction of cyclic AMP phosphodiesterase (Fig. 4) or for creatine phosphokinase (a omponent of the ATP regenerating system). Several other membrane marker enzymes do not show this change in energy of activation, including alkaline phosphatase (Fig. 5), 5′ nucleotidase, and Na^+, K^+ activated ATPase (24).

Short-chain alcohols have been found to produce a marked increase in glucagon-stimulated cyclase activity and a small increase in basal and epinephrine-stimulated activity

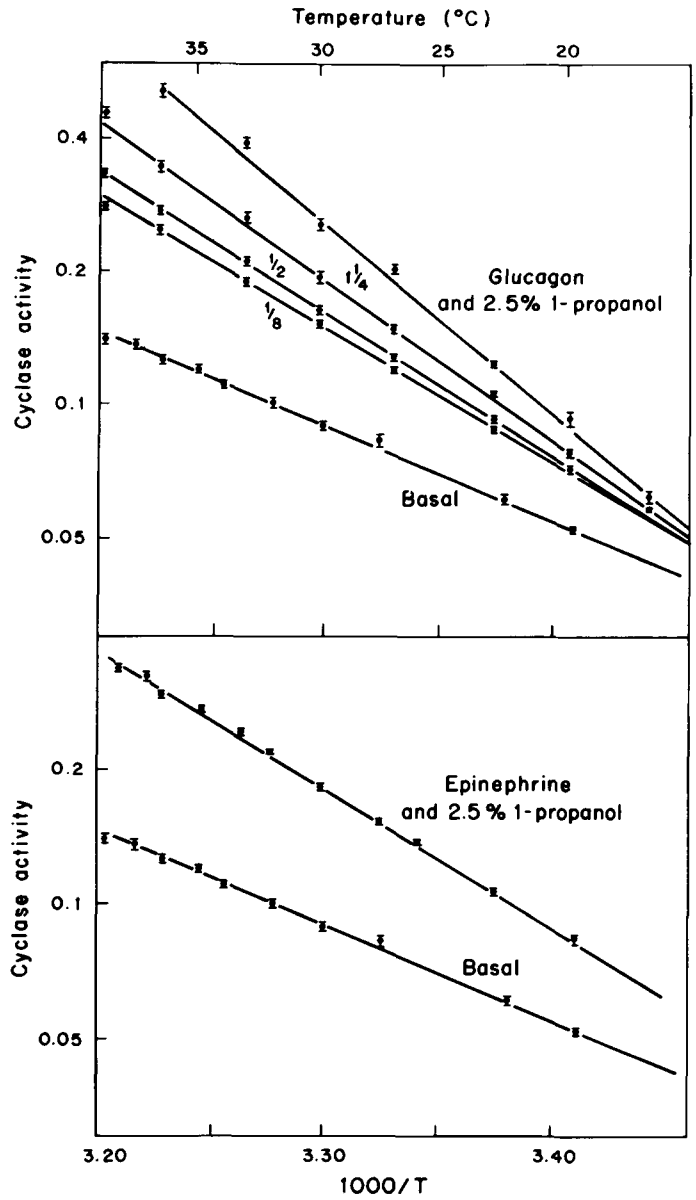

Fig. 5. Cyclase activity (nmole cAMP/min/mg. of protein) in the presence of 1-propanol and hormone. Top: with no added stimulator (basal) or with 1.6×10^{-5} M glucagon and 0.42, 0.21, 0.08, or 0.02 M 1-propanol. Bottom: with no added stimulator or with 2.5×10^{-5} M epinephrine and 0.42 M 1-propanol.

(25). The increases in activation energy with temperature in the presence of both epinephrine and glucagon were abolished by 1-propanol (Fig. 5). In the presence of 1-propanol, the energy of activation for the glucagon-stimulated liver cyclase reaction varies depending on the concentration of 1-propanol, but is similar to the value at high temperature in the absence of 1-propanol. For the epinephrine-stimulated enzyme, in the presence of 1-propanol (0.32 M), the energy of activation is intermediate between those found above and below the break point in the absence of 1-propanol (Table I).

DISCUSSION

The experiments described illustrate that the liver cyclase system in the presence of stimulators like epinephrine or glucagon exhibits an abrupt increase in the slope of the Arrhenius plot which occurs at 32°C. The exposure of the membranes to 1-propanol results in abolition of the abrupt changes in the energy of activation at 32°C. With nonhormonal stimulators such as prostaglandin E_1, fluoride, or cholera toxin (introduced in vivo), the break in energy of activation is not observed in spite of the fact that they are effective stimulators of hepatic cyclase activity. The change in the energy of activation appears neither to be associated with thermal effects on the availability of substrate or the destruction of product nor is it due to effects on the solubility of hormone stimulators or the number of available hormone binding sites. Further, the effect is not universal since a variety of other enzymes in the same membranes are not influenced in this manner (phosphodiesterase, alkaline phosphatase, 5′ nucleotidase, and ouabain-sensitive ATPase).

The effect does not appear to involve differences in the rate of formation, opening, or inversion of membrane vesicles, since the effect is reversible and is not influenced by prior incubation of the cyclase system. The fact that stimulation of cyclase by prostaglandin E_1, fluoride, or cholera toxin is not associated with this change in energy of activation suggests that the mechanism by which they stimualte cyclase is profoundly different from that for the hormonal stimulators.

In view of the large mole fraction of cholesterol in most mammalian membranes it is suggested that the changes in energy of activation observed here are probably occurring in protein components rather than lipid components of the cell membranes. The magnitude of thermal transitions in membrane lipids is markedly diminished when the mole fraction of cholesterol is above 0.20 and transitions are absent by 0.33 (26–28). The mole fraction of cholesterol in the purified preparations of liver plasma membranes is about 0.30 (29, 30), so that thermal transitions in bulk lipid would not occur. Under these conditions temperature-sensitive protein–protein or protein–lipid interactions are more likely to be involved in the cyclase stimulation sequence. However, we cannot exclude the possibility that cyclase resides exclusively in cholesterol-poor regions of the membrane and is affected by thermal transitions in the lipids of these regions. The ability of propanol to abolish the break in the Arrhenius plot and itself alter the energy of activation, and the absence of an Arrhenius plot break in the stimulation of cyclase by fluoride and prostaglandin E_1 suggest that the break is not an obligatory event in the cyclase stimulation sequence but rather is a feature of the interaction of some components in this sequence.

We observe that the stimulation of cyclase may be accomplished by such diverse reagents as fluoride, prostaglandin E_1, and cholera toxin. The processes for cyclase stimulation are presumably also quite different. [possibly in the case of cholera toxin involving protein synthesis (31)] ; For these stimulators, however, the energy of activation for the cyclase reaction appears similar and does not exhibit temperature-dependent changes. The stimulation sequence in the presence of epinephrine or glucagon appears very sensitive to membrane disturbances, as evidenced by the abolition of the change in energy of activation with these hormones in the presence of low concentrations of 1-propanol (too low to produce by themselves a measurable change from basal cyclase activity).

The question raised by these observations is whether the cyclase is restricted to very

special membrane regions which differ markedly in content of cholesterol and other lipids from the rest of the membrane or whether cyclase in fact is not in a special region so that the effect is a property of protein components of the cyclase apparatus. One way of exploring this question is to see the effects of such reagents as 1-propanol on other membrane enzymes. We will have to examine other kinetic characteristics of these enzymes since the other membrane enzymes examined do not exhibit changes in the energy of activation. Also, examination of the effects of detergents and, more importantly, of specific lipases on both physical properties of the membranes and on the energy of activation for the cyclase reaction might indicate whether their effects were specifically concerned with lipids. In these diverse ways the change in energy of activation of cyclase should provide insight both into the chemical structure of the membrane region which houses cyclase and possibly provide a sensitive tool for detecting the consequences of various effectors on membranes.

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