Adenylate Cyclase Activation by the β-Adrenergic Receptors as a Diffusion-Controlled Process†

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ABSTRACT: Previous results [Tolkovsky, A. M., & Levitzki, A. (1978) in *Hormones and Cell Regulation* (Dumont, J., & Nunez, J., Eds.) Vol. 2, pp 89–105, Elsevier/North Holland Publishing Co., Amsterdam; (1978) *Biochemistry 17*, 3795–3810] indicated to us that the activation of turkey erythrocyte adenylate cyclase by the hormone-bound β-adrenergic receptor is a bimolecular process: HR + E $\xrightarrow{k_1}$ (HRE) → HR + E′, where HR is the hormone-bound receptor, E the inactive form of adenylate cyclase, E′ the activated form of the enzyme, and k_1 the bimolecular rate constant of enzyme receptor collision. (HRE) is the short-lived complex which does not accumulate. In this study we have designed experiments aimed to examine the possibility that this bimolecular process is diffusion controlled. Turkey erythrocyte membranes were fluidized progressively by the insertion of increasing amounts of *cis*-vaccenic acid. The fluidity of the native and the *cis*-vaccenic acid treated membrane was monitored by fluorescence polarization using diphenylhexatriene as a fluorescence probe. In parallel, the rate constant of enzyme activation by the epinephrine-bound β receptor was measured. It was found that the bimolecular rate constant k_1 increases *linearly*, 20-fold, as a function of membrane fluidity. This dependence of k_1 on membrane fluidity strongly suggests that the process of adenylate cyclase activation by the β receptor is a diffusion controlled process. Applying the mathematical correlation between the bimolecular rate constant and the diffusion coefficient in two-dimensional space, one can calculate that the diffusion coefficient of the β-adrenergic receptor is in the range of 4.0×10^{-11} to 9.0×10^{-10} cm²/s at 25 °C. The increase in membrane fluidity also causes a maximal threefold increase of the adenylate cyclase specific activity. This effect is a direct effect on the catalytic unit, also found for the NaF-stimulated activity, and can be separated from the effect of membrane fluidity on the interaction between the receptor and the catalytic unit.

The hormone and the neurotransmitter dependent adenylate cyclases represent a transmembrane regulatory system. Upon the interaction of the receptor at the outer surface of the cell membrane, the catalytic unit of adenylate cyclase becomes activated and converts intracellular ATP to cAMP at the inner surface of the cell membrane. This sequence of events necessitates the interaction between the receptor moiety and the adenylate cyclase molecule, each of which represents independent macromolecules (Caron & Lefkowitz, 1976; Schramm et al., 1977). Recently, progress has been made in the understanding of the mechanistic aspects of the mode of coupling between the hormone receptor and adenylate cyclase. Thus, it is generally accepted that the activation of the adenylate cyclase unit requires the binding of intracellular GTP (Rodbell et al., 1971a,b, 1974a,b) to the GTP regulatory protein (Pfeuffer & Helmreich, 1975; Pfeuffer, 1977) which is tightly associated to the catalytic unit. Furthermore, in the β-adrenergic receptor adenylate cyclase, it was demonstrated directly that the hormonal signal is terminated concomitantly with the hydrolysis of GTP to GDP and inorganic phosphate (Cassel & Selinger, 1976). Therefore, the *continued* presence of *both* hormone and GTP is required to maintain a steady-state level of active adenylate cyclase (Levitzki, 1977; Sevilla & Levitzki, 1977; Tolkovsky & Levitzki, 1978a).

When a nonhydrolyzable analogue of GTP, such as GppNHp or GTPγS,[1] is used instead of GTP, the deactivation process is irreversibly blocked (Sevilla et al., 1976; Levitzki et al., 1976; Cassel & Selinger, 1976; Levitzki, 1977; Tolkovsky & Levitzki, 1978a,b) and all of the enzyme molecules are converted to a permanently active form. The availability of the nonhydrolyzable analogues of GTP proved useful in delineating the mode of coupling between the receptor and the enzyme. Hence, we have demonstrated that the kinetic pattern of adenylate cyclase activation by hormone, in the presence of GppNHp, depends strongly on the mode of receptor to enzyme coupling (Tolkovsky & Levitzki, 1978a,b; Levitzki, 1978). Thus we have demonstrated that the mode of coupling of the β-adrenergic receptor to adenylate cyclase in turkey erythrocytes is a bimolecular process and follows the scheme

$$H + R \underset{K_H}{\longrightarrow} H{\cdot}R \xrightleftharpoons[\leftarrow]{E\cdot GppNHp,\ k_1} [H{\cdot}R{\cdot}E{\cdot}GppNHp \rightleftharpoons H{\cdot}R{\cdot}E'{\cdot}GppNHp] \rightarrow HR + E{\cdot}GppNHp \quad (1)$$

where H is the hormone, R the receptor, K_H the hormone-receptor dissociation constant, E the enzyme, and E′ the activated form of the enzyme. It was found (Tolkovsky & Levitzki, 1978a,b) that the species H·R·E·GppNHp and H·R·E′·GppNHp are short-lived and do not accumulate.

The accumulation of the activated enzyme E′·GppNHp upon exposure of the system to hormone is given by

$$[E'] = [E_{max}]\left[1 - \exp\left(\frac{k_1[R_T][H]}{K_H + [H]}\right)t\right] = [E_{max}][1 - \exp(-k_{obsd}t)] \quad (2)$$

and at saturating hormone by

$$[E'] = [E_{max}]\{1 - \exp(-k_1[R_T])\} = [E_{max}](1 - \exp(-k_{obsd}t)] \quad (3)$$

where $[E_{max}]$ is the total enzyme concentration in the system and k_{obsd} the pseudo-first-order rate constant of activation.

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[1] Abbreviations used: GppNHp, guanylyl imidodiphosphate; DPH, 1,6-diphenyl-1,3,5(*all-trans*)-hexatriene; HYP, hydroxybenzylpindolol; $NaDodSO_4$, sodium dodecyl sulfate; TME, 50 mM Trizma base·HCl + 2 mM $MgCl_2$ + 1 mM EDTA buffer, pH 7.4; TM_7E, 50 mM Trizma base·HCl + 7 mM $MgCl_2$ + 1 mM EDTA buffer, pH 7.4; GTPγS, guanosine 5′(γ-thio)triphosphate.

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Equation 2 was found to be valid in the turkey erythrocyte system under all hormone concentrations for a variety of agonists. Furthermore, the values of K_H for different agonists obtained from the hormone dependence of k_{obsd} were shown to correspond very closely to the K_H values obtained from binding measurements and from the dependence of adenylate cyclase activity on agonist concentration (Tolkovsky & Levitzki, 1978a). The number of receptors remains constant in the turkey erythrocyte system since desensitization does not occur in these cells (Hanski & Levitzki, 1978). According to this scheme, the receptor activates the enzyme during its brief encounter with the enzyme; namely, the complexes H·R·E·GppNHp and H·R·E'·GppNHp never accumulate to significant concentrations. This mode of coupling was termed "collision coupling" (Levitzki, 1978; Tolkovsky, & Levitzki, 1978a,b) and accounts for the following findings.

(a) The binding of agonists and antagonists is noncooperative.

(b) The kinetics of enzyme activation by β agonists in the presence of GppNHp is first order.

(c) Progressive inactivation of the receptor results in a proportional decrease in the rate constant of enzyme activation by hormone but not in the maximum level of adenylate cyclase activity attainable. Thus, in the presence of l-epinephrine and GppNHp one can activate all the cyclase catalytic units even when more than 93% of the β receptors have been irreversibly inactivated by a β receptor directed affinity label.

The model described in eq 1 implies that the activation rate of adenylate cyclase is governed by the rate of random collision between the hormone-bound receptor and the catalytic unit. This bimolecular reaction is characterized by the rate constant k_1. Since every receptor molecule can activate every catalytic unit, it necessitates relative motion between these two units in the membrane matrix.

In this communication we examine the dependence of the rate of adenylate cyclase activation by the β receptor on membrane fluidity in order to explore the possibility that the process of activation is diffusion controlled. The suggestion of receptor mobility is in accordance with the findings of Schramm et al. (1977) that turkey erythrocytes' β receptors can be transferred to another cell upon fusion.

Materials and Methods

Materials. [α^{32}P]ATP and Na[^{125}I] were obtained from Radiochemical Centre (Amersham, England). GppNHp and l-epinephrine were obtained from I.C.N. (±)HYP was a gift from Dr. Hauser (Sandoz, Basel). *cis*-Vaccenic acid, creatine phosphokinase, creatine phosphate, theophylline, and Trizma base were obtained from Sigma. All other chemicals and biochemicals used were of the highest purity available.

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

Insertion of cis-Vaccenic Acid into the Membranes. The insertion of *cis*-vaccenic acid into the membranes followed essentially the procedure of Orly & Schramm (1975). Turkey erythrocyte membranes, washed twice and resuspended in TME buffer at concentrations of 3 mg of protein per mL, were incubated 30 min at 0 °C with different volumes (between 1 and 10 μL) of 100 mM *cis*-vaccenic acid dissolved in absolute ethanol. The ethanol volume was equalized in all samples prior to the incubation.

The amount of *cis*-vaccenic acid added varied in the range of 0.03 to 0.23 μmol of acid per mg of protein. More than 85% of the added fatty acids was incorporated into the membrane as was shown by Orly & Schramm (1975). The final volume of ethanol in adenylate cyclase assay due to this treatment did not exceed 0.5% (v/v). This amount of alcohol was found to be without effect on the adenylate cyclase activity.

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

Binding of [^{125}I]HYP to the β-Adrenergic Receptor. The binding of [^{125}I]HYP to the β receptor was assayed essentially according to Spiegel et al. (1976), as described by us earlier (Tolkovsky & Levitzki, 1978a). The volume of the reaction mixture was 500 μL, where each reaction mixture contained about 10–25 μg of membrane protein. 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.

Kinetics of cAMP Accumulation. cAMP production was initiated by mixing the assay ingredients and the activating ligands with the membranes, using the same procedure described earlier (Tolkovsky & Levitzki, 1978a). The activating ligands were 1.0×10^{-4} M l-epinephrine with 1.0×10^{-5} M GppNHp or 10 mM F^- ions. The assay components were 0.4 mg/mL of theophylline, 5 mM creatine phosphate, 50 U/mL of creatine phosphokinase, 2 mM ($2–10 \times 10^6$ cpm) [α^{32}P]ATP, and 6 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.4. The membranes' protein concentration was between 0.55 and 0.85 mg/mL, as determined according to Lowry et al. (1951), using bovine serum albumin as standard. All the concentrations mentioned above are final concentrations in the adenylate cyclase assay system.

At specific times, subsequent to mixing, samples of 150 μL were withdrawn from the reaction mixture and added to 100 μL of 0.2% $NaDodSO_4$ solution. The amount of cAMP produced was determined according to Salomon et al. (1974). All the components of the reaction mixture were preincubated for 5 min at 25 °C prior to mixing. The cAMP accumulation experiment was also performed at 25 °C. Further details are given in the legends to figures and in a previous study (Tolkovsky & Levitzki, 1978a).

Analysis of Kinetic Data. The initial estimates for the parameters characterizing the kinetics of cAMP accumulation, $k_{cat}[E_{max}]$, the specific activity of the enzyme, and the rate constant for enzyme, activation, k_{obsd}, were obtained by the method described in Results. The best fit parameters were computed by using a nonlinear curve fitting procedure, LSQ (Booth et al., 1959), obtained from the library of The Hebrew University of Jerusalem Computer Center.

Microviscosity Measurements. The apparent microviscosity of the membranes was measured by the fluorescence polarization technique, using DPH as the fluorescent probe. The fluorescent hydrocarbon 1,6 diphenyl-1,3,5-hexatriene (DPH) was found to be a very useful probe for measuring membrane fluidity (Shinitzky et al., 1971; Cogan et al., 1973; Rudy & Gitler, 1972; Shinitzky & Inbar, 1974).

Untreated membranes and *cis*-vaccenic acid treated membranes were incubated with 0.6 μM DPH for 30 min at 30 °C. The microviscosity was measured on the same membrane preparations which were used for the kinetic experiments. The quotient $I_\parallel/I_\perp$, fluorescence emitted in parallel to the exciting light divided by the fluorescence emitted in perpendicular to the exciting light, was monitored as a function of increasing temperature. The measurements were performed on the fluorescence polarization instrument built by Dr. Meir Shinitzky at the Department of Membranes and Bioregulation of the Weizmann Institute of Science, Rehovot, Israel. The

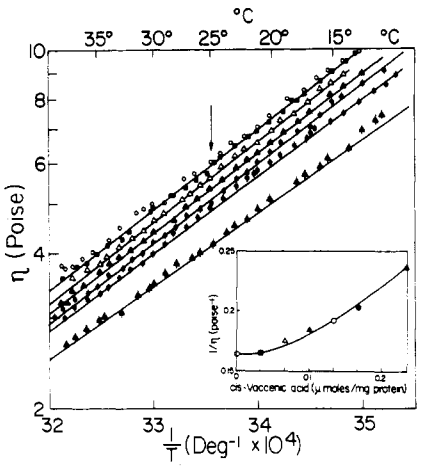

FIGURE 1: The dependence of turkey erythrocyte membrane apparent microviscosity on temperature as a function of *cis*-vaccenic acid content. The microviscosity of each sample was measured in the temperature range of 5–40 °C by the fluorescence polarization technique, using DPH as microviscosity probe. For details, see text. The membrane samples were treated with the following *cis*-vaccenic acid concentrations (μmol/mg of protein): (▲) 0.233; (●) 0.166; (◇) 0.133; (▲) 0.1; (△) 0.066; (■) 0.03; (○) 0.00. The apparent microviscosity values were calculated by using the approximated equation for η (eq 4). Inset: The dependence of membrane fluidity on *cis*-vaccenic acid incorporation. The reciprocal of the apparent microviscosity values at 25 °C derived from Figure 1 are shown as a function of the *cis*-vaccenic acid concentrations at which they were treated. As was pointed out in the text, over 85% of the fatty acid is incorporated into the membrane.

apparent microviscosity was calculated according to the approximate formula

$$\eta = \frac{I_{\parallel}/I_{\perp} - 1}{0.73 - 0.27 I_{\parallel}/I_{\perp}} \qquad (4)$$

where η is the apparent microviscosity. The derivation of the above formula (Shinitzky, 1979) is based on the results of Shinitzky & Inbar (1974) who showed that the term $c(r)T\tau$ for DPH in the Perrin equation (Perrin, 1934)

$$r_0/r = 1 + c(r)T\tau/\eta \qquad (5)$$

is approximately constant. This term obtains the value of 2.4 P for measurements using DPH in a series of liposomes and membranes over the temperature range of 0–40 °C. In eq 5 r is the fluorescence anisotropy, τ is the lifetime of the excited state, T is the absolute temperature, and r_0 is the upper limit of r. For DPH, $r_0 = 0.362$ (Shinitzky & Barenholz, 1974). $c(r)$ is a structural parameter of the probe which varies slightly with r and was calibrated for DPH using paraffin oil as the reference solvent (Shinitzky & Inbar, 1974).

Results

cis-Vaccenic Acid Induced Changes in Membrane Fluidity. Turkey erythrocyte membranes were treated with increasing concentrations of *cis*-vaccenic acid, as described in Materials and Methods. The fluidity of the *cis*-vaccenic acid treated membranes was measured using fluorescence polarization over the range of 5–40 °C. A plot of log (viscosity^{-1}) vs. $1/T$ yielded a set of parallel lines (Figure 1) with no discontinuity. A plot of membrane fluidity against the amount of *cis*-vaccenic acid, incorporated at constant temperature, revealed a parabolic dependence (Figure 1).

Absence of an Effect of cis-Vaccenic Acid on the β Receptor. Incorporation of increasing amounts of *cis*-vaccenic acid into the turkey erythrocyte membrane does not modify the properties of the β-adrenergic receptor. From Figures 2 and 3 it is apparent that the insertion of *cis*-vaccenic acid into the membrane does not alter either the number of receptors or their affinity toward β blockers or β agonists. Also, the kinetics of receptor–ligand interactions remain unaffected by the fluidization of the membrane (Figure 2). These findings are in agreement with previous observations (Hanski & Levitzki, 1978) where it was found that cholesterol depletion and cholesterol enrichment of intact turkey erythrocytes does not modify either the number of β-adrenergic receptors or their affinity toward [^{125}I]HYP. Similarly, the enrichment of intact turkey erythrocytes with phospholipids was found to be without effect on the properties of the β receptors (Hanski & Levitzki, 1978).

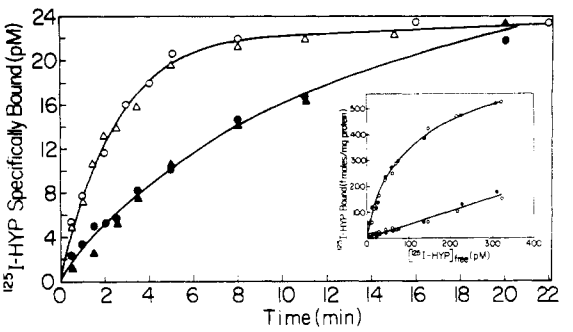

FIGURE 2: The time course of [^{125}I]HYP binding to β receptors in *cis*-vaccenic acid treated and native membranes. *cis*-Vaccenic acid treated membranes (○) and native membranes (△) were incubated at a concentration of 50 μg/mL of protein with 300 pM of [^{125}I]HYP in TM_7E buffer at 25 °C (empty symbols) and 15 °C (full symbols). At the indicated times samples of 500 μL were withdrawn and [^{125}I]HYP bound was determined by filtration techniques as described in the text. Inset: The equilibrium binding curve of [^{125}I]HYP to native and *cis*-vaccenic acid treated membranes. Binding assay of [^{125}I]HYP was performed on *cis*-vaccenic acid treated membranes (○) and on native membranes (●) at 25 °C as described in the text. Treated and untreated membranes at a concentration of 25 μg/mL of protein were incubated in the presence of [^{125}I]HYP concentration in TM_7E buffer. The straight line represents the nonspecific [^{125}I]HYP binding measured in the presence of 5×10^{-6} M propranolol. The saturative line represents total [^{125}I]HYP bound as a function of free [^{125}I]HYP.

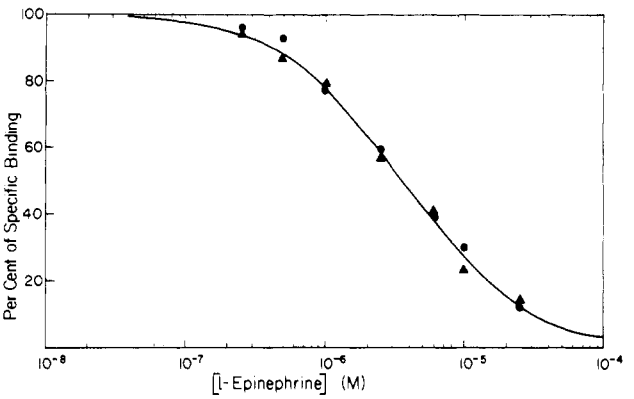

FIGURE 3: The displacement of [^{125}I]HYP by l-epinephrine in native and *cis*-vaccenic acid treated membranes. Treated (●) and untreated (▲) membranes at a concentration of 20 μg/mL of protein were incubated in the presence of 400 pM [^{125}I]HYP and increasing concentrations of l-epinephrine, as indicated. After 20 min of incubation at 25 °C, the specifically bound [^{125}I]HYP was determined.

Effect of Fluidity Changes on the Rate of Cyclase Activation. The process of turkey erythrocyte adenylate cyclase activation by *l*-catecholamines, in the presence of a nonhydrolyzable analogue of GTP, such as GppNHp, can be described by eq 2, as was shown by us earlier (Tolkovsky & Levitzki, 1978a,b; Levitzki, 1978).

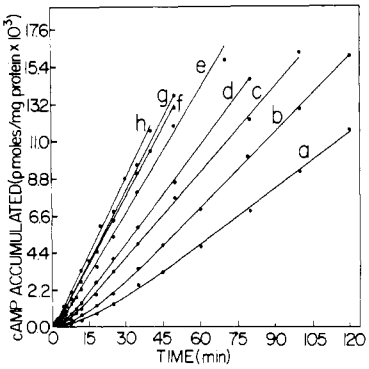

FIGURE 4: The time course of cAMP accumulation in the presence of hormone and GppNHp in *cis*-vaccenic acid treated membranes. Time dependence of cAMP accumulation was measured at 25 °C in *cis*-vaccenic acid treated membranes, as described in Materials and Methods. Each curve represents the optimized fit obtained using the integrated rate formula (eq 6) to the experimental points. The average deviation between duplicate determinations did not exceed 5%. a, b, c, d, e, f, g, and h curves were obtained from membranes with 0, 0.033, 0.05, 0.066, 0.1, 0.133, 0.166, and 0.233 μmol of *cis*-vaccenic acid/mg of membrane protein, respectively.

Since the activated form of the enzyme E′ is the species responsible for the production of cAMP from ATP, one can follow the time course of accumulation of E′ by following the accumulation of cAMP in the system (see also Tolkovsky & Levitzki, 1978a). The equation describing the accumulation of cAMP as a function of time is obtained by the integration of eq 2 and multiplying the integral with k_{cat}, the turnover number of adenylate cyclase. Thus, the product $k_{cat}[E_{max}]$ is the maximal specific activity attainable. The integrated equation, therefore, obtains the form (Tolkovsky & Levitzki, 1978a)

$$cAMP_t = k_{cat}[E_{max}]t + \frac{k_{cat}[E_{max}]}{k_{obsd}}[\exp(-k_{obsd}t) - 1] \qquad (6)$$

where $cAMP_t$ is the absolute amount of cAMP measured at each time point t.

Equation 6 predicts a lag time in the production of cAMP and a linear time dependence of cAMP production on time, when the term $\exp(-k_{obsd}t)$ becomes negligible. The slope of the linear portion yields the maximal specific activity, k_{cat}·$[E_{max}]$, and the intercept on time axis yields the value of $1/k_{obsd}$. Figure 4 shows the time dependence of cAMP accumulation at 25 °C, at saturating concentrations of l-epinephrine, for seven membrane preparations to which increasing amounts of *cis*-vaccenic acid were incorporated. The fluidity measurements described in Figure 1 were conducted on the same membranes on which the kinetic experiments were performed. The lines in Figure 4 represent a computer fit according to eq 6, where the points represent the experimental determinations. The kinetic model depicted in eq 6 was found earlier (Tolkovsky & Levitzki 1978a; 1979) to be the one which best fits the mode of adenylate cyclase activation by catecholamines in the turkey erythrocyte system. Other models predict kinetic patterns which deviate strongly from the experimentally observed patterns of cyclase activation. Furthermore, the *cis*-vaccenic acid induced fluidization does not change the kinetic pattern observed and therefore the mode of interaction between the receptor and the enzyme. From Figure 4 the values of k_{obsd} for each of the membrane preparations could be computed. A plot of k_{obsd} vs. the amount of *cis*-vaccenic acid incorporated is given in Figure 5. It can be seen from Figure 5 (inset) that k_{obsd} at 25 °C increases almost 20-fold over the range of *cis*-vaccenic acid incorporated and that the dependence of k_{obsd} on *cis*-vaccenic acid is similar

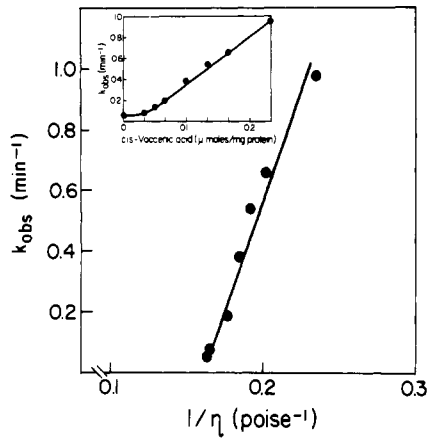

FIGURE 5: The dependence of k_{obsd} on membrane fluidity. k_{obsd} values at each *cis*-vaccenic concentration are plotted as a function of one over viscosity ($1/\eta$, fluidity). Experimental details are given in the text. Inset: The dependence of the activation rate constants, k_{obsd}, on the *cis*-vaccenic acid content of the membranes. The k_{obsd} values are shown as a function of the *cis*-vaccenic acid concentration at which the membranes were treated. The values of the k_{obsd} parameters were derived by fitting the integrated rate equation for cAMP accumulation for each curve in Figure 4.

to the dependence of membrane fluidity on *cis*-vaccenic acid (Figure 1). It can also be seen from Figure 4 that the increase of k_{obsd}, as a function of the amount of *cis*-vaccenic acid incorporated, shortens the lag period, as is indeed expected from eq 6.

When the values of k_{obsd} obtained from the data in Figure 4 were plotted against the fluidity of the membrane, a linear dependence was found (Figure 5). Interestingly, the intercept of the straight line describing the dependence of k_{obsd} vs. $1/\eta$ is at 0.16 P^{-1} and not at infinite viscosity (see Discussion).

Absence of an Effect of cis-Vaccenic Acid on the Basal Activity. Turkey erythrocyte membranes possess very low basal activity, 0.5–0.8 pmol of cAMP/(mg·min) in the regular cyclase assay. Also, in the presence of GppNHp alone, in the absence of an agonist, the rate of cyclase activation is insignificant and is less than 1500 pmol of cAMP accumulated in 120 min under the experimental conditions depicted in Figure 4. Similarly in the *cis*-vaccenic acid treated membranes the amount of cAMP accumulated during 120 min is less than 2000 pmol of cAMP. This finding eliminated the necessity to subtract the GppNHp induced activity from the GppNHp plus hormone activity. It must be emphasized that, in other systems such as the S49 β-receptor dependent cyclase, the basal activity is substantial (Ross et al., 1977).

Effect of cis-Vaccenic Acid on the Catalytic Unit of Adenylate Cyclase. The effect of *cis*-vaccenic acid on the maximal specific activity $k_{cat}[E_{max}]$ was determined at 25 °C from the limiting slopes of Figure 4, namely, from the same experiment in which the dependence of k_{obsd} on fluidity was measured. Figure 6 shows the dependence of the maximal specific activity of adenylate cyclase attainable with l-epinephrine and GppNHp ($k_{cat}[E_{max}]$) on the amount of *cis*-vaccenic acid incorporated into the membranes, and Figure 7 shows the dependence of the maximal specific activity of the enzyme on the fluidity of the membrane. It can be seen that the enzyme specific activity extrapolates to zero at a membrane fluidity of 0.164 P^{-1}. This value is identical with that obtained upon extrapolating the plot of k_{obsd} vs. $1/\eta$ to $k_{obsd} = 0$ (Figure 5). The dependence of the F^--stimulated adenylate cyclase specific activity on *cis*-vaccenic concentration (Figure 6) and on membrane fluidity (Figure 7) was found to be similar to the dependence of the hormone plus GppNHp stimulated

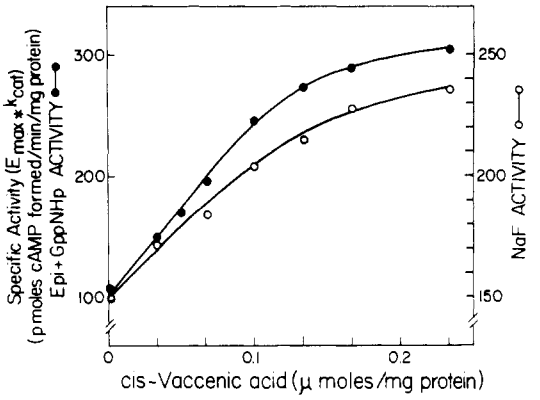

FIGURE 6: Adenylate cyclase specific activity $k_{cat}[E_{max}]$ in the presence of hormone and GppNHp and in the presence of F^- as a function of *cis*-vaccenic acid content. The values of $k_{cat}[E_{max}]$ for the 1-epinephrine plus GppNHp activity (●) were derived from each progress curve depicted in Figure 4. The F^- specific activity values (○) were determined from the linear slopes of the cAMP accumulation (see Figure 8).

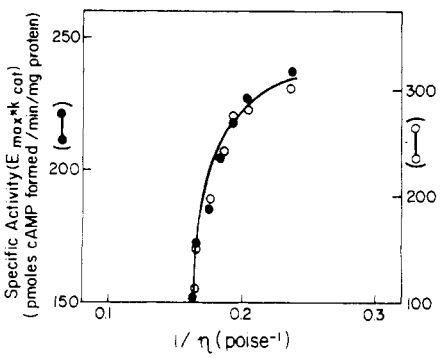

FIGURE 7: Adenylate cyclase specific activity $k_{cat}[E_{max}]$ in the presence of hormone and GppNHp (○) and in the presence of F^- (●) as a function of membrane fluidity.

adenylate cyclase on *cis*-vaccenic concentration (Figure 6) and on membrane fluidity (Figure 7). The lag time observed for the F^--stimulated cyclase activity was found to be independent on membrane fluidity (Figure 8). The steep dependence of $k_{cat}[E_{max}]$ on membrane fluidity, at the lower fluidity range (0.16–0.19 P^{-1}, Figure 7), accounts for the significant effect of *cis*-vaccenic acid on the rate of cAMP accumulation (Figure 4), at a concentration range that has a negligible effect on k_{obsd} (Figure 5).

In summary, these findings demonstrate that membrane fluidity affects the catalytic moiety directly in addition to the effect on the interaction between the receptor and the enzyme.

Discussion

In this study we have examined the effect of fluidity changes on the kinetic parameters characterizing the process of adenylate cyclase activation. The membrane fluidity was progressively increased by inserting *cis*-vaccenic acid which was demonstrated previously to be one of the most efficient fluidizing agents (Orly & Schramm, 1975). We have separated between the effect of membrane fluidity on the bimolecular rate constant which characterizes the rate of collision between the occupied receptor and the catalytic unit and the direct effect of membrane fluidity on the catalytic moeity.

Effect of Membrane Fluidity on the β Receptor. Increase of membrane fluidity has no effect on the mode of ligand binding to the β-adrenergic receptor. Both native and *cis*-vaccenic acid fluidized turkey erythrocyte membranes were found to possess an identical number of receptors representing

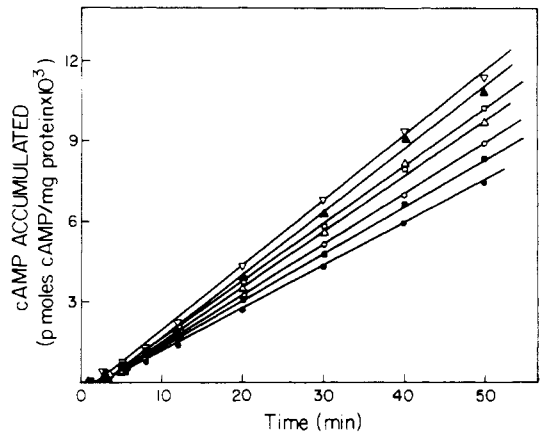

FIGURE 8: Time dependence of cAMP accumulation in the presence of fluoride in *cis*-vaccenic acid treated membranes. cAMP accumulation as a function of time was measured at 25 °C, as described in Materials and Methods, using 10 mM fluoride as an activating ligand. The symbols ●, ■, ○, △, □, ▲, and ▽ represent respectively 0, 0.033, 0.066, 0.1, 0.133, 0.166, and 0.233 μmol of *cis*-vaccenic acid incorporated per mg of membrane protein.

a single class of binding sites with respect to both β antagonists and β agonists (Figures 2 and 3). Furthermore, kinetics of ligand binding to the β receptor remain unaffected by fluidizing the membrane (Figure 2).

Effect of Membrane Fluidity on the Rate of Enzyme Activation. Whereas the properties of the β receptor per se do not change upon increasing membrane fluidity, a dramatic effect is observed in the kinetics of adenylate cyclase activation by the hormone. From Figure 5 it can be seen that the rate constant of enzyme activation (k_{obsd} in eq 2 and 3) is *directly proportional* to the membrane fluidity. A plot (Figure 5) of k_{obsd} vs. $1/\eta$ yields a straight line with an intercept at 0.160 P^{-1} (η = 6.25 P). The change in membrane fluidity also affects directly the catalytic unit of adenylate cyclase. The effect of increasing membrane fluidity on the hormone plus GppNHp specific activity and on the F^--independent specific activity (Figure 7) is found to be similar. In both cases the dependence of enzyme activity on membrane fluidity is saturable and extrapolates to zero at 0.162 P^{-1}.

The strong inhibition of enzyme activity as a result of changes in membrane organization at a certain temperature or at high membrane viscosity is a general phenomenon and was encountered in a number of membranal embedded systems such as Ca^{2+}–ATPase (Warren et al., 1974, 1975), (Na^+–K^+)ATPase (Kimelberg, 1975), and the β-glucoside transport system (Thilo et al., 1977).

From Figure 8 it is apparent that the lag period of adenylate cyclase activation by F^- ions is independent of the degree of membrane fluidity. This finding supports the commonly accepted view that the mode of F^- activation of adenylate cyclase is by the direct interaction of the latter with the catalytic moiety.

Diffusion-Controlled Reactions and the Viscosity of the Medium. Let us consider a chemical reaction between two reactants, R and E. Under steady-state conditions for diffusion one can express the bimolecular rate constant by (North, 1964)

$$k_b = cD_{RE}\frac{k}{k + cD_{RE}} \quad (7)$$

where k is the rate of the chemical reaction occurring between R and E during their encounter, D_{RE} is the coefficient of the relative diffusion of the two components, c is a constant determined according to the space dimensionality typical for that reaction, and k_b is the measured bimolecular rate constant.

When k is much larger than cD_{RE}

$$k_b = cD_{RE} \tag{8}$$

Namely, the reaction is diffusion controlled (or "diffusion limited"). Such reactions in three-dimensional space have been investigated (Amdur & Hammes, 1966; North, 1964). Since the diffusion coefficient is inversely proportional to the *viscosity* of the medium, or directly proportional to the *fluidity* of the medium, in which the reaction takes place, it follows that the bimolecular rate constant k_b is *inversely proportional* to the viscosity of the medium. Such inverse relation between the bimolecular rate constant and the viscosity of the medium was indeed found, for example, in the quenching of triplet naphthalene by 1-iodonaphthalene (Osborne & Porter, 1963).

Diffusion-Controlled Nature of Adenylate Cyclase Activation by the β Receptor. The inverse relationship between the rate constant of adenylate cyclase activation by the β receptor and the viscosity of the membrane (Figure 5) suggested to us that the reaction is diffusion controlled.

It should be stressed that the simple inverse relationship between the rate constant of a diffusion-controlled chemical reaction and the viscosity of the medium holds also for two-dimensional space, such as a membrane (Saffman & Delbrück, 1975).

Ideal dependence of k_{obsd} on a medium viscosity predicts that k_{obsd} approach zero at infinite viscosity value. From Figure 5 it is apparent, however, that the value of k_{obsd} approaches zero at the finite viscosity value of 6.1 P and not at infinite viscosity. Since the extrapolated value of the catalytic activity approaches zero also at 6.1 P, we would like to suggest that the deviation from the ideal case results from a local phase transition at the adenylate cyclase domain. In the ideal case it is expected that a diffusion-controlled process k_{obsd} should approach zero at infinite viscosity ($1/\eta \rightarrow 0$). Since, however, the phospholipids of biological membranes exhibit a phase transition from a crystalline–liquid phase to a crystalline phase at *finite* viscosity values, it is expected that the diffusion processes of membrane proteins will be completely inhibited at a finite value of viscosity, different from zero. This phase transition causes a strong inhibition of the adenylate cyclase system. As was indicated above, the strong inhibition of membranal systems of a finite viscosity is a widespread phenomenon.

Estimation of the Relative Diffusion Coefficient for Adenylate Cyclase System. The maximal rate of a steady-state diffusion-controlled reaction between substrate (S) and enzyme (E) in two-dimensional space was shown to be (Hardt & Cone, 1979)

$$v = \frac{[S][Enz]N_A 4\pi D_{s,E}}{\ln\left[\left(\frac{\pi}{4}[Enz]N_A\right)^{1/2}\frac{1}{a}\right]} \tag{9}$$

where N_A is Avogadro's number, $D_{s,E}$ is the relative diffusion coefficient, a is the length of the active site, v is the rate of the bimolecular reaction, [S] is the substrate concentration, and [Enz] is the enzyme concentration. Since in the system described here the receptor concentration remains constant throughout the activation process, but the inactive catalytic units are converted to active units and therefore depleted, one can consider the receptor, R, as the enzyme molecule and the inactive catalytic unit, E, as the substrate. As was shown previously, the activation rate can be described as (eq 1, Tolkovsky & Levitzki, 1978a,b) a bimolecular reaction

$$v = k_1[E][R_T] \tag{10}$$

where [E] is the concentration of cyclase and $[R_T]$ the total receptor concentration. Since the receptor concentration remains constant

$$v = k_{obsd}[E] \tag{11}$$

Equalizing eq 9 with eq 11 and substituting $[R_T]$ for [Enz] and [E] for [S] yields

$$k_{obsd} = \frac{N_A 4\pi D_{R,E}[R_T]}{\ln\left[\left(\frac{\pi}{4}[R_T]N_A\right)^{1/2}\frac{1}{a}\right]} \tag{12}$$

Since the diffusion coefficient is inversely proportional to the medium viscosity, one can write the Stokes–Einstein relation

$$D = \frac{kT}{6\pi r\eta} = \frac{c}{\eta} \tag{13}$$

namely

$$k_{obsd} = \left[\frac{N_A 4\pi[R_T]}{\ln\left[\left(\frac{\pi}{4}[R_T]N_A\right)^{1/2}\frac{1}{a}\right]}\right]\frac{c}{\eta} \tag{14}$$

The term in brackets is calculated from the slope of Figure 5. Since the number of β receptors per cell is known to be about 1200 per cell and the cell surface is 200 μm^2 (Levitzki et al., 1974; Atlas et al., 1977; Hanski & Levitzki, 1978), one can calculate $[R_T]$. a is taken as 10^{-8} cm. Thus from the slope of k_{obsd} vs. $1/\eta$ one can evaluate c and calculate $D_{R,E}$ by taking the ratio c/η (eq 13) for any desired temperature. Thus, the value for $D_{R,E}$ at 25 °C in native turkey erythrocyte membranes was found to be 4.2×10^{-11} cm^2/s.

In estimating $D_{R,E}$ we have implicitly assumed that every collision between occupied receptor and the inactive catalytic unit is productive. Obviously, if not every collision results in enzyme activation the real value of the diffusion coefficient will be higher. The highest diffusion coefficient reported for a membranal protein was found to be about 1×10^{-9} cm^2/s (Poo & Cone, 1974; Edidin, 1974; Edidin et al., 1976).

Adopting this value as the highest possible, one can immediately calculate that at least 1 out of 24 collisions between the hormone bound receptor and the catalytic unit must result in activation for a full agonist.

The possibility remains, however, that the receptor units as well as the cyclase units are clustered in domains (Atlas & Levitzki, 1978; Atlas et al., 1978). In this case one should compute $[R_T]$ (eq 12 and 14) by taking into account only a fraction of the cell surface area. Under these circumstances, the calculated value of $D_{R,E}$, assuming that every collision is fruitful, will attain a value lower than 4.2×10^{-11} cm^2/s. This conclusion can be deduced from eq 12 which shows that the experimental value for k_{obsd} is proportional to the product of $D_{R,E}$ and $[R_T]$. Since the upper limit for a diffusion coefficient characterizing the motion of a membrane protein is 1×10^{-9} cm^2/s, it is also possible to account for a collision efficiency lower than 1 out of 24 collisions without assuming a diffusion coefficient higher than the latter value if the receptors are localized in domains. Although the pattern of receptor distribution on the turkey erythrocyte is not known, it is likely that the receptors are randomly distributed. This assertion is based on the observation that, even when 93% of the receptor molecules are irreversibly inactivated, the remaining receptors are able to activate *all* the catalytic units on the cell membrane (Tolkovsky & Levitzki, 1978a,b).

Independence of NaF Activation on Membrane Fluidity. The activation of adenylate cyclase by NaF exhibits a lag time which is independent of membrane fluidity. This finding indicates that the rate-limiting step in the activation of

adenylate cyclase by NaF involves a structural transition within the enzyme which is independent on the surrounding membrane matrix.

Validity of the Approach to Other Adenylate Cyclases. The measurement of the dependence of the kinetic pattern of adenylate cyclase activation on membrane fluidity can be a useful tool to explore the mode of receptor to enzyme coupling also in other adenylate cyclase systems. The turkey erythrocyte system proved to be a relatively simple model system to study the mode of receptor to enzyme coupling since the properties of this system are less complex than those of other receptor dependent adenylate cyclases. (a) Turkey erythrocyte membranes do not demonstrate any dependence of the cyclase activity on externally added GTP. Furthermore, GTP does not induce a change in the receptor thus reducing its affinity to agonists, as reported for other receptor dependent adenylate cyclases (Rodbell et al., 1971a,b; Lad et al., 1977; Maguire et al., 1976a,b; Lefkowitz et al., 1976). (b) The binding of agonists to turkey erythrocyte membranes is noncooperative under all experimental conditions tested (Tolkovsky & Levitzki, 1978a, and references therein), whereas guanyl nucleotides were found to modulate agonist binding to S49 by lymphoma cell membranes (Ross et al., 1977). (c) The turkey erythrocyte system, unlike other β-adrenergic systems, does not (Hanski & Levitzki, 1978) undergo induced desensitization; namely, the concentration of receptors remains unchanged during the exposure to agonists. (d) Turkey erythrocyte membranes possess negligible basal activity and all of the cyclase activity measured results from either hormone or NaF stimulation. Therefore the factor of GppNHp activation is higher in turkey erythrocytes than in other membranes. These properties of the turkey erythrocyte adenylate cyclase allowed us to perform the analysis described in this study as well as in our previous studies. In fact the same approach used in this study as well as in other studies (Tolkovsky & Levitzki, 1978a,b) can be applied to the more complex systems which exhibit desensitization and changes in the properties of the receptor induced by guanyl nucleotides. In fact the difference between the turkey erythrocyte system and the other receptor dependent cyclases which exhibit the properties outlined above is quantitative but not qualitative. For example, it has been demonstrated (Cassel and Selinger, unpublished experiments) that turkey erythrocyte membranes possess tightly bound GDP which is phosphorylated to GTP upon the addition of ATP in the cyclase assay. Thus, sufficient endogenous GTP is available to the turkey erythrocyte cyclase such that external GTP is without effect. Other membranes such as rat liver membranes and S49 lymphoma cell membranes are probably devoid of tightly bound GDP and thus exhibit GTP dependence in their cyclase activity. Also, it seems that the level of basal activity depends on the efficiency of GTP to displace GDP at the regulatory site in the absence of agonists. In the turkey erythrocyte system this process is totally dependent on agonist, whereas in other systems the process takes place at a significant rate in the absence of agonist. The absence of a guanyl nucleotide induced decrease in the affinity of the receptor toward agonist in the turkey system as compared with other systems is not yet clear. This property, however, is probably not related to the mechanism of enzyme activation proper.

In conclusion, in the turkey system the number of parameters to be considered in a kinetic analysis such as the present one is small. In other systems more parameters must be taken into consideration and thus the mathematical model must be expanded. In principle, however, the methods applied in this study can be used to explore other receptor-dependent adenylate cyclases.

Conclusion

In this study it was demonstrated that the rate of adenylate cyclase activation by the catecholamine-bound adrenergic receptor is diffusion controlled and therefore depends linearly on the fluidity of the membrane. This finding is in accordance with the assumption that the receptor and the cyclase are separate units and diffuse freely in the membrane. It is expected that, when the receptor and the enzyme are permanently coupled, the rate of cyclase activation by the ligand bound receptor will be independent of membrane fluidity. Indeed, it has been recently shown (Braun & Levitzki, 1979) that the activation of turkey erythrocyte adenylate cyclase by the adenosine receptor is independent of membrane fluidity. These latter results confirm earlier kinetic data (Tolkovsky & Levitzki, 1978b) and biochemical data (Braun & Levitzki, 1979) which show that the adenosine receptor is permanently coupled to the cyclase catalytic unit. It appears therefore that a detailed study of the dependence of adenylate cyclase activation by its receptor on membrane fluidity can become a routine procedure for the determination of the mode of coupling between a receptor unit and its catalytic function.

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References

Amdur, I., & Hammes, G. G. (1966) in *Chemical Kinetics: Principles and Selected Tropics*, McGraw-Hill, New York.
Atlas, D., & Levitzki, A. (1978) *FEBS Lett.* 85, 158–162.
Atlas, D., Hanski, E., & Levitzki, A. (1977) *Nature (London) 268*, 144–146.
Atlas, D., Yaffe, D., & Skutelsky, E. (1978) *FEBS Lett. 85*, 158–162.
Booth, R., Box, G. W., Muller, M. E., & Peterson, T. I. (1959) in *Forecasting by Generalized Regression Methods. Nonlinear Estimation*, IBM Share Program No. 687, WL-NL1, Feb 1959, International Business Machines Corp., Princeton, N.J.
Braun, S., & Levitzki, A. (1979) *Biochemistry* (in press).
Caron, M. G., & Lefkowitz, R. J. (1976) *J. Biol. Chem. 251*, 2374–2384.
Cassel, D., & Selinger, Z. (1976) *Biochim. Biophys. Acta 452*, 538–551.
Cogan, U., Shinitzky, M., Weber, G., & Nishida, T. (1973) *Biochemistry 12*, 521–528.
Edidin, M. (1974) *Annu. Rev. Biophys. Bioeng. 3*, 179–201.
Edidin, M., Zagyansky, Y., & Lardner, T. J. (1976) *Science 191*, 466–468.
Hanski, E., & Levitzki, A. (1978) *Life Sci. 22*, 53–60.
Hardt, S., & Cone, R. A. (1979) *Biophys. J.* (in press).
Kimelberg, H. K. (1975) *Biochim. Biophys. Acta 413*, 143–156.
Lad, P. M., Welton, A. F., & Rodbell, M. (1977) *J. Biol. Chem. 252*, 5942–5946.
Lefkowitz, R. J., Mulikin, D., & Caron, M. G. (1976) *J. Biol. Chem. 251*, 4686–4692.

Levitzki, A. (1977) *Biochem. Biophys. Res. Commun. 74*, 1154–1159.
Levitzki, A. (1978) *Biochem. Pharmacol. 27*, 2083–2088.
Levitzki, A., Atlas, D., & Steer, M. L. (1974) *Proc. Natl. Acad. U.S.A. 71*, 2773–2776.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Rendall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maguire, M. E., Wiklund, R. A., Anderson, H. J., & Gilman, A. G. (1976a) *J. Biol. Chem. 251*, 1221–1231.
Maguire, M. E., Van-Arsdale, P. M., & Gilman, A. G. (1976b) *Mol. Pharmacol. 12*, 335–339.
North, A. M. (1964) in *The Collision Theory of Chemical Reactions in Liquids*, Wiley, New York.
Orly, J., & Schramm, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3433–3437.
Osborne, A. D., & Porter, G. (1963) *Int. Symp. Free Radicals*, quoted in detail in North, A. M. (1964) *The Collision Theory of Chemical Reactions in Liquids*, p 108, New York.
Perrin, F. (1934) *J. Phys. Radium 5*, 497.
Pfeuffer, T. (1977) *J. Biol. Chem. 252*, 7224–7234.
Pfeuffer, T., & Helmreich, E. J. M. (1975) *J. Biol. Chem. 250*, 59–65.
Poo, M. M., & Cone, R. A. (1974) *Nature (London) 247*, 438–440.
Rodbell, M., Birnbaumer, L., Pohl, S. L., & Krans, H. M. J. (1971a) *J. Biol. Chem. 246*, 1877–1882.
Rodbell, M., Krans, H. M. J., Pohl, S. L., & Birnbaumer, L. (1971b) *J. Biol. Chem. 246*, 1872–1876.
Rodbell, M., Lin, M. C., & Salomon, Y. (1974a) *J. Biol. Chem. 249*, 59–65.
Rodbell, M., Lin, M. C., Salomon, Y., London, C., Harwood, J. P., Martin, B. R., Rendall, M., & Berman, M. (1974b) *Acta Endocrinol., Suppl. 191*, 11–37.
Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L., & Gilman, A. G. (1977) *J. Biol. Chem. 252*, 5761–5775.
Rudy, B., & Gitler, C. (1972) *Biochim. Biophys. Acta 288*, 231–236.
Saffman, P. G., & Delbrück, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113.
Salomon, Y., London C., & Rodbell, M. (1974) *Anal. Biochem. 58*, 541–548.
Schramm, M., Orly, J., Eimerl, S., & Korner, M. (1977) *Nature (London) 268*, 310–313.
Sevilla, N., & Levitzki, A. (1977) *FEBS Lett. 76*, 129–133.
Sevilla, N., Steer, M. L., & Levitzki, A. (1976) *Biochemistry 15*, 3493–3499.
Shinitzky, M. (1979) *Biochim. Biophys. Acta Rev. Biomembr.* (in press).
Shinitzky, M., & Barenholz, Y. (1974) *J. Biol. Chem. 249*, 2652–2657.
Shinitzky, M., & Inbar, M. (1974) *J. Mol. Biol. 85*, 603–615.
Shinitzky, M., Dianoux, A. C., Gitler, C., & Weber, G. (1971) *Biochemistry 10*, 2106–2113.
Spiegel, A. M., Brown, E. M., Fedak, S. A., Woodard, C. J., & Aurbach, G. D. (1976) *J. Cyclic Nucleotide Res. 2*, 47–56.
Steer, M. L., & Levitzki, A. (1975) *J. Biol. Chem. 250*, 2080–2084.
Teichberg, V. I., & Shinitzky, M. (1973) *J. Mol. Biol. 74*, 519–531.
Thilo, L., Träuble, H., & Overoth, P. (1977) *Biochemistry 16*, 1283–1289.
Tolkovsky, A. M., & Levitzki, A. (1978a) in *Hormones and Cell Regulation* (Dumont, J., & Nunez, J., Eds.) Vol. 2, pp 89–105, Elsevier/North Holland Publishing Co., Amsterdam.
Tolkovsky, A. M., & Levitzki, A. (1978b) *Biochemistry 17*, 3795–3810.
Tolkovsky, A. M., & Levitzki, A. (1978c) *Biochemistry 17*, 3811–3817.
Tolkovsky, A. M., & Levitzki, A. (1979) in *Mathematical Models in Molecular and Cellular Biology* (Segel, L. A., Ed.) Cambridge University Press, Cambridge, MA (in press).
Warren, G. B., Bennett, J. P., Hesketh, T. R., Houslay, M. D., Smith, G. A., & Metcalfe, J. C., (1975) *Fed. Eur. Biochem. Soc., Meet., 10th*, 1974, 3-15.
Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalf, J. C. (1974) *Biochemistry 13*, 5501–5507.