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On the Kinetics and Temperature Dependence of Adrenaline–Adenylate Cyclase Interactions

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SUMMARY

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Adrenaline and *beta* adrenergic blocking drugs were shown to interact rapidly and reversibly with catecholamine-sensitive adenylate cyclases from Ehrlich ascites cells, rat liver, and rat fat cell ghosts within the temperature range of 15–37°. Rates of binding and dissociation of both adrenaline and *beta*-blocking drugs are faster than the time resolution of the methods (1–2 min). Arrhenius plots for the enzyme from Ehrlich cells showed inflection temperatures (about 24°) for basal and adrenaline-stimulated activities, energies of activation (E_a) being about 10 kcal/mole lower above this temperature. Differences in E_a between basal and adrenaline-stimulated activities were not apparent. Fluoride-stimulated activity did not show a clear inflection point. Unstimulated enzyme from rat brain cortex showed an inflection temperature at about 24°, similar to that for Ehrlich cell cyclase, and no inflection was seen for fluoride-stimulated activity. The activation constant of adrenaline for Ehrlich cell adenylate cyclase was 4–6-fold lower at 15° compared to 37°. This would not explain the inflection in Arrhenius plots, however, since the latter were obtained at maximally stimulating concentrations of adrenaline.

INTRODUCTION

Hormone-sensitive adenylate cyclase (EC 4.6.1.1) represents a fascinating enzyme system comprising aspects of a pharmacological receptor, i.e., high selectivity or specificity of interaction with an extracellular signal (first messenger) and subsequent generation of an intercellular response by way of synthesis of cyclic AMP from ATP. Consequently the action of hormones upon adenylate cyclases from several tissues has been the subject of intensive research. Since enzyme activity and its stimulation by hormones can be measured *in vitro* in tissue homogenates or suitable membrane fractions, it should be possible eventually to purify and isolate receptors for peptide hormones or biogenic amines. A major step in this direction has been the development of hormone binding studies using radioactive isotope-labeled hormones and adenylate cyclase-containing particles. Such studies should allow evaluation of kinetic and thermodynamic parameters of receptor-hormone interaction and the correlation of such data with those obtainable from direct measurements of hormone action on adenylate cyclase *in vitro*, as well as on the whole tissue or organ.

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It is thus necessary to study in greatest possible detail the stimulatory effect of hormones upon adenylate cyclase activity *in vitro* in order to establish a basis for correlations between this effect and hormone binding characteristics. Useful experiments may include studies of the dependency of hormonal stimulation on factors such as temperature, pH, and ions, as well as measurement of kinetic parameters and, in turn, their dependence on these factors. As part of such a project, the present work deals with an attempt to measure temperature effects on adenylate cyclase activity in the presence and absence of adrenaline and on the time course of *beta* adrenergic drug action. A portion of these results has been presented elsewhere (1).

METHODS AND MATERIALS

Adenylate cyclase from Ehrlich ascites cells was prepared as described previously (2). Rat liver enzyme was obtained from livers of newborn animals (3), and fat cell ghosts were prepared as described by Rodbell (4). Adenylate cyclase from rat brain cortex was obtained in the form of mitrochondrial pellets (20,000 × *g*) from homogenates of 1 g of tissue in 5 volumes of 20 mM Tris-HCl–1 mM $MgCl_2$ (pH 7.5). Adenylate cyclase assays contained, in a total volume of 0.05 ml, the following: 40 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.1 mg/ml of creatine phosphokinase, 10 mM sodium creatine phosphate, 1 mM sodium ethylenebis(oxyethylenenitrilo)tetraacetate, 0.6 mM $CaCl_2$, 0.5 mM cyclic AMP, and 0.1 mM [α-^{32}P]ATP (600,000 cpm). Radioactive cyclic AMP was separated from ATP by chromatography on polyethylenimine-impregnated cellulose thin-layer plates as described elsewhere (5, 6). When cyclic AMP formation was measured as a function of time, total assay volumes were increased to 0.1 or 0.2 ml, and 2–3-μl aliquots were removed at suitable time intervals and applied directly to thin-layer plates to which chromatographic carriers, including cyclic AMP and ATP, had been applied. Temperatures were controlled in circulating water baths with mereury contact thermometers and measured with a precision mercury thermometer.

Since adenylate cyclase activity following thawing of enzyme preparations does not remain constant (2), small batches of 0.1 ml were kept frozen under liquid nitrogen. For each series of incubations (triplicates) at a given temperature in the presence and absence of stimulants, a single batch of enzyme was thawed, and aliquots were added to incubation vials immediately to start the reactions.

All biochemicals, including adrenaline bitartrate, were purchased from Sigma Chemical Company or Boehringer/Mannheim. The *beta* adrenergic blocking drug 1-(3-methylphenoxy)-3-isopropylaminopropranol-2 (Kö 592) was a gift from Boehringer-Sohn-Ingelheim; and propranolol, from Imperial Chemical Industries, Ltd.

RESULTS

Initial experiments showed that full stimulation of catecholamine-sensitive adenylate cyclases, obtained from various tissues, occurred whether or not adrenaline was mixed with the enzymes before or after the addition of substrate ATP. This conclusion was reached because the rate of cyclic AMP production remained linear from the start of incubation. Such experiments, however, provided no evidence whether interaction of adrenaline with adenylate cyclase and stimulation of enzyme activity were readily reversible. When various *beta* blocking drugs were added to assays at intervals following the start of incubations in the presence of adrenaline, virtually immediate reduction of the rate of cyclic AMP production to basal levels could be seen. This effect is demonstrated in Fig. 1 with enzyme from Ehrlich ascites cells. Identical results were obtained with cyclase preparations from rat liver and rat fat cell ghosts, also involving adrenaline as the stimulant, and either propranolol or Kö 592 as the *beta* blocking agent.

The reversibility of the blockade produced by *beta* blocking drugs was not directly investigated. However, prior incubation of Ehrlich cyclase at 0–4° with various concentrations of propranolol had no effect on subsequent stimulation of enzyme activity at 37° by doses of adrenaline (0.01–0.1 mM) sufficient to overcome propranolol inhibition. Presuming that essentially all receptor sites

had been occupied by the *beta* blocking drug during prior incubation in the cold, this observation indicates reversibility of propranolol binding and blocking action as well. In adenylate cyclase assays containing maximally effective doses of adrenaline, partial blockade of stimulation of activity could be obtained with low doses of propranolol (Fig. 2), and it appears again that this effect was rapid, since rates of cyclic AMP formation seemed essentially linear following addition of the blocking drug. However, the limits of accuracy in this type of assay obviously do not permit detection or elimination of a possible latency of action, or of a curvature in the rate of cyclic AMP production, within 1–2 min or less following addition of propranolol.

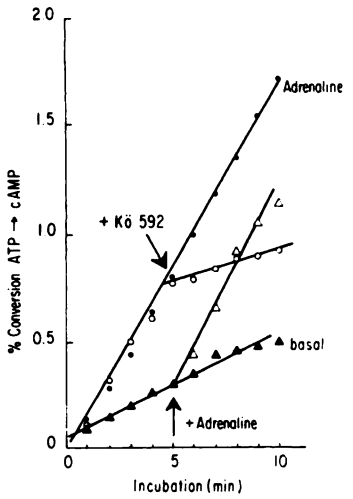

FIG. 1. *Cyclic AMP formation by adenylate cyclase from Ehrlich tumor cells at 37° in the absence (▲) and presence (●) of 0.1 mM adrenaline*

At the arrows (i.e., at 5 min) either 0.5 mM Kö 592 (○) or 0.1 mM adrenaline (△) was added to parallel incubations. Volume changes due to removal of aliquots for chromatography and addition of Kö 592 or adrenaline were in the range of 5–8%. Basal activity was about 10 pmoles/mg of protein per minute.

At temperatures as low as 15°, where enzyme activity and hormone effects could still be clearly demonstrated, both the stimulating effect of adrenaline upon Ehrlich ascites adenylate cyclase and the blocking action of propranolol were virtually immediate upon addition of the drugs, as they were when the incubation temperature was 37° (Fig. 1).

The above experiments indicated that the action of adrenaline as well as of the *beta* blocking drugs (which have no stimulatory action of their own but which are considered to act as competitive inhibitors of catecholamines) is readily reversible and occurs too rapidly to allow measurement of the rate of association of adrenaline by the present technique of monitoring the rate of cyclic AMP production.

The effects of temperature on adenylate cyclase activity were measured in more detail. Enzyme from Ehrlich ascites cells was incubated at temperatures between 15° and 37° in the absence and presence of 10 mM sodium fluoride or 0.1 mM adrenaline as

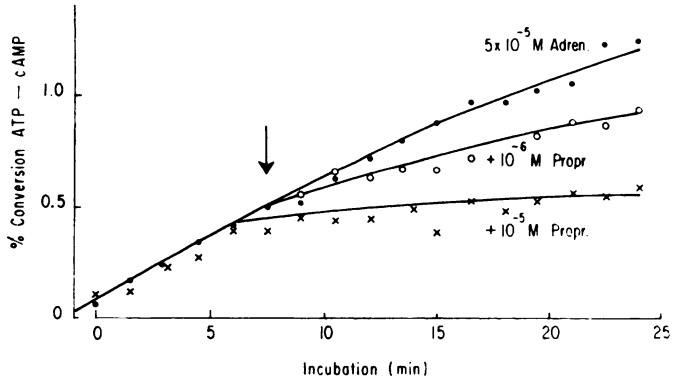

FIG. 2. *Effect of propranolol (Propr.) on adrenaline (Adren.)-stimulated Ehrlich cell adenylate cyclase*

At the time indicated by the arrow (7 min) propranolol was added at the concentrations indicated, volume changes being about 5%. cAMP, 3′,5′-cyclic AMP.

stimulant. When the logarithms of rates were plotted against the reciprocals of the absolute temperatures (Arrhenius plots), characteristic inflection points were seen in the mid-20° range with both basal and adrenaline-stimulated enzyme, while inflection was less evident in the presence of sodium fluoride (Fig. 3). Temperatures of inflection in Arrhenius plots from three experiments, including the one in Fig. 3, are summarized in the last two columns of Table 1. In both the presence and absence of adrenaline the temperatures of inflection were within the range of 23–27°. Although inflection points were not clearly evident upon visual inspection of Arrhenius plots of fluoride-stimulated enzyme, values for these and for apparent activation energies obtained by mathematical analysis are included in Table 1.

In addition to the procedure of Bogartz (7) referred to in Table 1, rate data were also subjected to tests of significance of regression as outlined by Sokal and Rohlf (8). In general, points of straight-line segments could be fitted by linear regressions, but in some experiments (and particularly in the low temperature region) strict analysis indicated that experimental points could not be fitted by straight lines. Since this finding was not consistent, and since low temperature rate data were subject to higher experimental errors, more intensive and precise measurements should be performed to see whether or not the segments in Arrhenius plots of Ehrlich cell adenylate cyclase below and above inflection temperatures are linear or not. This present uncertainty does not, however, affect the existence of inflections in the plots.

Limited studies were also performed with an adenylate cyclase of higher specific activity obtained from rat brain cortex. Meas-

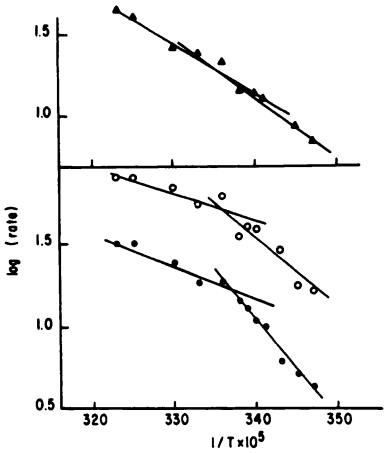

FIG. 3. *Arrhenius plots for basal (●), adrenaline-stimulated (○), and fluoride-stimulated (▲) adenylate cyclase from Ehrlich cells*

Data are from experiment 2 in Table 1. The adrenaline concentration was 0.1 mM, and that of sodium fluoride was 10 mM. Rates are expressed as picomoles per milligram of protein per minute.

TABLE 1

Inflection temperatures and energies of activation from Arrhenius plots

The data points in the Arrhenius plots were fitted by two-line segments (linear regressions) by the method of Bogartz (7), which minimizes the sum of squared deviations from the regressions fitted to all possible two-line combinations and thus arrives at a "best" separation. Subsequently slopes and intersections of the optimal two-line segments yielded the apparent activation energies and inflection temperatures, respectively.

Addition	Inflection temperature	$-E_a$ inflection temperature	
		Above	Below
		kcal/mole	
Ehrlich cell enzyme (expt. 1)			
No addition	23°	6	10
Adrenaline	24°	6	16
Fluoride	33°	10	14
Ehrlich cell enzyme (expt. 2)			
No addition	23°	11	28
Adrenaline	26°	6	20
Fluoride	27°	11	17
Ehrlich cell enzyme (expt. 3)			
No addition	25°	6	15
Adrenaline	27°	4	12
Fluoride	28°	13	18
Rat brain cortex (expt. 4)			
No addition	24°	1.6	8
Fluoride		13	13
Rat brain cortex (expt. 5)			
No addition	24°	2.8	8
Fluoride		13	13

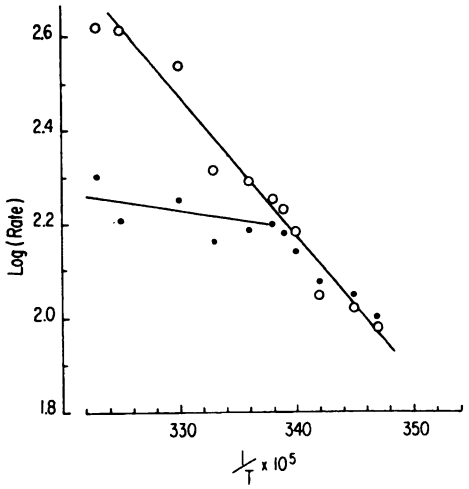

FIG. 4. *Arrhenius plot for basal (●) and fluoride-stimulated (○) adenylate cyclase from rat brain*
The sodium fluoride concentration was 10 mM. Rates are expressed as picomoles per milligram of protein per minute.

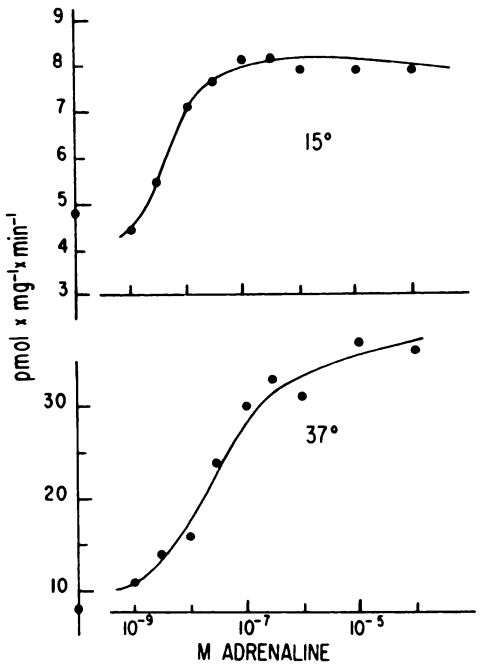

FIG. 5. *Dose-response curves for ardrenaline with Ehrlich cell adenylate cyclase at 15° and 37°*

urements were carried out in the absence and presence of 10 mM fluoride. It appeared that an inflection in Arrhenius plots around 24° was present with basal but not with fluoride-stimulated enzyme (Fig. 4). In addition, no stimulatory effect of fluoride was obtained at or below the temperature of inflection; thus progressive stimulation of activity by fluoride above this temperature was observed. Data for inflection temperatures and energies of activation from two experiments with rat brain enzyme are listed in Table 1. The slopes of straight-line regression segments above and below 23° in Arrhenius plots of fluoride-stimulated enzyme were not significantly different, confirming the absence of an inflection temperature in this range.

In the above studies with enzyme from Ehrlich cells, constant amounts of adrenaline (0.1 mM) were included in all measurements of stimulated enzyme activity, and it became necessary to assure that this concentration provided optimal stimulation throughout the temperature range studied. Dose-response curves were therefore determined at different temperatures. Results obtained at 37° and 15° (Fig. 5) show that in both cases maximal cyclase stimulation could be achieved at concentrations above 0.01 mM adrenaline. With sodium fluoride as the stimulant, dose-response curves at 15°, 25°, and 37° were virtually identical; i.e., maximal stimulation occurred at 8–10 mM, and half-maximal stimulation at 2–3 mM.

From a series of dose-response curves at different temperatures the concentrations of adrenaline producing half-maximal stimulation (K_a) were determined graphically from plots such as shown in Fig. 5. Data from three series of experiments (Table 2) indicate that K_a values tend to decrease with lower temperature, indicating a stronger binding of adrenaline to its receptor site.

All measurements of cyclase activity were carried out at a substrate concentration of 0.1 mM ATP, which is close to the K_m of Ehrlich cell adenylate cyclase (2). Discontinuities in Arrhenius plots could be related to changes of initial velocities or changes in K_m as a function of temperature. Although K_m measurements of adenylate cyclases are subject to large errors, particularly at the low reaction rates obtained at low temperature, attempts were made to determine these values at different temperatures. Results obtained in two series of experi-

TABLE 2

Adrenaline K_a values at different temperatures for Ehrlich cell adenylate cyclase

Temperature	K_a		
	Expt. 1	Expt. 2	Expt. 3
		$M \times 10^7$	
37°	1	2	3
30°	0.5	2	1
25°	0.3	0.5	0.6
20°	0.4	0.5	0.3
15°	0.2	0.5	0.5

TABLE 3

Effect of temperature on rate parameters of Ehrlich cell adenylate cyclase

Six different substrate concentrations, between 0.02 and 2 mM, were used in each rate curve, and results were processed by the computer program HYPER by W. W. Cleland (9), which makes a weighted fit to the reciprocal form of the rate equation $v = V_{max} \cdot S(k_m + S)^{-1}$. Standard errors of K_m estimates are listed; standard errors for V_{max} were below 10%. Two different enzyme preparations were used for experiments 1 and 2.

Temperature	K_m		V_{max}	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
	mM		*pmoles/mg protein/min*	
37°	0.10 ± 0.03	0.13 ± 0.02	20	43
32°		0.33 ± 0.06		47
28°	0.39 ± 0.15		31	
22°	0.13 ± 0.05	0.34 ± 0.10	14	23
19°	0.08 ± 0.03		9	
18°		0.25 ± 0.06		21
15°	0.19 ± 0.09	0.42 ± 0.09	9	16

ments, involving different batches of enzyme, are listed in Table 3. As expected, errors for K_m values are large and results of individual experiments are variable. However, it seems there is no major change in K_m in the mid-20° range. On the basis of the present data it is thus likely that discontinuities in Arrhenius plots in Fig. 3 were due to changes in initial velocities (proportional to V_{max}) and not to changes in the K_m or the affinity of the substrate for the enzyme.

DISCUSSION

The present studies show that adrenaline stimulation of adenylate cyclase *in vitro* is a rapid process, the time course of which cannot be measured by monitoring the progress of the catalytic reaction under physiologically relevant temperature conditions. The effects of *beta* adrenergic blocking drugs also involve rapid kinetic steps. Adrenaline action is readily reversible within the 15–37° temperature range studied, and reversal of *beta* blocking drug action likewise seems to be a fast process. Although only a limited number of enzyme systems have been used in this work and systematic data have been obtained only with enzyme from Ehrlich tumor cells, it seems reasonable to assume that the findings with respect to the rate and reversibility of *beta* adrenergic drug action are representative of other adrenaline-sensitive adenylate cyclases as well.

The time course of *beta* adrenergic drug interaction with adenylate cyclase-related receptor sites is of considerable relevance to binding studies involving suitable membrane preparations (containing adenylate cyclase) and radioactive catecholamines. Thus one would expect only equilibrium techniques to yield meaningful data, while others, involving precipitation and washing of membranes, would bear the risk of disturbing the binding equilibrium and of leading to extensive dissociation of radioactive hormone from the receptor. However, it is possible that at temperatures below 15° the dissociation of the drug-receptor complex is sufficiently slow to allow, for example, washing of membranes retained on filters. Schramm *et al.* (10) studied [^{3}H]adrenaline binding activity in membranes from turkey erythrocytes, using an equilibrium precipitation method that did not require any washing steps. In this system complete binding equilibrium was obtained at 37° within 1 min or less, which is compatible with the rate of adrenaline-cyclase interaction observed in the present study. On the other hand, Marinetti *et al.* (11) found a latency of

several minutes for the detection of any binding of radioactive adrenaline to a liver membrane preparation. This finding would indicate that the binding observed was unrelated to adenylate cyclase, and the trichloracetic acid precipitation procedure used by those authors makes it even more doubtful that they were dealing with the cyclase-related *beta* receptor in their studies.

Lefkowitz and Haber (12) measured [^{3}H]-noradrenaline binding to cardiac microsomes. The incubations were carried out at 37° over 2 hr, and the resulting complex appeared to be stable to a washing procedure on Millipore filters (not detailed). The slow establishment of complete binding appears incompatible with the conclusions reached in the present study; however, close inspection of their data indicates that a more rapidly occurring binding component might have been present. Subsequently Lefkowitz *et al.* (13) reported that a detergent-solubilized "receptor" fraction showed a more rapid establishment of binding equilibrium with [^{3}H]noradrenaline over a 10-min period, but this still appears slow in relation to the time course of adenylate cyclase activation observed in the present study and presumed to be similar in the case of heart membranes. Rapid dissociation of [^{3}H]noradrenaline from the binding complex, however, has been demonstrated (14).

In a further study on turkey erythrocyte membranes, Bilezikian and Aurbach (15) reported that isopropylnoradrenaline binding became maximal after a 10-min incubation period at 37°. This contrasts with parallel adenylate cyclase measurements in the absence and presence of catecholamine carried out within a 10-min incubation period. Clearly a general discrepancy exists between the time course of catecholamine binding and the stimulation of adenylate cyclase in the systems referred to above, begging more detailed studies.

The molecular basis of discontinuities in Arrhenius plots cannot be deduced from the studies carried out so far. Dixon and Webb (16) enumerated several theoretical possibilities. In addition to considering a possible transition (conformational change) of the enzyme itself, one should, in the case of membrane-bound adenylate cyclase, also expect that a phase or structural change of the membrane, of which the enzyme is an integral part, could affect rate parameters and the energy of activation of the over-all process. Such a possibility has also been considered by Keirns *et al.* (17) in a study on the temperature dependence of adenylate cyclase from liver. Small changes in pH as a function of temperature would not be expected to influence activity appreciably, since the pH optimum of the Ehrlich cell enzyme is sufficiently broad (2).

The observation that adrenaline does not seem to affect the slopes of the straight-line segments in Arrhenius plots further indicates that both transition temperature and activation energies for the cyclase reaction are determined by the catalytic unit. Stimulation by adrenaline then is probably expressed only on the level of the rate-limiting step of catalysis.

In a study of adenylate cyclase from frog bladder epithelium, Bockaert *et al.* (18) found that in the presence of oxytocin or fluoride the activation energy for the cyclase reaction was approximately doubled. The authors interpreted this finding to mean that a high-activation-energy step is involved for stimulation of the enzyme by fluoride or oxytocin. However, only kinetic analysis of the coupling step between the hormone receptor and adenylate cyclase at different temperatures will yield information on the activation energy for hormonal stimulation. These measurements would be very difficult, indeed, both technically as well as with respect to the problem of attributing kinetic data to molecular events. Bockaert *et al.* (18) did not observe a discontinuity in Arrhenius plots with adenylate cyclase from frog bladder epithelium, with or without stimulants. It is conceivable that there are differences in this respect between mammalian and nonmammalian enzymes, or between those stimulated by peptide hormones and adrenaline.

Following completion of the present study, Kreiner *et al.* (19) and Keirns *et al.* (17) reported on temperature effects on basal and hormone-stimulated rat liver adenylate cyclase and observed that in the presence of

glucagon or adrenaline a discontinuity in Arrhenius plots around 32° occurred, with an increase in activation energies from about 10 kcal/mole below 32° to 17 kcal/mole above that temperature. This change was absent in basal cyclase as well as in the presence of fluoride, propranolol, or prostaglandin E_1. Those authors did not extend their studies to temperatures below 20°, and it is conceivable that a further discontinuity, analogous to the one reported here, was missed near or below 20°. Conversely, present studies with Ehrlich cell enzyme have not focused on the 30–40° interval, and a discontinuity with an increase in activation energy may likewise have been missed. In view of the differences in the temperature dependence of basal and stimulated adenylate cyclases evident from the present work and that by Bockaert *et al.* (18) and Kreiner *et al.* (17, 19), it seems appropriate to caution against generalizing data obtained in one enzyme system only.

It is interesting that in the presence of fluoride ion the inflection point in Arrhenius plots of Ehrlich adenylate cyclase is uncertain or that, at least, differences in activation energies above and below such an inflection point are smaller than in the case of basal or adrenaline-stimulated enzyme. With fluoride-stimulated adenylate cyclase from rat brain the absence of a discontinuity in the Arrhenius plot is more clearly indicated. One is tempted to interpret the observations that activation by sodium fluoride is observed only above 24° in the latter system, and that no discontinuity in the Arrhenius plot exists, to mean that the agent stabilizes a "low-temperature form" of the enzyme.

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