Temperature Dependence of β Receptor, Adenosine Receptor, and Sodium Fluoride Stimulated Adenylate Cyclase from Turkey Erythrocytes†

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ABSTRACT: The individual temperature dependencies of the processes which control the activity of turkey erythrocyte adenylate cyclase have been determined. The temperature dependence of the fraction of activable cyclase units experiences a thermal transition at 24 °C for all three modes of enzyme activation: *l*-epinephrine, adenosine, and NaF. This thermal transition probably reflects the phase transition in the inner monolayer of the membrane which influences the behavior of the GTP regulatory unit which is involved in all three modes of enzyme activation. The "rate constant" of enzyme activation by adenosine reflects two thermal transitions, at 24 and at 35 °C; the apparent rate constant of cyclase activation by NaF activation experiences a transition only at 24 °C whereas the rate constant of the β-receptor-bound agonist decreases monotonously with no "breaks" on the Arrhenius plot. Following the temperature dependence of the fluorescence intensity of dansylphosphatidylethanolamine embedded in both sides of the membrane and exclusively in the outer monolayer, one can assign the thermal transition of 24 °C to the inner monolayer and the other two transitions to the *outer monolayer (10 and 35 °C)*. We interpret these results as follows. (a) The monomolecular rate constant characterizing the activation of cyclase by the precoupled adenosine receptor experiences both the transition at 24 and 35 °C, indicating that the latter may span the bilayer. (b) The β receptor activates the cyclase units only in fluid areas since it can diffuse exclusively in the fluid areas of the membrane and is unable to interact with cyclase units in "frozen" areas. The linear dependence of the logarithm of the rate constant on $1/T$ for the β receptor reflects the change of membrane fluidity as a function of temperature.

In a variety of systems it has been demonstrated that the physiochemical properties of membranal lipids strongly influence the activity and efficiency of membranal systems (Cuatrecasas, 1974) such as the galactose transport system (Thilo et al., 1977), (Na^+,K^+)-ATPase (Kimelberg & Papahadjopoulos, 1972, 1974; Wheeler et al., 1975), Ca^{2+}-ATPase (Warren et al., 1974, 1975), and the adenylate cyclase system (Rubaclava & Rodbell, 1973; Puchwein et al., 1974; Orly & Schramm, 1975; Rimon et al., 1978; Hanski et al., 1979; Bakardjieva et al., 1979). Perturbation of the chemical composition of the membrane by the insertion of fatty acids (Orly & Schramm, 1975) or by changing the composition of the phospholipids (Zenser et al., 1976; Klein et al., 1978; Engelhard et al., 1976; Bakardjieva et al., 1979; Levey, 1973; Dipple & Houslay, 1979) modulates the activity of the above-mentioned systems. For all of these systems a good correlation was found to exist between the inflection temperature observed in the Arrhenius plot of the temperature dependence of the enzyme activity and the transition temperature of the membranal phospholipids (Houslay et al., 1976; Kimelberg & Papahadjopoulos, 1974; Wisnieski et al., 1974). Below the transition temperature the phospholipid molecules tend to acquire a crystalline organization at which their translational and rotational movements are restricted (Lee, 1977; Hubbell & McConnel, 1971; Levine et al., 1972). Above the transition temperature the phospholipids are in a fluid state. In this study we present an attempt to analyze in detail the temperature dependence of the parameters which determine the steady-state activity of the turkey erythrocyte adenylate cyclase and explore the origins of the discontinuities observed in the Arrhenius plot of the steady-state adenylate cyclase activity of this system (Kriner et al., 1973; Counts & Jutisz, 1977; Bar, 1974).

Turkey erythrocyte adenylate cyclase is converted from its inactive state to its cAMP producing state in the presence of a β agonist and GTP (Sevilla et al., 1976; Tolkovsky & Levitzki, 1978a,b) or in the presence of adenosine and GTP (Sevilla et al., 1977; Tolkovsky & Levitzki, 1978b; Braun & Levitzki, 1979b).

GTP, at its regulatory site, has a finite life time as it hydrolyzes to GDP and inorganic phosphate (Cassel & Selinger, 1976), concomitantly with the deactivation of the enzyme (Levitzki, 1977). Reactivation of the enzyme requires the removal of GDP from the regulatory site and the replenishment of the latter with GTP, a process which requires the presence of a β agonist on the receptor. It can be shown (Levitzki, 1977) that in the presence of GTP and β agonist the activation (k_{on})–deactivation (k_{off}) cycle can be described as

$$E \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E' \qquad (1)$$

where E is the inactive form of the enzyme and E′ is the activated form of the enzyme. It follows that the steady-state activity of the system at saturating hormone and GTP concentrations is given by the multiplication of the steady-state concentration of the active enzyme by the turnover number of the catalytic moiety:

$$V = \frac{[E_T]}{1 + (k_{off}/k_{on})} k_{cat} \qquad (2)$$

where $[E_T]$ is the total enzyme concentration, k_{cat} is the turnover number of the catalytic moiety, and V is the steady-state adenylate cyclase specific activity.

Each of the three parameters, k_{on}, k_{off}, and k_{cat}, is temperature dependent, and therefore an understanding of the temperature dependence of adenylate cyclase activity requires the detailed knowledge of the dependence of each of these three parameters on temperature. In the present study we have explored these temperature dependencies in order to gain more

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insight into the mode of coupling between the adenylate cyclase and its regulatory ligands: the agonists and GTP.

Materials and Methods

Materials. [α-^{32}P]ATP and Na^{125}I were obtained from Radiochemical Centre (Amersham, England). GppNHp[1] and *l*-epinephrine were obtained from ICN. (±)-HYP was a generous gift from Dr. Hauser (Sandoz, Basel). Creatine phosphokinase, creatine phosphate, theophylline, and Tris base were obtained from Sigma. Dns-PE was a generous gift from Dr. Shinitzky (Weizmann Institute of Science, Rehovot, Israel). All other chemicals and biochemicals used were of the highest chemical purity available.

Preparation of Turkey Erythrocyte Plasma Membranes. Turkey erythrocyte membranes were prepared and stored as previously described (Steer & Levitzki, 1975).

Iodination of HYP. Iodination of HYP and purification of [^{125}I]HYP were performed according to the procedure of Maguire et al. (1976).

Measurement of the Temperature Dependence of the Adenylate Cyclase Activity. The temperature dependence of the adenylate cyclase activity was measured by two different methods. In the first method the membranes were preactivated at 45 °C by saturating concentrations of agonist and GppNHp in order to activate maximally all catalytic units, and then the temperature dependence of the maximal specific activity was measured at a range of temperatures. In the second method both the activation process and the resultant specific activity were measured simultaneously at a range of temperatures by incubating the enzyme with saturating agonist and GppNHp at different temperatures. The maximal specific activity obtained by the first and the second methods is designated by the terms $(k_{cat}[E_T])_1$ and $(k_{cat}[E_T])_2$, respectively.

Measurement of the Adenylate Cyclase Specific Activity According to Method 1: $(k_{cat}[E_T])_1$. Membranes suspended in TME buffer at protein concentrations in the range of 2–3.5 mg/mL were preincubated at 45 °C in the presence of the following ligands: (a) 1.0×10^{-4} M *l*-epinephrine and 1.0×10^{-4} M GppNHp and (b) 1.0×10^{-4} M adenosine and 1.0×10^{-4} M GppNHp. After 5 min in the case of (a) and after 15 min in the case of (b), 1.0×10^{-5} M propranolol and 5×10^{-3} M theophylline were added to (a) and (b), respectively. The additions terminate the activation process as was demonstrated earlier (Sevilla et al., 1976, 1977; Tolkovsky & Levitzki, 1978a,b). Adenylate cyclase specific activity was then determined at the desired temperatures.

The time course of cAMP accumulation was followed by taking many data points. The limiting slopes and their standard deviation were obtained by processing a linear regression procedure to the experimental data, at each temperature, as described earlier (Hanski et al., 1979).

Measurement of the Adenylate Cyclase Activity and of the Activation Rate Constant According to Method 2: $(k_{cat}[E_T])_2$. cAMP production was initiated by mixing the assay ingredients and the activating ligands with membranes, using the same procedure described earlier (Tolkovsky & Levitzki, 1978a,b). The activating ligands were as in method 1. The assay contained 2.22 mM theophylline when the activating ligands were those described in (a), whereas when the activating ligand was adenosine, 0.3 mM R020-1724 was used instead of theophylline. All the assay systems included 5 mM creatine phosphate, 50 units/mL creatine phosphokinase, 2 mM ($2-10^6$ cpm) [α-^{32}P]ATP, 6 mM $MgCl_2$, 0.5 mM DTT, and 0.1 mM cAMP, all in 50 mM Tris-HCl buffer, pH 7.4. The protein concentration was always between 0.5 and 1 mg/mL.

At specific times subsequent to mixing, samples of 150 μL were withdrawn from the reaction mixture and added to 100 μL of 2% $NaDodSO_4$ solution. cAMP was measured according to Salomon et al. (1974). All the components of the reaction mixture were preincubated at the desired temperatures for 5 min prior to mixing. The reaction was initiated by adding the membranes to the cocktail.

Analysis of the Kinetic Data. The process of turkey erythrocyte adenylate cyclase activation by *l*-catecholamines or adenosine in the presence of GppNHp fits the equation (Tolkovsky & Levitzki, 1978a,b; Hanski et al., 1979)

$$[cAMP]_t = k_{cat}[E_T]t + \frac{k_{cat}[E_T]}{k_{obsd}}[\exp(-k_{on}t) - 1] \quad (3)$$

where $[cAMP]_t$ is the measured amount of cAMP produced at time t, k_{obsd} is the first-order rate constant of enzyme activation, and $k_{cat}[E_T]$ is the maximal specific activity attained.

Initial values for the parameters describing the kinetics of cAMP accumulation, $k_{cat}[E_T]$ and k_{obsd}, were obtained from the slope of the linear part and from the reciprocal value of the intercept on the time axis, respectively, of the curve describing the time course of cAMP accumulation at each temperature. By use of these parameters, experimental data were processed by utilizing a nonlinear curve-fitting procedure (Booth et al., 1959) as described also earlier (Hanski et al., 1979).

Measurement of Enzyme Activity in the Presence of Fluoride. Activation of the adenylate cyclase system by fluoride was initiated at each temperature by mixing an assay solution, containing 10 mM NaF and 2.22 mM theophylline, with membranes as described above. The resultant time dependence of the cAMP accumulation revealed a lag time in the cAMP appearance and a linear time dependence of cAMP accumulation at longer times. From the slope of the linear portion of the curve a specific activity in the presence of fluoride was calculated at each temperature. The lag time in the cAMP appearance at each temperature was obtained from the extrapolation of the linear portion of the curve with the time axis.

Dependence of the [^{125}I]HYP-β Receptor Dissociation Constant on Temperature. The dependence of the specifically bound [^{125}I]HYP on the free [^{125}I]HYP concentration at each temperature was measured as previously described (Spiegel et al., 1976; Hanski et al., 1979). The values of the dissociation constant K_d at each temperature were obtained by fitting the data of the specifically bound [[^{125}I]HYP] at each temperature to

$$[[^{125}I]HYP]_{bound} = \frac{[R_T][[^{125}I]HYP]_{free}}{K_d + [[^{125}I]HYP]_{free}} \quad (4)$$

by the nonlinear curve-fitting procedure. $[R_T]$ is the total concentration of receptors and K_d is the receptor–[^{125}I]HYP dissociation constant. Specific binding is defined as the difference in the amount of [^{125}I]HYP bound in the presence and in the absence of 1.0×10^{-5} M *dl*-propranolol. Because of the small changes in the receptor–[^{125}I]HYP dissociation constant over the temperature range 5–52 °C (see below), it was feasible to measure the number of receptors accurately at all temperatures.

[1] Abbreviations used: HYP, hydroxybenzylpindolol; $NaDodSO_4$, sodium dodecyl sulfate; TME, 50 mM Trizma base hydrochloride + 2 mM $MgCl_2$ + 1 mM EDTA buffer, pH 7.4; GTPγS, guanosine 5′-(γ-thio)-triphosphate; Dns-PE, dansylphosphatidylethanolamine; R020-1724, *d*-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; GppNHp, guanylyl imidodiphosphate; DTT, dithiothreitol.

Measurements of the Number of Binding Receptors as a Function of Temperature. At any desired temperature, membranes in TME solution were incubated with saturating [^{125}I]HYP concentrations (1.0×10^{-9} M) in eight test tubes, four of which contained 1.0×10^{-5} M propranolol. At the end of the incubation period, four test tubes, two with propranolol and two without, were taken for measurement of the specific binding at that temperature, as described by us previously (Hanski et al., 1979). The remaining four systems (two with and two without 1.0×10^{-5} M propranolol) were incubated for 2 h at 10 °C. The ratio of specific binding of the warmed membranes to that of membranes incubated with [^{125}I]HYP at 10 °C without preincubation at the higher temperature is proportional to the fraction of the binding receptors that survive irreversible denaturation during the incubation at the higher temperature. The equilibration times were determined by preliminary experiments of the time dependence of [^{125}I]HYP binding. At each temperature it was found that at 1.0 nM [^{125}I]HYP the binding was linear with receptor concentration in the assay.

Measurement of the Temperature Dependence of the Off-Rate of Specifically Bound [^{125}I]HYP. Turkey erythrocyte membranes and [^{125}I]HYP were incubated for 30 min at 25 °C with and without 5×10^{-5} M propranolol in TME buffer. This length of time was found to be sufficient for the system to reach equilibrium. The membrane protein concentration was 0.18 mg/mL. The free concentration of [^{125}I]HYP was equal to its K_d constant (0.07 nM). Samples of 1 mL were withdrawn and delivered into 5 mL of stirred TME solution containing 5.0×10^{-5} M propranolol. The 1-mL sample and the TME–propranolol solution were preequilibrated at the chosen temperature before mixing. Ten samples of 0.5 mL were transferred, after mixing at successive times, directly to the filtration apparatus in order to determine bound [^{125}I]HYP, as previously described by us (Hanski et al., 1979). By subtraction of the off-rate of the nonspecifically bound HYP from the off-rate of the total bound HYP, the off-rate of the specifically bound [^{125}I]HYP could be calculated. The first-order off-rate constant was obtained by linear regression of ln (specifically bound [^{125}I]HYP) vs. time. At all temperatures measured, linear slopes were obtained; namely, the kinetic patterns were first order.

Measurement of the Fluorescence Temperature Dependence of Dns-PE Embedded in Turkey Membranes. For the purpose of detection of discontinuous changes in the organization of the membranal phospholipids, turkey membranes were labeled with Dns-PE and the temperature dependence of the resultant membrane fluorescence was measured at the range 45–5 °C. The labeling of the membranes was performed by two different methods. (a) Membranes in TME buffer, prepared as described above, were incubated for 30 min at 37 °C with Dns-PE dissolved in ethanol. The final concentrations of the membrane protein and the Dns-PE were 0.5 and 0.04 mg/mL, respectively. The concentration of alcohol did not exceed 0.5% (v/v). (b) A 12.0-mL amount of packed intact washed turkey erythrocytes (Steer & Levitzki, 1975) was resuspended in 50 mL of isotonic medium (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) as described above, to which 200 μL of Dns-PE in alcohol (20 mg/mL) was added. After incubation for 15 min at 35 °C, the labeled cells were washed 3 times in isotonic solution. Membranes were then prepared from the washed labeled cells according to the usual method (Steer & Levitzki, 1975).

Fluorescence Measurements. After the labeled membranes were washed 3 times with TME buffer, equal volumes of 0.5 mg/mL membrane protein suspension and sucrose (50% w/v) in TME solution were mixed in order to prevent membrane precipitation during the fluorescence measurement. The membrane mixture was kept at 4 °C in the dark. For every measurement, 3 mL of the membrane mixture was transferred to a 1-cm light path quartz cuvette which had been already kept at the desired temperature. The fluorescence measurement started after 7 min of temperature equilibration. The measurements were performed between 5 and 50 °C with a Hitachi Perkin-Elmer MPF-4 fluorescence spectrophotometer. It was found that the effect of Dns-PE in the aqueous solution on the total fluorescence intensity was less than 1%. Also, the fluorescence intensity of Dns-PE in ethanol is temperature independent in the same range.

In all the temperature experiments, the reaction temperature was kept constant within ±0.25 °C by using a Hetofrig thermostatically controlled temperature bath.

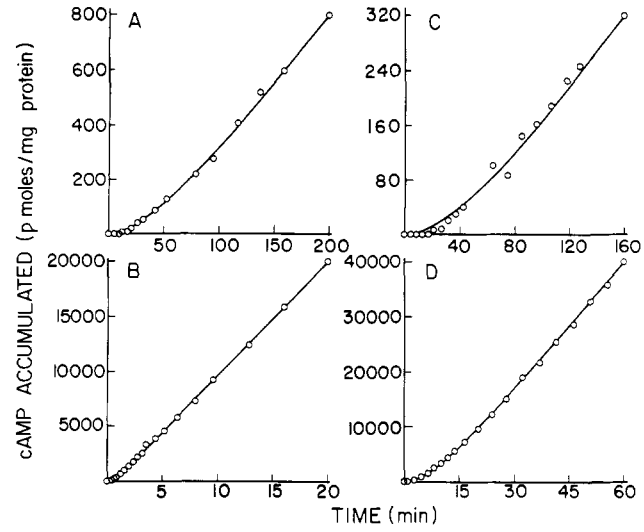

FIGURE 1: Time course of cAMP accumulation in the presence of activating ligands at two different temperatures. The time course of cAMP accumulation in the presence of *l*-epinephrine and GppNHp (A and B) and in the presence of adenosine and GppNHp (C and D) was measured as described in the text. The temperature of the kinetic determination was 14 °C (A and C) and 36 °C (B and D). Each curve represents the optimized fit obtained by using eq 3 for fitting the experimental points.

Results

As was described under Materials and Methods, the temperature dependence of the adenylate cyclase specific activity was determined according to two methods. The difference between the methods is as follows. In the first method, the catalytic units are preactivated at 45 °C and the resultant specific activity is measured at a series of lower temperatures. The formation of cAMP is a linear function of time because at each measured temperature the catalytic units are already fully activated and produce cAMP according to the temperature dependence of the turnover number. In the second method, the formation of cAMP and the activation of the catalytic units occur simultaneously, directly after mixing the assay ingredients containing the activating ligands: *l*-epinephrine plus GppNHp or adenosine plus GppNHp.

The time course of cAMP formation at each temperature was found to follow eq 3, confirming previous results at one temperature (Tolkovsky & Levitzki, 1978a,b). Equation 3 predicts a lag time in cAMP formation at short times and a linear dependence on time at long times when the term $\exp(-k_{obsd}t)$ nullifies. Parts A–D of Figure 1 depict the time course of cAMP accumulation at two different temperatures,

Table I: Maximal Adenylate Cyclase Specific Activities at Different Temperatures[a]

temp of determination (°C)	maximal sp act. [pmol of cAMP formed)/(min mg of protein)]	
	$(k_{cat}[E_I])_1$	$(k_{cat}[E_T])_2$
45	1665	1666
42	1500	1470
39	1233	1134
36	1037	913
32	703	567
28	436	308
25	290	186
22	203	101
18	109	27
14	58.7	5.2
10	30.7	1.2

[a] $(k_{cat}[E_T])_1$ represents the specific activity at different temperatures subsequent to the preactivation of the membrane in the presence of saturating epinephrine and GppNHp at 45 °C. $(k_{cat}[E_T])_2$ represents the maximal specific activity attained at each temperature for enzyme which was not preincubated at higher temperature. Further details are given in the text.

in the presence of *l*-epinephrine plus GppNHp (A and B) and in the presence of adenosine plus GppNHp (C and D), respectively. The maximal enzyme specific activity is given by the slope of the linear portion of the curve at each temperature. The accurate values of these slopes were obtained by fitting the experimental data to eq 3, as described under Materials and Methods. The enzyme maximal specific activity obtained by this method is designated by the term $(k_{cat}[E_T])_2$. It was found that at high temperatures, the values of the enzyme maximal specific activity, $(k_{cat}[E_T])_2$, and the maximal specific activity, $(k_{cat}[E_T])_1$, obtained by a preheating step at 45 °C (method 1) are identical. As one proceeds, however, to lower temperatures, the $(k_{cat}[E_T])_2$ values become lower than the corresponding $(k_{cat}[E_T])_1$ values.

Table I describes the values of enzyme maximal specific activities obtained by the two methods at different temperatures, in the presence of *l*-epinephrine and GppNHp. Each number is the mean of two separate determinations where the standard deviation from the mean did not exceed 7% for $(k_{cat}[E_T])_1$ and 12% for $(k_{cat}[E_T])_2$. The difference between the maximal activities obtained by the two methods results from the fact that the *number* of catalytic units which can be activated by the β receptors and by adenosine receptors is a function of temperature and decreases as a function of decreasing temperature. Therefore, membranes preexposed to GppNHp and *l*-epinephrine or adenosine at 45 °C exhibit specific activities higher or equal to those which were never exposed to the high temperature; namely, $(k_{cat}[E_T])_1 \geq (k_{cat}[E_T])_2$.

Temperature Dependence of the Enzyme Maximal Specific Activity $(k_{cat}[E_T])_1$. The temperature dependence of the maximal specific activity $(k_{cat}[E_T])_1$ is depicted in Figure 2. The maximal enzyme specific activities attained by GppNHp plus *l*-epinephrine or adenosine or by NaF all exhibit an inflection temperature around 35 °C. The activation energies above 35 °C were found to be $E_a = 10 \pm 1$ kcal/mol for all modes of cyclase activation. Below the inflection temperature the activation energy was found to be 25.5 ± 1.4 kcal/mol for the GppNHp plus epinephrine specific activity and 30 ± 1 for the adenosine specific activity and the NaF-dependent maximal activities.

Dependence of $(k_{cat}[E_{max}])_2$ *on Temperature.* The Arrhenius plot of enzyme maximal specific activities obtained by the cAMP accumulation method is shown in Figure 2

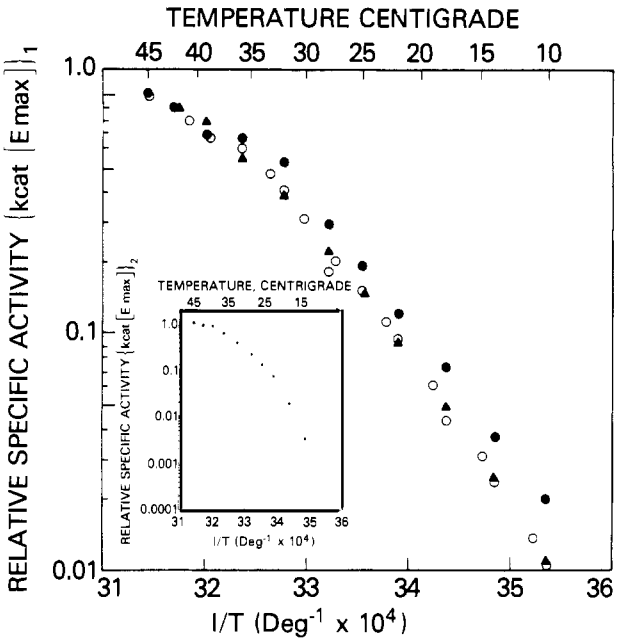

FIGURE 2: Dependence of enzyme maximal specific activity $(k_{cat}[E_T])_1$ on temperature. Membranes were preactivated at 45 °C with 0.1 mM *l*-epinephrine and 0.1 mM GppNHp (●) or with 0.1 mM adenosine and 0.1 mM GppNHp (○), as described in the text (method 1). The specific activities were then determined at different temperatures, as indicated in the figure. The maximal specific activities of the enzyme were also determined in the presence of F^- (▲). Inset: Dependence of the maximal specific activity $(k_{cat}[E_T])_2$ on temperature. The maximal specific activity was obtained by activating the enzyme at each temperature, as described in the text (method 2). Maximal specific activities obtained in the presence of *l*-epinephrine and GppNHp (●). Maximal specific activities obtained in the presence of adenosine and GppNHp (○).

(inset). There are three linear regions in the Arrhenius plot, separated by two inflection temperatures, around 35 and 24 °C. The apparent energies of activation for each of the three regions increases as the temperature decreases as follows: E_a = 12 ± 1.3, 31 ± 1.5, and 62 ± 2 kcal/mol.

Temperature Dependence of the Fraction of Activated Catalytic Units. The ratio between the maximal specific cyclase activities, $(k_{cat}[E_T])_2/(k_{cat}[E_T])_1$, represents the fraction of activated catalytic units at each temperature. Namely, from the data in Figure 2 and its inset, one can calculate the fraction of activated catalytic units at each temperature. The temperature dependence of this fraction represented as an Arrhenius plot is depicted in Figure 3 and demonstrates that the fraction of activated catalytic units through the adrenergic receptor and through the adenosine receptor decreases in an *identical* fashion as a function of decreasing temperature. The Arrhenius plots deviate from linearity, and it is possible to divide the temperature range into at least three linear regions. These regions are separated by two inflection temperatures: one around 35 °C and the other around 24 °C. The apparent activation energies for the three different regions increase as the temperatures decrease: 3 ± 0.5, 12 ± 1, and 37 ± 2 kcal/mol, respectively.

NaF-Dependent Activity. The NaF-dependent adenylate cyclase activity was also examined as a function of temperature (Figure 4). Both the temperature dependence of the lag time and of the maximal specific activity were analyzed in detail.

Reversible Decrease in the Number of Binding β Receptors as a Function of Increasing Temperature. The fraction of binding β receptors at each temperature was derived from the levels of maximal specific binding, as described under Materials and Methods. The fraction of binding receptors decreases

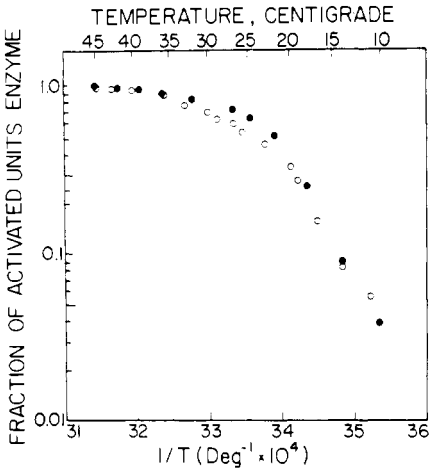

FIGURE 3: Temperature dependence of the fraction of activated catalytic units. The fraction of activated units at each temperature is expressed by the term $(k_{cat}[E_T])_2/(k_{cat}[E_T])_1$, as explained in the text. The full symbols (●) represent the fraction of catalytic units activated by *l*-epinephrine and GppNHp, and the empty symbols (○) represent the fraction of units activated by adenosine and GppNHp.

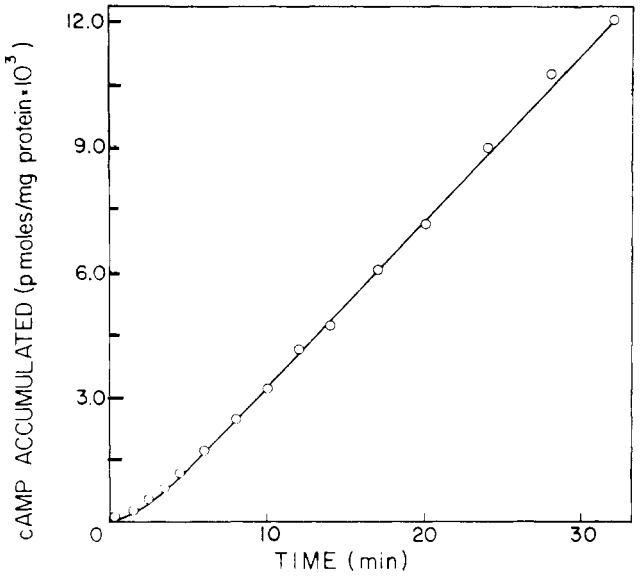

FIGURE 4: Time course of cAMP accumulation in the presence of NaF. cAMP accumulation in the presence of fluoride was determined as explained in the text. The cAMP accumulation in this particular experiment was measured at 28 °C.

as a function of increasing temperature (Figure 5). In the temperature range between 10 and about 35 °C, an increase of each 10 °C results in a decrease of 13% of the total amount of binding receptors. In the temperature range between 35 and 52 °C, an increase of each 10 °C results in a decrease of 28% in the total amount of binding receptors.

The following experiment was performed in order to explore whether the decrease in the number of binding receptors is due to an irreversible denaturation of the receptor protein. Membranes were incubated at different temperatures in the range of 5–52 °C with saturating amounts of [^{125}I]HYP. At the end of the time periods required to obtain equilibrium at each incubation temperature, samples from each solution were cooled or heated to 10 °C. The relationship between the number of binding receptors in the various samples to the number of binding receptors in membranes incubated at 10 °C and which were not exposed to higher temperatures yields the fraction of undenatured receptors. It was found that over the temperature range of 5–52 °C irreversible loss of receptor

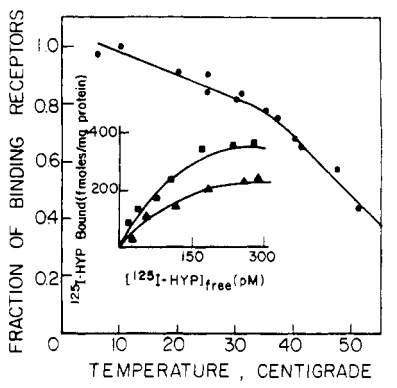

FIGURE 5: Temperature dependence of the fraction of binding β receptors. The number of binding accessible β receptors at each temperature was measured by using saturating concentrations of [^{125}I]HYP, as determined from the binding curves for each temperature. Every point in the curve is the mean of three independent measurements. The specific binding of [^{125}I]HYP as a function of its free concentration at 20 °C (■) and at 40 °C (▲) is shown in the figure. Experimental details concerning the binding of [^{125}I]HYP were published elsewhere (Hanski & Levitzki, 1978; Tolkovsky & Levitzki, 1978a,b; Hanski et al., 1979). The fraction of binding receptors as a function of temperature is also depicted (●).

Table II: Values of [^{125}I] HYP-Receptor Dissociation Constant at the Indicated Temperatures

temp (°C)	[^{125}I] HYP-receptor dissociation constant (M)
10	$(0.7 \pm 0.3) \times 10^{-10}$
20	$(0.7 \pm 0.25) \times 10^{-10}$
30	$(0.9 \pm 0.3) \times 10^{-10}$
40	$(1.6 \pm 0.5) \times 10^{-10}$
50	$(1.6 \pm 0.6) \times 10^{-10}$

[a] The values represent the mean plus the standard error of the mean of triplicate determinations.

increases linearly to up to 7% over the whole temperature range. Thus, the decrease in the number of binding receptors, as a function of increasing temperature, is a reversible process. The total reversible "loss" of receptors over the temperature range 5–50 °C amounts to 60%.

Dependence of [^{125}I]HYP Dissociation Constant on Temperature. The dissociation constant of the receptor–[^{125}I]HYP complex at various temperatures was derived as described under Materials and Methods. As shown in Table II, the dissociation constant at 0 °C is only 2.5-fold lower than the dissociation constant at 50 °C.

Effect of Temperature on the Off-Rate Constant of Specifically Bound [^{125}I]HYP. The small change in the [^{125}I]-HYP–receptor dissociation constant as a function of temperature in Table II indicates that if a temperature-dependent change occurs in the on-rate and the off-rate of [^{125}I]HYP binding, both must occur in the same direction and thus compensate for each other. Indeed, the off-rate constant of specifically bound [^{125}I]HYP is changed about 20-fold within the temperature range of 10–37 °C. The Arrhenius plot of the off-rate constant of [^{125}I]HYP (Figure 6) is linear, indicating that there is no discontinuous change in the kinetic properties of the functionally binding β receptors as a function of temperature.

Temperature Dependence of the Rate Constant of Cyclase Activation by l-Epinephrine-Bound Receptor. The rate constant of enzyme activation, k_{obsd}, at each temperature was determined by fitting the time course of cAMP accumulation in the presence of saturating concentrations of *l*-epinephrine and GppNHp to eq 3, as explained under Materials and Methods. Since during the kinetic determinations of cAMP

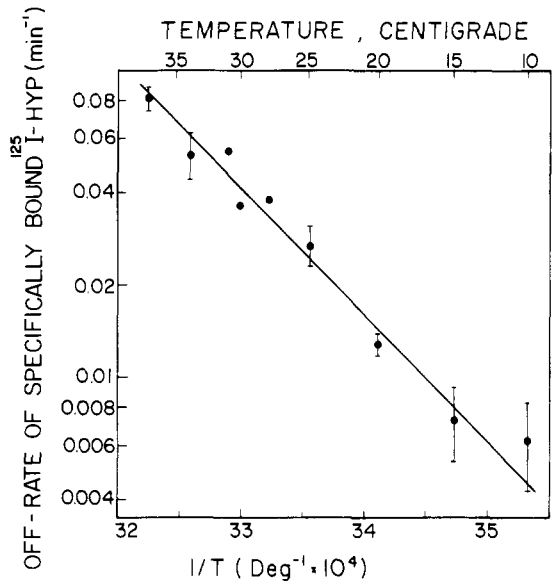

FIGURE 6: Dependence of the off-rate constant of specifically bound [^{125}I]HYP on temperature. The vertical lines demonstrate the standard error of the measurement of triplicates. Points with no error bars represent single samples.

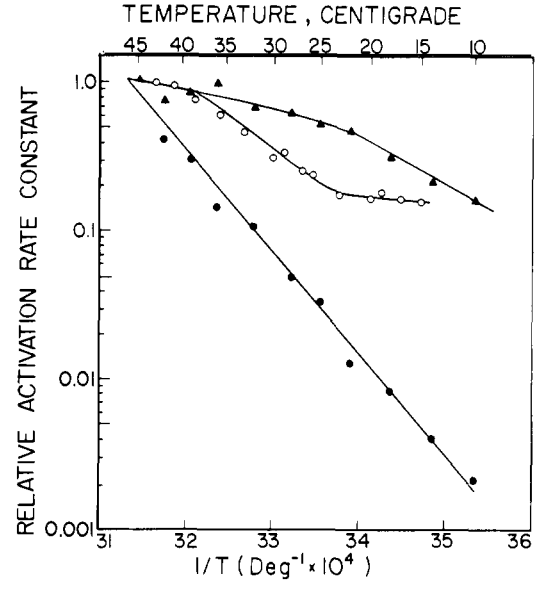

FIGURE 7: Temperature dependence of the rate constant of activation of adenylate cyclase by β receptors, adenosine receptors, and NaF. The first-order rate constant of activation was obtained from fitting the time course of cAMP accumulation in the presence of *l*-epinephrine and GppNHp (●) and adenosine and GppNHp (○) to eq 6 at different temperatures. The temperature dependence of the reciprocal of the fluoride lag time (▲) was obtained as described under Materials and Methods.

production the occupancy of the receptor is constant and maximal, the observed rate constant of activation of adenylate cyclase by the β receptors can be written as (Tolkovsky & Levitzki, 1978a)

$$k_{obsd} = k_1[R_T] \quad (5)$$

where k_1 is the bimolecular rate constant describing the collision between the cyclase complex and the hormone-bound receptor, $[R_T]$ is the total concentration of β receptors, and k_{obsd} is the pseudo-first-order rate constant of activation. Thus, the temperature dependence of k_{obsd} reflects both the dependences of $[R_T]$ and of k_1 on temperature. Since the dependence of $[R_T]$ on temperature is completely accounted for (Figure 5), it was feasible to determine the temperature dependence of k_1. Interestingly, the dependence of k_{obsd} on temperature was found to be linear (Figure 7) with an activation energy of 32 ± 2 kcal/mol. The discontinuity in the dependence of $[R_T]$ on temperature is not manifested in the Arrhenius plot of k_{obsd} because the activation energy characterizing the temperature change in $[R_T]$ is much smaller (2–5 kcal/mol) (Figure 6) than the activation energy of k_1 (32 kcal/mol) (see also Appendix).

Temperature Dependence of the Rate Constant of Enzyme Activation by Adenosine. The activation process of adenylate cyclase in the presence of adenosine and GppNHp obeys the precoupled mechanism (Tolkovsky & Levitzki, 1978b; Braun & Levitzki, 1979b). According to this mechanism, the observed rate constant of activation is proportional to the occupancy of the adenosine receptors, as described by

$$k_{obsd} = \frac{k[A]}{[A] + K_A} \quad (6)$$

where [A] is the free concentration of adenosine, K_A is the dissociation constant of the adenosine–receptor complex, and k represents the rate constant of formation of the active enzyme AR_AE' from the inactive ternary complex AR_AE, where R_A represents the adenosine receptor.

Due to the lack of a binding probe to the adenosine receptor, at this point, it was not possible to perform direct binding studies in order to determine the temperature dependence of receptor binding properties. Since all the activation experiments were performed in the presence of adenosine concentrations 2 orders of magnitude higher than the apparent dissociation constant for adenosine at 37 °C (Tolkovsky & Levitzki, 1978b; Braun & Levitzki, 1979b), it is most likely that the occupancy of the adenosine receptor at each temperature was maximal.

The observed activation rate constant of enzyme in the presence of adenosine and GppNHp was obtained by fitting the time course of cAMP production at each temperature to eq 3, as explained under Materials and Methods.

The Arrhenius plot of k_{obsd} for adenosine has a complex behavior (Figure 7). It is possible to divide the temperature range into three linear regions, separated by two inflection temperatures, 35 and 24 °C. The activation energies at the range 45–35 °C and 24–14 °C are about 2 ± 1.0 kcal/mol. At the intermediate temperature range of 35–24 °C, the rate constant of activation has a much greater dependence on temperature and the activation energy for this range is 18 ± 1 kcal/mol.

Temperature Dependence of the NaF-Stimulated Cyclase Activity. The kinetics of cAMP formation in the presence of fluoride ions reveal a lag time in cAMP production at short times and a linear dependence on time at long times, as shown in Figure 4. As the temperature decreases, the lag time increases. The reciprocal of the lag time at each temperature was calculated and plotted against the reciprocal temperature in order to express the temperature dependence of the lag time in cAMP production in (time)$^{-1}$ units. The resultant Arrhenius plot of the reciprocal apparent lag time can be divided into two regions (see Figure 7), separated by an inflection temperature at 24 °C. The apparent activation energies above and below 24 °C are 5 ± 2 and 17 ± 1 kcal/mol, respectively.

Temperature Dependence of the Fluorescence Intensity of Membranes Labeled with Dns-PE. The fluorescence intensity of fluorescent phospholipids embedded in membranes was found to be a sensitive tool for detecting (Christian et al., 1977) transitions in the membranes as a function of temperature.

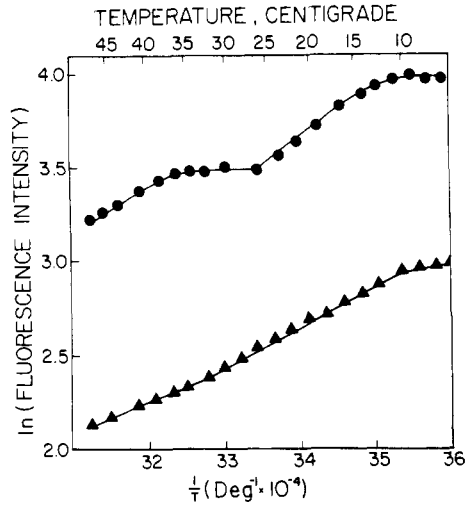

FIGURE 8: Temperature dependence of the fluorescence intensity of membranes labeled with Dns-PE. Fluorescence intensity of membranes labeled by Dns-PE at both the outer and inner monolayers [see method a under Materials and Methods (●)]. Fluorescence intensity of membranes labeled by Dns-PE exclusively at the outer monolayer [see method b under Materials and Methods (▲)].

Inflection points in the Arrhenius plot of the fluorescence intensity appear at 10, 24, and 35 °C when the turkey membranes are labeled with Dns-PE on both sides (method 1) (see Figure 8). When membranes are labeled exlusively with Dns-PE at the external side (method 2), the transition at 24 °C completely disappears (Figure 8).

Discussion

The temperature dependence of adenylate cyclase systems is complex and probably reflects the fact that the enzyme system is a multicomponent system which is embedded in the asymmetric membrane bilayer. One usually finds in an Arrhenius-type plot that the temperature dependence of the adenylate cyclase specific activity has one or more transition points. In the present study we have attempted to study in detail the transitions in the temperature dependence of the individual parameters which determine the steady-state level of adenylate cyclase activity. The system we chose is the turkey erythrocyte adenylate cyclase. The latter enzyme can be activated either by *l*-epinephrine through β receptors or by adenosine through adenosine receptors and like all eukaryotic adenylate cyclases by NaF. In parallel, a fluorescent probe, Dns-PE, was inserted into the *outer* monolayer of the membrane by treating the intact cells with Dns-PE prior to preparation of the plasma membranes and into both the outer and the inner monolayer by treatment of the *membranes* with Dns-PE subsequent to the preparation of the membranes. By following the fluorescence change in both types of labeled membranes as a function of temperature, one can tentatively assign the temperature transitions to either the outer or the inner membrane layers and correlate them with the transition temperatures of the individual parameters which determine the adenylate cyclase activity.

Temperature Dependence of the Fraction of Activatable Cyclase Molecules. Figure 3 reveals that the fraction of catalytic units which can be activated by the agonist-bound receptors decreases as a function of decreasing temperature. This reduction is probably due to the temperature-dependent distribution of the total pool of catalytic units between two forms, E_{act} and E_{inact}, where E_{act} and E_{inact} are the forms which can and cannot be activated by the receptor-bound agonist, respectively. Clearly

$$[E_T] = [E_{act}] + [E_{inact}] \quad (7)$$

From the experimental results, it appears that dynamic equilibrium between the two forms of the catalytic units *does not exist*. A dynamic equilibrium between E_{act} and E_{inact} necessitates that every catalytic unit spends a fraction of the time as E_{act}. In such a case, all catalytic units would have been activated by GppNHp in the presence of agonist, at all temperatures. From the observation that the temperature dependence of the enzyme fraction which can be activated by either adenosine or epinephrine is identical (Figure 3), one can conclude that the activation process by the two receptors possesses a common and crucial step which is strongly dependent on temperature. This step could involve the GTP regulatory step which was demonstrated to be the common link between these two receptors and the cyclase (Sevilla et al., 1977; Tolkovsky & Levitzki, 1978b). The transition occurs within the entire measured range of temperatures (10–45 °C). At 10 °C the process is nearly completed and more than 98% of the population of catalytic units are in the E_{inact} form. Thus, neither adenosine nor *l*-epinephrine is capable of activating E_{inact}. The discontinuity at 24 °C of the transition of E_{act} to E_{inact} (Figure 3) is identical with the transition temperature exhibited by the apparent rate constant of NaF activation (Figure 7), indicating that the mode of NaF activation also involves the GTP regulatory unit as does adenosine and *l*-epinephrine. The transition temperature of 24 °C is the one characteristic of the inner monolayer of the membrane as indicated from fluorescence measurements (Figure 8). To summarize this point, we would like to suggest that upon freezing of the inner monolayer the GTP regulatory unit which is associated with the inner membrane becomes nonfunctional. Equally feasible is the alternative possibility that the transition at 24 °C reflects a temperature-dependent structural transition within the GTP regulatory unit. According to this explanation the thermal transition at 24 °C reflected in the Dns-PE fluorescence may therefore not be related to the structural change in the GTP binding protein. In summary, all three modes of activation of cyclase, by *l*-epinephrine, adenosine, and NaF, reveal discontinuity at 24 °C. The fraction of activable catalytic units is also sensitive to the state of the outer monolayer of the membrane, since it is also sensitive to a transition which occurs at 35 °C (Figure 8, Table II). It is therefore suggestive that the catalytic moiety is in close contact with *both* layers of the membrane. That this may be the case is also indicated by the finding that the turnover number of adenylate cyclase, when all the GTP regulatory units are occupied by GppNHp (or in the presence of NaF), also exhibits a transition temperature at 35 °C (Figure 2). The preactivated enzyme (either by GppNHp or NaF) does not exhibit the transition at 24 °C, since GppNHp occupies and activates permanently the system and the enzyme activity is independent of the process of GTP replenishment occurring at the inner monolayer.

Similar phenomena have been shown in the galactose transport system of *Escherichia coli* where the "break" in the Arrhenius plot results from the temperature-dependent reduction in the number of functional transport units (Therisol et al., 1977; Letellier et al., 1977).

Temperature Dependence of the Rate Constant of Cyclase Activation by Adenosine-Bound Receptors. Evidence has been provided that the adenosine receptor is permanently associated with the adenylate cyclase (Tolkovsky & Levitzki, 1978b; Rimon et al., 1978; Braun & Levitzki, 1979b). The rate-limiting step in the activation of the catalytic unit by the adenosine receptors is the conversion of the inactive catalytic

unit E to its active form E′ according to the scheme

$$R_AE \underset{+A}{\overset{-A}{\rightleftharpoons}} AR_AE \xrightarrow{k_2} AR_AE'$$

where A, R_A, and k_2 represent adenosine, the receptor, and the first-order rate constant of enzyme activation. At saturating adenosine concentrations the activation rate constant k_{on} is equal to k_2.

The Arrhenius plot of the adenosine rate constant for activation reveals equal apparent activation energies (Figure 7) above 35 °C and below 24 °C (2 kcal/mol), with a steep temperature dependence between 35 and 24 °C. Such a triphasic Arrhenius plot has been explained by Thilo et al. (1977) as follows. Above 35 °C the activation rate constant characteristic for the population of catalytic units is $k = P_2 \exp(-E_a/R_T)$, where E_a is the apparent activation energy and P_2 is a temperature-independent factor. Under 24 °C, $k = P_1 \exp(-E_a/R_T)$. Between 35 and 23 °C there is a transition of the P factor from P_2 to P_1. This transition causes the appearance of the two "breaks" in the Arrhenius plot. The activation energy between 24 and 35 °C is determined by the temperature dependence of the transition in the population of catalytic units activated according to P_2 to the population activated according to P_1. Since the activation rate constant for adenosine is sensitive only to changes which occur in the E_{act} form (see eq 7) of the catalytic units at each temperature, the changes of P suggest a concomitant change in the fluid area of the membrane between 24 to 35 °C. The suggested changes in the properties of the fluid area might be the result of a prefreezing phenomenon. The occurrence of the prefreezing phenomenon in organic solutions and phospholipids, as well as the sensitivity of membranal systems to the prefreezing stage, has already been demonstrated (Davies & Matheson, 1967; Elert & Keith, 1972; Ertl & Dullien, 1973; Ubbelehde, 1965; Lee et al., 1974).

The adenosine mode of activation is sensitive to the properties of the external monolayer which exhibits the transition at 35 °C and the internal monolayer which exhibits the transition at 24 °C. Since the complex adenosine receptor with the catalytic unit traverses both the internal and external monolayers, it is expected that the adenosine activation rate constant will reflect events that take place on both sides of the membrane. A similar situation was observed recently for the glucagon-stimulated adenylate cyclase (Dipple & Houslay, 1979).

Temperature Dependence of the Rate Constant of Cyclase Activation by Epinephrine. Evidence has been presented that the β-adrenergic receptors are not physically associated with the catalytic units in turkey erythrocytes (Tolkovsky & Levitzki, 1978a; Hanski et al., 1979). The process of activation is biomolecular and occurs during a brief encounter between the catalytic unit and the hormone-bound receptor, according to the scheme

$$R + H \rightleftharpoons HR + E \xrightarrow{k_1} HRE \xrightarrow{k_2} E' + HR \qquad (8)$$

The rate-limiting step in the activation process is k_1 ($k_2 \gg k_1$). At saturating hormone concentrations k_{on} for the activation process is given by $k_{on} = k_1[R_T]$ (eq 8, see above) where $[R_T]$ is the total receptor concentration and k_1 is the bimolecular rate constant describing the frequency of the fruitful encounters between the hormone-bound receptor and the adenylate catalytic units.

The linearity of the Arrhenius plot of the epinephrine activation bimolecular rate constant (Figure 7) in the temperature range of 10–45 °C indicates that the ln of the rate constant k_1 decreases linearly as a function of decreasing temperature. Since the ln of membrane viscosity increases linearly as a function of T^{-1}, it is in fact not surprising that ln k_{on} decreases linearly with T^{-1}. The rate constant seems to be completely insensitive to the phase transitions which occur in the inner and outer monolayers. The interpretation of these seemingly contradictory findings is as follows. Upon membrane freezing, β receptors "caught" in frozen areas become immobile and functionally silent, since they are unable to move and therefore cannot encounter a cyclase moiety. Receptors still in fluid areas, however, remain functional, but their mobility diminishes with increasing viscosity which in turn increases as a function of $1/T$. Namely, only functional receptors within the fluid areas of the outer monolayers are expressed in the measurement of the parameter k_1.

Temperature Dependence of the Steady Adenylate Specific Activity. A number of parameters which determine adenylate cyclase activity were shown to be temperature dependent: (a) the number of receptors accessible to the ligand; (b) the fraction of enzyme molecules which can be activated by agonist-bound receptors; (c) the rate constant of cyclase activation by the agonist-bound receptor; (d) the catalytic efficiency of the adenylate cyclase. All these parameters enter and, therefore, determine the temperature dependence of the overall steady-state adenylate cyclase activity. It can be shown (see Appendix) that the overall apparent activation energy of the steady-state adenylate cyclase specific activity is *approximately equal to the algebraic sum of the activation energies of each parameter appearing in the steady-state equation.* Therefore, only a significant change in a parameter possessing a large apparent activation energy is expected to contribute substantially toward the appearance of a "break" in the Arrhenius plot of the steady-state activity. Thus, the contribution of the apparent activation energy to the temperature dependence of the number of binding of β receptors is small, relative to the apparent activation energies of the other processes which determine the steady-state specific activity. Therefore, the temperature-dependent transition in this process (Figure 5) is not manifested in the Arrhenius plot of the β-receptor-dependent steady-state activity of the system (Figure 9), although a large number of the receptors become cryptic over the temperature range tested (Figure 5). Our data and analysis suggest that no measurable steady-state activity will be revealed under 10 °C for either adenosine- and epinephrine-dependent cyclase activity. This is due to the observation that under 10 °C all the catalytic units are in the inactivated form E_{inact} (eq 7). For both adenosine and epinephrine steady-state activities it is also expected that the apparent activation energy in the temperature range 35–45 °C will be lower than that under 35 °C. This is because the activation energy of the transition of E_{act} to E_{inact} increases with decreasing temperature. One can also predict that under 24 °C the apparent activation energy for the adenosine-stimulated activity will be lower than the apparent activation energy of the epinephrine-stimulated activity. This is due to the observation that under 24 °C the activation energy for the adenosine activation rate constant largely decreases (Figure 7) whereas the epinephrine activation rate constant remains constant throughout the temperature range (Figure 7). Figure 9 reveals that these qualitative predictions are indeed fulfilled. At the present stage, however, accurate predictions as to the exact temperature dependence of the steady-state activity must await the detailed temperature dependence of k_{off} (see also Appendix). The values of k_{off}, as already pointed out in a previous study (Arad & Levitzki, 1979), can only be obtained thus far within 30–40% accuracy. It is therefore not possible at this stage to

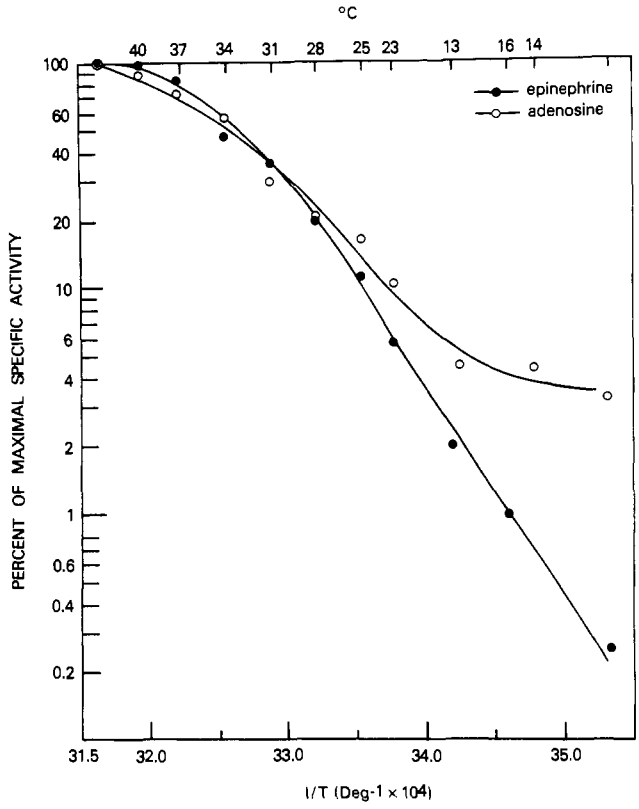

FIGURE 9: Steady-state adenylate cyclase activity. Adenylate cyclase activity was measured as described previously (Steer & Levitzki, 1975). The assay mixture contained 1 mM [α-^{32}P]ATP, 1 mM phosphocreatine, 5 units/assay of creatine phosphokinase, 5.0 mM $MgCl_2$, 1 mM EDTA, 1 mM R020-1724, and 50 mM Tris-HCl, pH 7.4. The data are presented as percent. The absolute specific activites at 37 °C were as follows: basal activity, 1.2 ± 0.3 (pmol of cAMP)/(min mg), *l*-epinephrine (0.1 mM), 83 ± 5 pmol/(min mg), and adenosine, 10.8 ± 1.0 pmol/(min mg). All determinations were carried out in triplicate, and the data are presented as the average ± SEM. (○) *l*-Epinephrine activity; (●) adenosine activity.

fully reconstruct the temperature dependence of the steady-state cyclase activity (Figure 9) from the temperature dependence of the individual parameters.

Modes of Cyclase to Receptor Coupling. The different modes of cyclase activation and the difference in the temperature dependence of the rate constant of cyclase activation by epinephrine as compared to that by adenosine reflects the difference in the mode of coupling between adenosine receptors and β receptors to the enzyme. The adenosine receptor is permanently coupled to the cyclase system (Tolkovsky & Levitzki, 1978b; Rimon et al., 1978; Braun & Livitzki, 1979a,b) and therefore monitors the temperature transitions which the complex experiences. The β receptor diffuses in the fluid areas of the outer monolayer and is therefore sensitive to the average fluidity changes of these areas. The NaF mode of activation reflects only a transition at 24 °C (Figure 7). This is not unexpected in view of the general belief that NaF activates cyclase in a receptor-independent mechanism and presumably through the GTP regulatory protein. Namely, the activation by NaF is independent of the outer monolayer and therefore is not sensitive to the temperature transition at 35 °C. Our findings are similar to those of Houslay et al. (1976), who showed, in liver membranes, that the NaF mode of cyclase activation is independent of the properties of the outer monolayer.

Does the Cyclase Moiety Traverse the Bilayer? The temperature-dependent transition at 35 °C in the accessible β receptors (Figure 5) and in a number of kinetic parameters

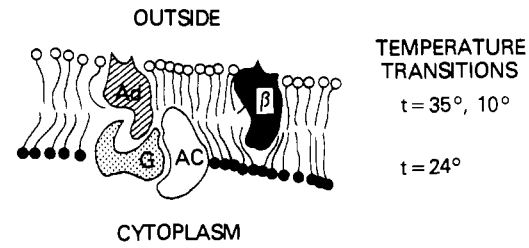

FIGURE 10: Behaviour of the turkey adenylate cyclase system. The β-adrenergic receptor is functional only at the fluid areas of the outer monolayer of the membrane. The cyclase complex consists of the adenosine receptor (Ad), the GTP regulatory unit (G), and the catalytic unit E. The fraction of activable enzyme by either epinephrine and adenosine or NaF (Figure 3) experiences a sharp transition at 24 °C. This fraction is determined by the interaction between G and E which occurs at the inner monolayer which indeed experiences a transition at 24 °C. The transitions at the outer monolayer are manifested by the rate constant of cyclase activation by the adenosine receptor (10 and 35 °C) and by the accessibility of the β receptors to [^{125}I]HYP (35 °C).

reflects a temperature-dependent transition in the structure of the outer monolayer as confirmed by the transition in the fluorescence intensity of the Dns-PE embedded in the outer monolayer (Figure 8). Interestingly, the turnover number of the catalytic moiety of cyclase also exhibits a temperature transition at 35 °C (Figure 2). This can be taken to mean that the catalytic moiety traverses the bilayer and is therefore sensitive to structure transitions within both the inner (24 °C) and the outer (35 °C) monolayers.

Overall Picture. The overall behavior of the turkey erythrocyte adenylate cyclase as deduced from the temperature studies is summarized in Figure 10.

Acknowledgments

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Appendix

The purpose of this appendix is to demonstrate that the apparent activation energy of the steady-state activity equals approximately the algebraic sum of the apparent activation energies of each of the parameters in the steady-state equation. The specific activity of hormone-dependent activity is given by eq 2. In turkey erythrocyte membranes the k_{off} of the steady state of epinephrine-stimulated activity is much higher than the k_{on} (Arad & Levitzki, 1979). Therefore, the steady-state equation for the specific activity of the enzyme can be written as

$$V = k_{cat}[E_T]k_{on}/k_{off} \quad \text{(A1)}$$

where k_{cat} represents the turnover number of the cyclase system, $[E_T]$ is the total concentration of catalytic units from the rate constant of cyclase activation, and k_{off} is the rate of the turnoff reaction which converts the cAMP producing state to the inactive state. The temperature dependence of every parameter in eq A1 can be written as

$$(k_{cat})_T = (k_{cat})_o \exp(-E\alpha_1/RT) \quad \text{(A2)}$$

$$(E_T)_T = (E_T)_o \exp(-E\alpha_2/RT) \quad \text{(A3)}$$

$$(k_{on})_T = (k_{on})_o \exp(-E\alpha_3/RT) \quad \text{(A4)}$$

$$(k_{off})_T = (k_{off})_o \exp(-E\alpha_4/RT) \quad \text{(A5)}$$

$E\alpha_1$, $E\alpha_2$, $E\alpha_3$, and $E\alpha_4$ are the apparent activation energies of each parameter in the equation. $(k_{cat})_o$, $(E_T)_o$, $(k_{on})_o$, and $(k_{off})_o$ are temperature independent factors.

By inserting the temperature-dependent terms of eq A2–A5 into A1 and expressing the resultant equation in a logarithmic form, one obtains

$$\ln V = \ln \left[\frac{(k_{cat})_o (E_T)_o (k_{on})_o}{(k_{off})_o} \right] - \frac{1}{RT}[E\alpha_1 + E\alpha_2 + E\alpha_3 - E\alpha_4] \quad \text{(A6)}$$

Equation A6 reveals the temperature dependence of the steady-state specific activity determined by the *algebraic* sum of the activation energies of the different processes involved in the determination of the steady-state activity. Clearly, a discontinuity in the temperature dependence in one of the parameters is a necessary condition but not sufficient for generating a discontinuity in the steady-state cyclase activity. The relative contributions of each of the activation energies to each of the processes involved will determine whether the "breaks" in the temperature dependencies of each of the parameters reappear in the overall temperature dependence of the steady-state activity.

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