540.
Yarbrough, L. R., & Wu, C.-W. (1974) *J. Biol. Chem. 249,* 4079.
Zillig, W., Zechel, K., Rabussey, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., & Seifert, W. (1971) *Cold Spring Harbor Symp. Quant. Biol. 35,* 47.

A Kinetic Study of Cyclic Adenosine 3′:5′-Monophosphate Binding and Mode of Activation of Protein Kinase from *Drosophila melanogaster* Embryos†

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ABSTRACT: Cyclic AMP-dependent protein kinase and its regulatory subunit were isolated from *Drosophila melanogaster* embryos. The profiles of cyclic AMP binding by these proteins were significantly different. In order to explain such a difference and to find the mode of enzyme activation by cyclic AMP, a kinetic study of cyclic AMP binding was carried out. First, the association rate constant k_1 and dissociation rate constant k_{-1} in the cyclic AMP-regulatory subunit interaction at 0 °C were estimated to be 2.3×10^6 M^{-1} s^{-1} and 1.1×10^{-3} s^{-1}, respectively. Secondly, the three possible modes of enzyme activation by cyclic AMP were mathematically considered and could be described by a unique formula: $r = AP^t + BQ^t$ ($A + B = 1$) in which the parameters A, B, P, and Q are equivalent to rate constants in the sense that the rate constants are simply expressed by these parameters. Thirdly, the values of the parameters and subsequently the values of rate constants involved in the possible mechanisms were evaluated using a curve-fitting technique and compared with experimental observation. It was then found that the following mechanism was the only one which fitted the experimental observations. Namely,

$$RC + L \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} LRC \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} RL + C$$

where R, C, and L represent the regulatory and catalytic subunits and cyclic AMP as a ligand. Thus, our results indicate that in the presence of cyclic AMP the active enzyme (C) is released from a ternary intermediate which is the primary product of the cyclic AMP-holoenzyme interaction. The estimated values of the rate constants are: $k_3 = 3.5 \times 10^6$ M^{-1} s^{-1}; $k_{-3} = 7.3 \times 10^{-1}$ s^{-1}; and $k_4 = 3.8 \times 10^{-2}$ s. These estimates indicate that the reaction LRC → RL + C is relatively slow and limits the rate of the overall reaction. By comparing k_{-3} and k_4, it is apparent that a large part of newly formed ternary intermediate reverts to the holoenzyme.

Activity of cAMP-dependent protein kinase (EC 2.7.1.37; ATP: protein phosphotransferase) seems to mediate the effect of cAMP on many eucaryotic processes (for review, see Krebs, 1972; Langan, 1973). From our present knowledge, cAMP-dependent protein kinases, as found in a variety of eucaryotic sources, are all composed of two distinct moieties, i.e., the regulatory (R) and catalytic (C) subunits. This enzyme is therefore a heterodimeric (RC) or heterotetrameric (R_2C_2) holoenzyme (Krebs, 1972; Langan, 1973; Beavo et al., 1975; Rosen and Erlichman, 1975; Hofmann et al., 1975). It is known that cAMP is exclusively bound to the R moiety of a holoenzyme with concomitant release of C, the catalytically active form of the enzyme (Krebs, 1972; Langan, 1973). The mechanism of activation is schematically described as follows:

$$cAMP + RC \rightleftarrows cAMP\text{-}R + C$$

Although the above general paradigm is generally accepted, the precise mode of activation has yet to be demonstrated. Theoretically, a variety of models can be postulated (Ogez and Segel, 1976; Boeynaemes and Dumont, 1977). Furthermore, there exist a number of factors, in addition to cAMP, which are capable of interacting with the enzyme or its subunits and thereby affecting the enzyme activity (Krebs, 1972; Langan, 1973; Haddox et al., 1972; Ashby and Walsh, 1973; Donnelly et al., 1973; Corbin et al., 1973; Rangel-Aldao and Rosen, 1976; Demaille et al., 1977; Szmigielski et al., 1977). It is possible that there exist additional undetermined factors controlling intracellular regulation of protein kinase activity. It is therefore of special importance to learn the mode of activation of the enzyme by cAMP in order to fully understand how the regulation and biological role of protein kinase can involve various interacting factors. In the attempt to study the mode of activation of protein kinase, there are numerous difficulties when the analysis is based upon observations of the relationship between added cAMP and elicited enzyme activity, since the enzyme activation might be considerably affected by reactants such as Mg-ATP (Brostrom et al., 1971; Haddox et al., 1972) and substrate protein (Langan, 1973; Reiman et al., 1971; Miyamoto et al., 1971; Tao, 1972) or where the enzyme itself could be self-phosphorylated and therefore bias activity measurements (Rosen and Erlichman, 1975; Erlichman et al., 1974; Maeno et al., 1974; Hofmann et al., 1975). Observations obtained under such diverse circumstances seem unlikely to permit the recognition of one valid model among the large number of possibilities. Alternatively, several authors have attempted to construct diagnostic models of protein kinase activation based upon binding equilibrium (Ogez and Segel, 1976; Boeynaemes and Dumont, 1977;

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Swillens et al., 1974). But when a reaction involves more than one binding equilibrium constant as in the case of cAMP-protein kinase interaction, constructed theoretical curves inevitably tend to become less distinctive from one another reducing their diagnostic values or rendering experimental conditions difficult to achieve.

These circumstances require a methodology based neither upon the direct measurement of enzyme activity nor upon tenuous differences in equilibrium states. Employment of rate constants to describe an activation model is possible, but the algebraic solution of its expression as a set of simultaneous differential equations is not feasible even for the most simple case. But in some cases these equations may be reduced to simpler forms when experimental conditions are adequately predetermined. Since it is not generally possible to obtain an estimation of the kinetic constants for cAMP-holoenzyme interaction where the only exploitable data are the time courses of formation of protein-bound cAMP, we have developed a method to estimate the kinetic constants by evaluating certain parameters by curve fitting. The validity of these values is then examined here in comparison with empirically derived determinations.

The protein kinase and its subunits have been isolated from the early embryos of *Drosophila melanogaster* in which we have been studying the cAMP system (Tsuzuki and Kiger, 1975). The embryos contain three distinct proteins capable of binding cAMP. One of these is a cAMP-binding protein which is unrelated to protein kinase. [Existence of such a cAMP-binding protein has been confirmed in other organisms (Yuh and Tao, 1974; Ueland and Døsekeland, 1977).] The other two are protein kinase and its free regulatory subunit. The protein kinase is cAMP-dependent and recovered as a dimeric holoenzyme (RC) in the embryonic supernatant. The catalytic subunit (C) and cAMP-binding subunit (R) of this enzyme can be separated by gradient sedimentation in the presence of cAMP. The molecular weights of these components, measured by sedimentation with reference proteins, are 49 000 for C and 21 000 for R. While the molecular weight of C falls in a range observed for those from other sources (38 000–60 000), that of R is extremely small as compared with its counterparts from other sources (40 000–100 000) (Tao et al., 1970; Gill and Garren, 1971; Labrier et al., 1971; Corbin et al., 1972; Beavo et al., 1974a; Rosen and Erlichman, 1975). Thus, C is larger than R in the *Drosophila* protein kinase, while the order is usually reversed in other organisms. One exception is the yeast protein kinase that possesses relatively small R (mol wt 28 000) which is slightly smaller than C (mol wt 30 000) (Takai et al., 1974). Taking advantage of such marked difference in molecular size, R (completely free of RC and C) and RC (completely free of R) can be separated by single conventional gradient sedimentation (Tsuzuki and Kiger, 1975). Using R and RC thus purified from *D. melanogaster* embryos, we have carried out a kinetic study of cAMP binding as the simplest system of protein kinase activation consisting solely of R, C, and RC and cAMP. Three models can be postulated for this system. The binding kinetics should be consistent with one of the models unless concurrence of these is the case. The results of our experiments and comparison with theory will be presented below.

Materials and Methods

Collection of Embryos. Adult *D. melanogaster* (Oregon R-C) were maintained in population cages at 25 °C and 60% relative humidity on a 12-h day–12-h night light cycle and fed freshly fermented bakers yeast. Embryos were harvested from the food trays which had been exposed to the flies for 4 h. Thus, the embryos used in the present experiment ranged between 0 and 4 h postoviduction. The embryos were extensively washed on a fine screen with deionized water before they were stored at −80 °C.

Assay of cAMP Binding. Cyclic [^{3}H]AMP (New England Nuclear, sp act. 22.1 or 33.2 Ci/mmol) binding was carried out in buffer A followed by filtration through a Millipore HAW025 under a mild vacuum as described by Gilman (1970). The filter was successively washed with 5, 4, and 3 mL of cold buffer A, dried, and dissolved in 10 mL of Bray's solution (Bray, 1960) for determination of radioactivity. The values were corrected for the blanks. Other conditions are specified in the legends for figures and tables.

Assay of Protein Kinase. [γ-^{32}P]ATP was prepared according to a method slightly modified from Glynn and Chappel (1964) (sp act. 3.7 Ci/mmol at the time of preparation). Protein kinase activity was assayed as follows: 0.05 mL of sample in buffer A was mixed with 0.02 mL of protamine sulfate (Sigma, 10 mg/mL), 0.02 mL of 0.2 mM [γ-^{32}P]ATP, 0.1 mL of 100 mM Tris-HCl[1] (pH 7.5), 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol, and 0.01 mL of water or 2×10^{-5} M cAMP. Incubation was carried out at 30 °C for 15 min and was stopped by the addition of 5 mL of cold 20% trichloroacetic acid. The resulting precipitate was collected on a glass-fiber filter (Whatman GF/A), washed with 30 mL of the same acid, dried, and counted for radioactivity in 5 mL of toluene-based scintillator.

Sucrose Density Gradient Electrophoresis. A method of density gradient electrophoresis was devised during the course of our study which permitted the loading of a relatively large sample amount with good separation and complete recovery under mild conditions. The details of the method have been described elsewhere (Tsuzuki and Kiger, 1974).

Preparation of Protein Kinase Holoenzyme and Its Regulatory Subunit. Pooled frozen embryos were weighed and suspended at about 10% (w/v) in ice-cold homogenizing solution (0.25 M sucrose, 3 mM $MgCl_2$, 1.5 mM 2-mercaptoethanol). The suspension was gently agitated for dispersion of embryos before it was subjected to homogenization with a Virtis high-speed blender at 4 °C. Under the experimental conditions, no disruption of the nuclei was detected by microscopic examination. The homogenate was centrifuged at 30 000*g* for 20 min at 4 °C, and the resultant supernatant was filtered through Miracloth (Calbiochem) to remove debris. The clear solution thus obtained was then centrifuged at 105 000*g* for 120 min at 4 °C. The resultant supernatant was filtered as above to remove unsedimentable lipoidic debris before it was dialyzed to buffer A (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1.5 mM 2-mercaptoethanol). The dialysate was transferred to a Whatman DE-52 column (2 × 15 cm; equilibrated with buffer A) and washed with 50 mL of buffer A. Gradient elution was performed with 0–0.5 M NaCl in buffer A (total volume 200 mL) and fractions were assayed for protein kinase activity and cAMP binding. Protein kinase appeared as a single peak superimposed on cAMP-binding activity, which itself was split into two peaks, D1 and D2. The pooled fractions of D1 and D2 were concentrated in dialysis tubing covered with polyvinylpyrrolidone (Sigma) and dialyzed to 50 mM glycine–50 mM imidazole containing 1.5 mM 2-mercaptoethanol. About 3 mL of sample solution was applied over 10–50% sucrose gradient (80 mL) in the same buffer. Electrophoresis was carried out at 1000 V for 3 h at 0 °C. Details of the method have been

[1] Abbreviation used: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

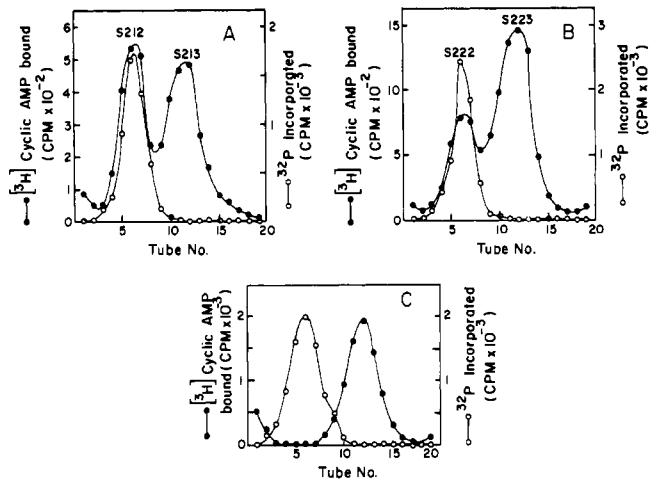

FIGURE 1: Sucrose gradient sedimentation of components obtained after successive DEAE chromatography and sucrose gradient electrophoresis. D2 component obtained after DEAE (DE52) chromatography was further fractionated by sucrose gradient electrophoresis. The resultant two peak components (E21 and E22) were separately pooled, dialyzed against buffer A, and concentrated as described previously (Tsuzuki and Kiger, 1975). A 0.2-mL sample of E21 or E22 was layered on a 5–20% sucrose gradient in buffer A and centrifuged in Spinco SW 65 rotor at 55 000 rpm for 20 h at 4 °C. Separated components were collected dropwise and aliquots were assayed for cyclic [^{3}H]AMP binding (●-●) and [γ-^{32}P]ATP incorporation (○-○) as described under Materials and Methods: (A and B) sedimentation of E21 and E22, respectively; (C) E21 was pretreated with 5×10^{-7} M cyclic [^{3}H]AMP in buffer A at 0 °C before the sample was sedimented in sucrose gradient containing 1×10^{-7} M cyclic [^{3}H]AMP. cAMP binding was assayed without further incubation.

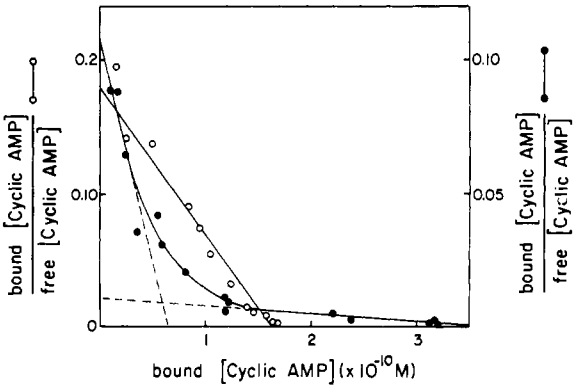

FIGURE 2: Scatchard plots of cAMP binding by holoenzyme and by regulatory subunit. 50 μL of protein kinase holoenzyme or regulatory subunit in buffer A was mixed with 50 μL of buffer A containing various amounts of cyclic [^{3}H]AMP in a small glass vial. The reaction mixture was equilibrated at 0 °C for 60 min in a melting-ice bath. To the mixture was then added 5 mL of cold buffer A and immediately filtered on a Millipore HAW 025 placed on a precooled filter apparatus. The filter was washed and cyclic [^{3}H]AMP binding was counted as described under Materials and Methods: (●-●) cAMP binding by holoenzyme; (○-○) cAMP binding by regulatory subunit.

described elsewhere (Tsuzuki and Kiger, 1974). After completion of electrophoresis, fractions were collected dropwise and assayed for protein kinase activity and cAMP binding. Two major peaks of cAMP binding were observed for both D1 and D2. In both cases one of the two peaks was accompanied by protein kinase activity (E11 and E21) while the other was completely free of the enzyme activity (E12 and E22). The details up to this stage have been described elsewhere (Tsuzuki and Kiger, 1975). Peak components were separately pooled, dialyzed to buffer A, and concentrated as above. Samples (ca. 0.2 mL) were sedimented through 5–20% sucrose in buffer A at 55 000 rpm for 20 h in Spinco SW 65 rotor. E21 yielded a large cAMP-binding peak with protein kinase activity (S212) and a smaller peak of cAMP binding alone (S213). From sedimentation of E22, a small cAMP-binding peak with protein kinase activity (S222) and a large peak of cAMP-binding activity alone (S223) were obtained (Figure 1A,B). Sedimentation of E11 produced a similar pattern to Figure 1A but with much less protein kinase holoenzyme. The component E12 contained a large amount of cAMP-binding protein which was unrelated to protein kinase and its subunit (Tsuzuki and Kiger, 1975). When the sample S212 was pretreated with cyclic [^{3}H]AMP, sedimentation through sucrose gradient containing labeled nucleotide (1×10^{-7} M) resulted in a complete separation of enzyme activity from cAMP-binding activity which now shifted to the position where S213 and S223 were found (Figure 1C), indicating that S213 and S223 were indeed the free regulatory subunits of the protein kinase holoenzyme. In the following studies, S212 and S223, both originally from D2 in DE-52 chromatography, were used as the holoenzyme of protein kinase and its regulatory subunit, respectively.

Results

Comparison of cAMP Binding by Holoenzyme and Regulatory Subunit. When cAMP binding was measured at various concentrations of the nucleotide at 0 °C, a considerable difference was evident between the resulting apparent K_d (half-maximum binding) of the holoenzyme (2.7×10^{-8} M) and K_d of the regulatory subunit (9.3×10^{-10} M) as compared to the similarity between the apparent K_d (2.7×10^{-8} M) of cAMP binding by the holoenzyme and K_m (5.0×10^{-8} M) for cAMP stimulation of enzyme activity. Figure 2 shows Scatchard plots of the data. While cAMP binding by the regulatory subunit yielded a straight line, cAMP binding by the holoenzyme resulted in an apparently biphasic curve, indicating that the latter binding involved more than one reaction whereas the former binding was the result from one unique binding. Similarly, a Hill plot of the binding by the regulatory subunit yielded a straight line with a slope of 0.9–1.1, but the binding by the holoenzyme brought about a curved line in which only the middle portion approximated a straight line with a slope of 0.8–0.6 (data not shown), again indicating complexity of the reaction.

Activation of Protein Kinase. Before the analysis of mode of activation, the effect of cAMP on the enzyme activity of the holoenzyme was examined. Our holoenzyme preparation exhibited approximately a fivefold stimulation in the presence of cAMP ($>1 \times 10^{-7}$ M) when protamine (1 mg/mL) was present. A double-reciprocal plot of the cAMP-dependent portion of the enzyme activity yielded a straight line with apparent K_m for cAMP equal to 5×10^{-8} M, which is similar to K_m values of protein kinases from other sources (Langan, 1968; Garren et al., 1971; Walsh et al., 1971; Corbin and Krebs, 1969; Takai et al., 1974). A cAMP-independent portion should be attributed to the presence of the free catalytic subunit. In view of our present study, the method of holoenzyme preparation eliminated the free regulatory subunit but it was important to eliminate the possibility that the observed cAMP-independent activity was due to spontaneous dissociation of the holoenzyme after its purification with implication of the release of free regulatory subunit as the counterpart of the catalytic subunit. In order to rule out the latter possibility, first the holoenzyme preparation was resedimented in a sucrose gradient similar to Figure 1, resulting in no detectable cAMP-binding activity in the position where free regulatory subunit would appear. Secondly, the effect of protamine which was used as substrate in the enzyme activity assay was closely

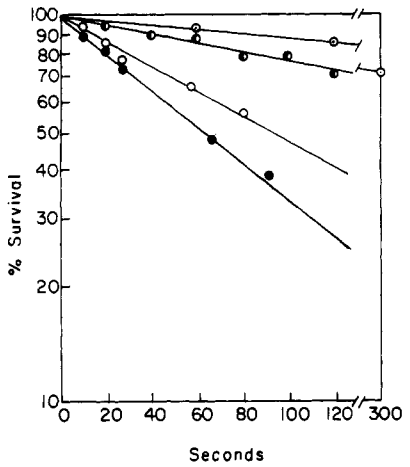

FIGURE 3: Kinetics of dissociation of cAMP-regulatory subunit complex at various temperatures. Regulatory subunit was equilibrated with 1×10^{-7} M cyclic [^{3}H]AMP in 100 μL of buffer A at a temperature indicated. A 10-μL aliquot from the equilibrate was mixed with 100 μL of the buffer containing 1×10^{-5} M unlabeled cAMP and incubated at the same temperature for the desired time before the reaction mixture was diluted with 5 mL cold buffer and immediately poured onto membrane filter for determination of remaining bound cyclic [^{3}H]AMP as described under Materials and Methods: (⊙) 0; (●) 10; (○) 20; (●) 30 °C.

examined, since the activation of cAMP-dependent protein kinase by protamine and other basic proteins has been reported by many authors (Reiman et al., 1971; Miyamoto et al., 1971; Tao, 1972). When enzyme activity was measured with or without histone (1 mg/mL) in the presence of increasing amounts of protamine up to the concentration used for the enzyme activity assay (1 mg/mL), a progressive increase in phosphorylation was observed (the blank value was about 3% compared to the fully activated activity). Further, cAMP binding by the holoenzyme was examined at various nucleotide concentrations in the presence of protamine at the concentration identical to that used for enzyme activity assay (1 mg/mL). The result was that the apparent K_d (concentration of cAMP to give half-maximum binding) shifted from 4.5×10^{-8} M in the absence of protamine to 1.0×10^{-8} M in its presence (double-reciprocal plot was still nonlinear). Such a shift has been reported in rabbit skeletal muscle protein kinase in the presence of histone (Beavo et al., 1974b). Conversely, the K_d of cAMP binding by the regulatory subunit shifted from 1.5×10^{-9} M in the absence of protamine to 1.2×10^{-8} M in its presence. These results indicated that the cAMP-independent portion observed in the enzyme activity was not due to spontaneous dissociation of the purified holoenzyme but due to partial activation of the enzyme by added protamine. Consequently, it was concluded that the holoenzyme used in the present study was essentially free of the free regulatory subunit and thus could be employed in the following binding study.

Kinetics of cAMP Binding by the Regulatory Subunit. A binding involving a ligand (L) and a receptor (R) with a unique binding site, namely

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} RL \qquad (1)$$

can be described by the following equation

$$\frac{d[RL]}{dt} = k_1[R][L] - k_{-1}[RL] \qquad (2)$$

When experimental conditions permit measurement of the reverse reaction alone avoiding the recurrence of association reaction, the above equation becomes

$$\frac{d[RL]}{dt} = -k_{-1}[RL]$$

When $[RL] = [RL]_0$ at $t = 0$, the integrated form is

$$\ln \frac{[RL]}{[RL]_0} = -k_{-1}t \qquad (3)$$

and k_{-1} can be estimated as the slope. In order to obtain such experimental conditions, the regulatory subunit was first equilibrated with cyclic [^{3}H]AMP, and then nonlabeled nucleotide was added in excess at the beginning of the experiment so that formation of radioactive RL complex was abolished. Figure 3 shows the results obtained at various temperatures. The value of k_{-1} for 0 °C reaction has been estimated to be $1.12 \pm 0.41 \times 10^{-3}$ s^{-1}. As the temperature was raised, the value of k_{-1} increased. From an Arrhenius plot of the data, E_a (activation energy) for k_{-1} has been estimated to be 13.5 kcal mol^{-1}.

The association rate constant may be determined in several ways. We employed three methods and the results were compared. The first method was the direct determination of k_1 using an experimental condition where disturbance by concurrent reverse reaction was negligible during the experimental time. In this case, the reaction could be approximated by the equation

$$\frac{d[RL]}{dt} = k_1[R][L]$$

By integrating

$$A(t) = \frac{1}{[L]_0 - [R]_0} \ln \frac{[L]_0 - [RL]}{[R]_1 - [RL]} = k_1 t + \frac{1}{[L]_0 - [R]_0} \ln \frac{[L]_0}{[R]_0} \qquad (4)$$

where $[L]_0$ and $[R]_0$ are the initial concentrations of L and R. In order to evaluate k_1, the time course of the overall reaction of cAMP binding by the regulatory subunit was examined at 0 °C with various initial ligand concentrations. The reaction proceeded extremely fast and the plateau was reached within 10 s at 1×10^{-7} M. A linear increase was observed at ligand concentration less than 4×10^{-9} M for the first 10 s (Figure 4). From the result of the dissociation experiment, practically no effect of the reverse reaction could be expected during such short experimental time at 0 °C. Indeed, plotting of the data of Figure 4 according to eq 4 yielded straight lines, from which k_1 values could be estimated as the slope. The value of k_1 thus obtained was $2.5 \pm 0.6 \times 10^6$ M^{-1} s^{-1}. The second method was based on our knowledge of the value of k_{-1} from the previous experiment. When [RL] is small and practically $[L] \doteq [L]_0$ during experimental time, the integrated form of eq 2 becomes

$$[RL] = \frac{k_1[L]_0[R]_0}{k_1[L]_0 + k_{-1}} [1 - \exp\{-(k_1[L]_0 + k_{-1})t\}] \qquad (5)$$

When k_{-1} is known, the value of k_1, as a parameter, should be obtained by fitting experimental time course to eq 5. We have evaluated k_1 values as least-square values best fit to a time

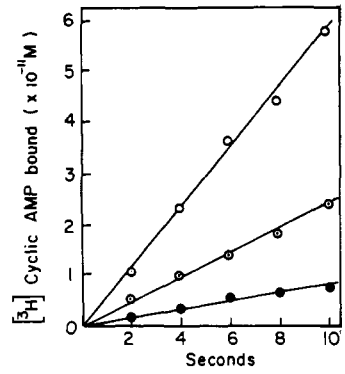

FIGURE 4: Kinetics of cAMP binding by regulatory subunit at 0 °C. Reaction was started by mixing 20 μL of regulatory subunit in buffer A with 20 μL of cyclic [^{3}H]AMP in buffer A. At the end of incubation, 5 mL of ice-cold buffer A containing 1×10^{-5} M unlabeled cAMP was flushed into the reaction mixture so that further cyclic [^{3}H]AMP binding could be halted. The solution was immediately filtered on a membrane filter placed on a precooled filter apparatus. Cyclic [^{3}H]AMP binding was determined as described under Materials and Methods: (○-○) 4×10^{-9}; (⊙-⊙) 2×10^{-9}; (●-●) 1×10^{-9} M.

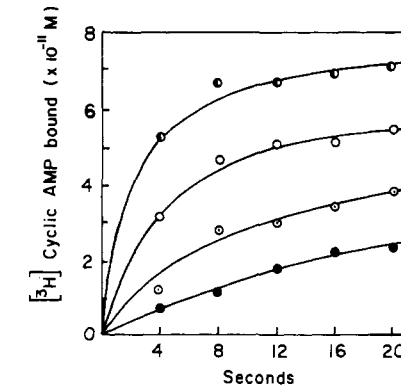

FIGURE 5: Kinetics of cAMP binding by holoenzyme at 0 °C. Reaction was started by mixing 20 μL of the holoenzyme in buffer A and 80 μL of buffer A containing various amounts of cyclic [^{3}H]AMP. Incubation was continued at 0 °C for the time indicated. Bound cAMP was determined as in Figure 4. Cyclic [^{3}H]AMP concentrations: (◐-◐) 4×10^{-8}; (○-○) 2×10^{-8}; (⊙-⊙) 1×10^{-8}; (●-●) 4×10^{-9} M.

course, such as shown in Figure 4 using an electronic calculator, and the value of k_1 thus obtained was $3.8 \pm 0.6 \times 10^6$ M^{-1} s^{-1}. The third method was the computation of k_1 from the binding equilibrium data such as used for Figure 2 according to the equation:

$$k_1 = k_{-1}\left(\frac{[RL]_e}{([R]_0 - [RL]_e)([L]_0 - [RL]_e)}\right) \qquad (6)$$

where $[RL]_e$ represents the concentration of RL at equilibrium. The resulting value of k_1 was $1.5 \pm 0.1 \times 10^6$ M^{-1} s^{-1}. By comparing the results with the different methods, it has been concluded that the value of k_1 for 0 °C reaction is in the order of 10^6 M^{-1} s^{-1} with the average of the three methods being 2.3×10^6 M^{-1} s^{-1}. When the reaction temperature was raised, the value of k_1, determined by method I, increased and the E_a has been estimated by an Arrhenius plot to be 7.9 kcal mol^{-1}.

Kinetics of cAMP Binding by the Holoenzyme. Figure 5 shows the time course of cAMP binding by the holoenzyme at various nucleotide concentrations. In contrast to the cAMP binding by the regulatory subunit, the binding by the holoenzyme proceeded remarkably slowly. Indeed, the determination of binding was only possible at ligand concentrations above 2×10^{-9} M under the experimental conditions employed in the present work. As seen in the figure, unlike the case of the regulatory subunit, a linear increase was not observed even at a ligand concentration as low as 4×10^{-9} M, indicating the occurrence of more complex reactions than a simple ligand–unique binding site interaction. Accordingly, application of the data to eq 4 no longer yielded straight lines.

Theoretical Consideration of Possible Modes of Activation of cAMP-Dependent Protein Kinase. In the following section, several possible mechanisms of activation of cAMP-dependent protein kinase are first theoretically considered with special reference to rate constants instead of equilibrium constants, and the experimental data will be used to test each mechanism for validity in the next section. The first possibility to be considered here is the one where an intermediate does not exist. Namely:

$$RC + L \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} RL + C \qquad (7)$$

where R, C, and L represent, as before, the regulatory and catalytic subunit and the ligand (cAMP). The equation for this mechanism is

$$\frac{d[RL]}{dt} = k_2[RC][L] - k_{-2}[RL][C]$$

When $[L]_0$ and $[RC]_0$ are the initial concentrations of each reactant,

$$[L]_0 = [L] + [RL]$$
$$[RC]_0 = [RC] + [RL]$$
$$[RC]_0 + [C]_0 = [RC] + [C]$$

When $[L] \gg [RL]$ as in the beginning of reaction,

$$[L] \doteqdot [L]_0$$

Then the above equation becomes

$$\frac{d[RL]}{dt} + (k_2[L]_0 + k_{-2}[C]_0)[RL] + k_{-2}[RL]^2 = k_2[RC]_0[L]_0$$

Since $[RL] = 0$ and $d[RL]/dt = k_{-2}[L]_0[RC]_0$ at $t = 0$, the solution is

$$[RL] = p_1\left(\frac{1 - \exp[-k_{-2}(p_1 - q_1)t]}{1 - \frac{p_1}{q_1}\exp[-k_{-2}(p_1 - q_1)t]}\right) \qquad (8)$$

where

$$p_1 = 0.5\left[\sqrt{D_1} - \frac{k_2[L]_0 + k_{-2}[C]_0}{k_{-2}}\right]$$
$$q_1 = -0.5\left[\sqrt{D_1} + \frac{k_2[L]_0 + k_{-2}[C]_0}{k_{-2}}\right]$$
$$D_1 = \left(\frac{k_2[L]_0 + k_{-2}[C]_0}{k_{-2}}\right)^2 + \frac{4k_2[L]_0[RC]_0}{k_{-2}}$$

As evident in eq 8, p_1 is the value of [RL] at equilibrium when

[L] ≫ [RL]. Here the fraction $1/r_1$ is defined as:

$$\frac{1}{r_1} = \frac{p_1 - [RL]}{p_1} = \frac{\left(1 - \frac{p_1}{q_1}\right) \exp[-k_{-2}(p_1 - q_1)t]}{1 - \frac{p_1}{q_1} \exp[-k_{-2}(p_1 - q_1)t]}$$

Consequently

$$r_1 = A_1 P_1{}^t + B_1 \tag{9}$$

where

$$A_1 = -\frac{q_1}{p_1 - q_1}$$

$$B_1 = \frac{p_1}{p_1 - q_1} = 1 - A_1$$

$$P_1 = \exp[k_{-2}(p_1 - q_1)]$$

According to eq 9, a plot of r_1 vs. reaction time t, if the reaction follows mechanism 5, should yield a concave upward curve which starts from the point (0,1) on the ordinate with the asymptote of $r_1 = 1$.

The second possible mechanism may be

$$RC + L \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} LRC \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} RL + C \tag{10}$$

This mechanism involves an intermediate LRC. Complete solution of the differential equation for this mechanism is infeasible. However, under the experimental condition where the reverse reaction RL + C → RCL can be neglected and the dissociation RL → R + L is negligible, mechanism 10 can be reduced to:

$$RC + L \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} LRC \xrightarrow{k_4} RL + C \tag{11}$$

Then mechanism 11 can be described by the equations:

$$\frac{d[LRC]}{dt} = k_3[RC][L] - (k_{-3} + k_4)[LRC] \tag{12}$$

$$\frac{d[RL]}{dt} = k_4[LRC] \tag{13}$$

When $[RC]_0$ and $[L]_0$ are the initial concentrations of RC and L,

$$[RC]_0 = [RC] + [LRC] + [RL]$$

$$[L]_0 = [L] + [LRC] + [RL]$$

If [L] ≫ [LRC] + [RL], as in the beginning of reaction,

$$[L] \doteq [L]_0$$

Subsequently, eq 12 is reduced to

$$\frac{d[LRC]}{dt} = k_3[L]_0([RC]_0 - [RL]) - (k_3[L]_0 + k_{-3} + k_4)[LRC]$$

and eq 13 can be rewritten as:

$$[LRC] = \left(\frac{1}{k_4}\right)\left(\frac{d[RL]}{dt}\right)$$

By differentiating

$$\frac{d[LRC]}{dt} = \left(\frac{1}{k_4}\right)\left(\frac{d^2[RL]}{dt^2}\right)$$

Combining the above three equations, the following differential equation is obtained

$$\frac{d^2[RL]}{dt^2} + (k_3[L]_0 + k_{-3} + k_4)\frac{d[RL]}{dt} + k_3k_4[L]_0[RL] = k_3k_4[L]_0[RC]_0$$

Since [RL] = 0 and $d[RL]/dt = 0$ at $t = 0$, the solution is

$$[RL] = [RC]_0\left[\frac{q_2}{\sqrt{D_2}}\exp(p_2t) - \frac{p_2}{\sqrt{D_2}}\exp(q_2t) + 1\right]$$

where

$$p_2 = -0.5(k_3[L]_0 + k_{-3} + k_4 + \sqrt{D_2})$$

$$q_2 = -0.5(k_3[L]_0 + k_{-3} + k_4 - \sqrt{D_2})$$

$$D_2 = (k_3[L]_0 + k_{-3} - k_4)^2 + 4k_{-3}k_4$$

also,

$$[LRC] = \left(\frac{1}{k_4}\right)\left(\frac{d[RL]}{dt}\right) = \left(\frac{[RC]_0}{k_4}\right)\left(\frac{p_2q_2}{\sqrt{D_2}}\right)[\exp(p_2t) - \exp(q_2t)]$$

When an experimental method of assay gives the sum of RL and LRC, as the membrane filter method used in the present work, [T] is defined as,

$$[T] = [RL] + [LRC] = [RC]_0[1 - (A_2P_2{}^t + B_2Q_2{}^t)]$$

where

$$A_2 = -\frac{q_2}{\sqrt{D_2}}\left(1 + \frac{p_2}{k_4}\right)$$

$$B_2 = \frac{p_1}{\sqrt{D_2}}\left(1 + \frac{q_2}{k_4}\right) = 1 - A_2$$

$$P_2 = \exp p_2$$

$$Q_2 = \exp q_2$$

Here the fraction r_2 is defined as

$$r_2 = \frac{[RC]_0 - [T]}{[RC]_0} = A_2P_2{}^t + B_2Q_2{}^t \tag{14}$$

With A_2, B_2, P_2, and Q_2, the rate constants k_3, k_{-3}, and k_4 are described as follows

$$k_3 = -\frac{\ln(P_2{}^{A_2}Q_2{}^{B_2})}{[L]_0} \tag{15}$$

$$k_4 = -\frac{\ln P_2 \times \ln Q_2}{\ln(P_2{}^{A_2}Q_2{}^{B_2})} = \frac{\ln P_2 \times \ln Q_2}{k_3[L]_0} \tag{16}$$

$$k_{-3} = \ln(P_2{}^{A_2}Q_2{}^{B_2}) - k_4 = k_3[L]_0 - k_4 \tag{17}$$

The third possible mechanism may be that in which the ligand L (cAMP) binds to free regulatory subunit R but does not interact with the holoenzyme RC, and the activation of the holoenzyme (production of free catalytic subunit C) is brought about by dissociation of RC thereby favored.

$$\begin{aligned} R + L &\underset{k_{-1}}{\overset{k_5}{\rightleftarrows}} RL \\ RC &\underset{k_{-6}}{\overset{k_6}{\rightleftarrows}} R + C \end{aligned} \tag{18}$$

Complete solution of the differential equation for this mechanism is not feasible. However, under experimental conditions in which the reverse reactions R + C → RC and RL → R +

L are negligible, mechanism 18 can be reduced to

$$R + L \xrightarrow{k_5} RL$$

$$RC \xrightarrow{k_6} R + C$$

Then the mechanism can be described by the following equations

$$\frac{d[RL]}{dt} = k_5[R][L]$$

$$\frac{d[R]}{dt} = k_6[RC]$$

When the initial concentrations of RC, R, and L are $[RC]_0$, $[R]_0$, and $[L]_0$

$$[RC]_0 + [R]_0 = [RC] + [R] + [RL]$$

$$[L]_0 = [L] + [RL]$$

When $[L] \gg [RL]$ as in the beginning of reaction,

$$[L] \doteqdot [L]_0$$

Combining these equations, the following differential equation is obtained,

$$\frac{d^2[RL]}{dt^2} + k_6\left(\frac{d[RL]}{dt}\right) + k_5k_6[L]_0[RL] = k_5k_6[L]_0([RC]_0 + [R]_0)$$

The solution is

$$[RL] = h_1 \exp(p_3t) + h_2 \exp(q_3t) + h_3$$

where

$$p_3 = -0.5(k_6 + \sqrt{D_3})$$

$$q_3 = -0.5(k_6 - \sqrt{D_3})$$

$$D_3 = k_6^2 - 4k_5k_6[L]_0$$

Since $[RL] = 0$ and $d[RL]/dt = k_5[L]_0[R]_0$ at $t = 0$

$$h_1 = \frac{q_3([RC]_0 + [R]_0) + k_5[L]_0[R]_0}{\sqrt{D_3}}$$

$$h_2 = -\frac{p_3([RC]_0 + [R]_0) + k_5[L]_0[R]_0}{\sqrt{D_3}}$$

$$h_3 = [RC]_0 + [R]_0$$

Here the fraction r_3 is defined as

$$r_3 = \frac{([RC]_0 + [R]_0) - [RL]}{[RC]_0 + [R]_0} = A_3P_3^t + B_3Q_3^t \quad (19)$$

where

$$A_3 = -\frac{h_1}{[RC]_0 + [R]_0}$$

$$B_3 = -\frac{h_2}{[RC]_0 + [R]_0}$$

$$P_3 = \exp(p_3)$$

$$Q_3 = \exp(q_3)$$

With A_3, B_3, P_3, and Q_3, the rate constants k_5 and k_6 are described below.

$$k_6 = -\ln(P_3Q_3) \quad (20)$$

$$k_5 = -\left(\frac{1}{[L]_0}\right)\left(\frac{\ln P_3 \times \ln Q_3}{\ln(P_3Q_3)}\right) = \left(\frac{1}{[L]_0}\right)\left(\frac{\ln P_3 \times \ln Q_3}{k_6}\right) \quad (21)$$

As shown above, if the actual mode of activation of protein kinase by cAMP is represented by either mechanism 7, 10, or 18, the experimental data should be expressed in the form of eq 9, 14, or 19 when the experiment to be examined was carried out under the conditions described above. The r_1, r_2, and r_3 are all expressed in the form of

$$r = AP^t + BQ^t \quad (22)$$

where $A + B = 1$ should hold in all cases. Furthermore, the rate constants peculiar to each mechanism can be described by the parameters A, B, P, and Q.

Examination of Experimental Data with Reference to Theoretical Possibilities. As shown in the foregoing section, the three possible mechanisms of protein kinase activation can be expressed in the form of $r = AP^t + BQ^t$, where $A + B = 1$ and r, which is experimentally measurable, is a function of time t via the parameters A, B, P, and Q, which are equivalent to kinetic constants in the sense that the kinetic constants can be computed from these parameters when their values are known.

If r values at different times are measured under the experimental conditions assumed in each derivation of the above formula, the values of the parameters A, B, P, and Q can be estimated by determining the values of these parameters which best fit to the experimental curve of r plotted vs. time.

Figure 6A shows an example from time courses of cAMP binding by the holoenzyme at 0 °C. In this example, the initial concentration of cAMP ($[L]_0$) was 4.0×10^{-8} M, and the receptor concentration ($[RC]_0 + [R]_0$) measured by cAMP binding at 5×10^{-6} M was 4.61×10^{-10} M, and 13% of the total receptor was bound by the nucleotide after 10 s. Therefore, $[L]/[L]_0$ at 10 s was 0.998. Such conditions obviously satisfied the assumption made in the previous section (that $[L] \doteqdot [L]_0$). The values of r_1, r_2, and r_3 obtained from the above time course are shown in Figure 6B. The definite inconsistency between the r_1 plots and the theoretical curve for mechanism 7 ($RC + L \rightleftarrows RL + C$) is immediately apparent, ruling out this mechanism. Indeed, it is not possible to estimate reasonable values of the parameters in eq 9 to fit the empirical values of r_1. Apparently, the curve for r_2 and r_3 is consistent with the equations and the parameters should be evaluated for the fitting of eq 14 and 19 to the empirical data. Because of the multidimension and multipower of the equation, an ordinary computer program for curve fitting such as the ravine search method is impractical. Accordingly, a computer program was designed for the least-squares estimation of parameters whose principle was computation of the lattice points yielded by $^1/_{1000}$ division of A, B, P, and Q, followed by search for the least-squares best fit. The values of the parameters, thus obtained, and the comparison of the experimental observations with the r values calculated from these parameter values are given in Table I. A good agreement between two values is evident at different reaction times. In Table II are listed the estimated values of the parameters from five independent experiments, and the values of the apparent rate constants are computed by using eq 15–17, 20, and 21.

If mechanism 18 is the case, on the one hand, the value of k_5 ($9.98 \pm 2.72 \times 10^4$ M^{-1} s^{-1}) in Table II should be expected to be identical with the value of k_1 in eq 1 which was estimated in the previous section to be in the order of 10^6 M s^{-1}. Such a

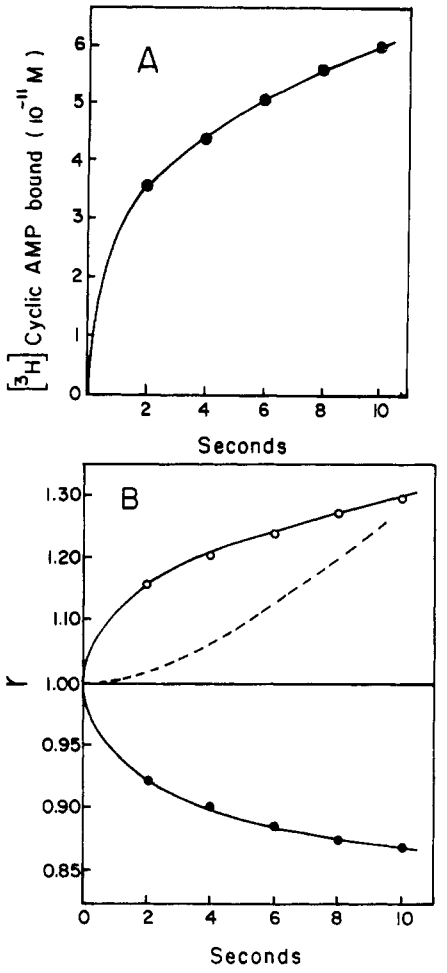

FIGURE 6: (A) Kinetics of the initial phase of cAMP binding by holoenzyme. Reaction was started by mixing 20 μL of holoenzyme in buffer A with 80 μL of buffer A containing cyclic [^{3}H]AMP (final concentration $[L]_0 = 4.0 \times 10^{-8}$ M) at 0 °C. cAMP binding was determined as in Figure 4. (B) Plots of cAMP binding by holoenzyme in the forms of r_1, r_2, and r_3. The data given in A were plotted in the forms of r_1, r_2, and r_3 according to eq 9, 14, and 19 described in the text. The initial receptor concentration ($[RC]_0 + [R]_0 = 4.61 \times 10^{-10}$ M) and the ligand-equilibrated receptor concentration ($[RL]_e + [LRC]_e = 2.62 \times 10^{-10}$ M) were determined by incubation at 0 °C for 60 min with 5×10^{-6} and 4×10^{-8} M cyclic [^{3}H]AMP, respectively: (○-○) plot of observed r_1; (●-●) plot of observed r_2 and r_3; (···) theoretical curve for r_1 according to eq 9.

TABLE I: Values of Parameters Obtained by a Least-Squares Curve-Fitting Method and Comparison of Calculated r with Observed r.[a]

parameters	values by curve fitting
A	0.077
B	0.923
P	0.393
Q	0.994

time (s)	r obsd	calculation AP^t	calculation BQ^t	calculation r
0	1	0.077	0.923	1.000
2	0.923	0.012	0.912	0.924
4	0.906	0.002	0.901	0.903
6	0.890	0.000	0.890	0.890
8	0.875	0.000	0.880	0.880
10	0.871	0.000	0.869	0.869

[a] The parameters A, B, P, and Q in eq 22 were estimated for the best-fit values to the data given in Figure 6B by a least-squares curve-fitting method using an electronic computer. Values of r_2 or r_3 ($= AP^t + BQ^t$) computed with the parameters thus estimated are compared with r experimentally observed. The values of AP^t and BP^t are also given in order to show the degree of their contribution to the sum at different reaction times.

TABLE II: Estimation of Rate Constants Appearing in Mechanisms 10 and 18.[a]

parameter	mean value
A	0.083 ± 0.014[b]
B	0.917 ± 0.012
P	0.306 ± 0.134
Q	0.997 ± 0.001

rate constants	calculated value
k_3	3.54 ± 1.17[b] $\times 10^6$ M^{-1} s^{-1}
k_{-3}	$7.26 \pm 5.13 \times 10^{-1}$ s^{-1}
k_4	$3.77 \pm 1.76 \times 10^{-2}$ s^{-1}
k_5	$9.98 \pm 2.72 \times 10^4$ M^{-1} s^{-1}
k_6	1.18 ± 0.42 s^{-1}

[a] Parameters A, B, P, and Q are mean values of estimations obtained in five independent experiments. Kinetic rate constants k_3, k_{-3}, k_4, k_6, and k_5 were computed by replacing the parameters by these values in eq 15-17, 20, and 21. [b] Standard deviations.

significant discrepancy between k_5 and k_1 should be interpreted as an indication that mechanism 18 is not applicable to the activation of protein kinase by cAMP. Further, an extremely large value of the estimated k_6 (1.18 ± 0.42 s^{-1}) would imply rapid spontaneous degradation of the protein kinase holoenzyme (RC) to its subunits, which is also inconsistent with experimental observation. Thus, mechanisms 18 should be eliminated.

On the other hand, there seems to be nothing unreasonable about the estimated values of k_3, k_{-3}, and k_4 as far as the present work concerns. The values of k_{-3} and k_4 differ by the ratio of about 19 favoring the reverse reaction LRC → RC + L, which implies that less than 5% of the degradation of the intermediate LRC contributes to production of the free catalytic subunit as the active form of the enzyme, while more than 95% of the degradation product is the restored holoenzyme. Since the value of k_3 is relatively close to k_1, evidently the reaction governed by k_4 is the rate-limiting step of the enzyme activation. Also, the relatively large value of k_{-3} explains the marked slow down in cAMP binding by the holoenzyme at the beginning of the reaction (Figures 5 and 6A), while the equilibrium is still far to the left, as the result of extensive reverse reaction due to k_{-3}.

Discussion

In order to avoid the incidental involvement of substrates and other reactants in enzyme activation which are required to detect enzyme activity, we have attempted to determine the mode of activation of protein kinase by cAMP from interaction of the cyclic nucleotide with the protein kinase holoenzyme and its regulatory subunit. When the holoenzyme is given as a heterodimer (RC), three models can be postulated as given above (mechanisms 7, 10, and 18). Even in such simple systems, curves of equilibrium binding vs. ambient nucleotide concentration are not sufficient to test the validity of each model. We have attempted, therefore, to exploit rate constants in lieu of equilibrium constants. It is evident, however, that experimentally measurable rate constants are limited to the R-L (cAMP) interaction. To overcome the difficulty, we have carried out mathematical transformations of differential equations for each model and have succeeded in expressing the

three different mechanisms by the same form of equation $r = AP^t + BQ^t$ ($A + B = 1$), where the parameters A, B, P, and Q are equivalent to rate constants in the sense that the rate constants can be expressed by these parameters. Instead of carrying out measurement of rate constants, the value of these parameters has been estimated as the least-squares fit value for the experimental kinetics by a computer-aided curve-fitting method. In combination with the measured values of kinetic constants for R–L interaction, we have concluded that only one of the three models can explain our experimental observation:

$$\mathrm{RC} + \mathrm{L} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \mathrm{LRC} \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} \mathrm{RL} + \mathrm{C}$$

$$\mathrm{R} + \mathrm{L} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{RL}$$

The estimated values of the rate constants are: $k_3 = 3.5 \times 10^6$ $\mathrm{M^{-1}\,s^{-1}}$; $k_{-3} = 7.3 \times 10^{-1}\ \mathrm{s^{-1}}$; $k_4 = 3.8 \times 10^{-2}\ \mathrm{s^{-1}}$; $k_1 = 2.3 \times 10^6\ \mathrm{M^{-1}\,s^{-1}}$; and $k_{-1} = 1.1 \times 10^{-3}\ \mathrm{s^{-1}}$.

Korenman and his colleagues (Sanborn et al., 1973) were aware of the importance of knowing the rate constants involved in the activation of cAMP-dependent protein kinase in order to recognize the activation mechanism among others. These authors studied the interaction between cAMP and the holoenzyme and R subunit from bovine endometrium protein kinase and estimated K_a, k_1, and k_{-1} for R to be $1 \times 10^8\ \mathrm{M^{-1}}$, $10^5\ \mathrm{M^{-1}\,s^{-1}}$ and $10^{-5}\ \mathrm{s^{-1}}$, respectively. The values of k_1 and k_{-1} significantly differ from our results, but the value of K_a is not far from ours after all. Their plots of binding data with the holoenzyme according to an equation similar to eq 4 were, in agreement with our result, no longer linear, but further effort to recognize the activation mechanism was not made.

The most important aspect of our results is the existence of the intermediate LRC which is the primary product of the cAMP–holoenzyme interaction and from which the active enzyme is released. Comparing k_3 with k_1, it can be said that the rate of cAMP binding by the holenzyme is almost as fast as that by the regulatory subunit, but, unlike LR complex, the intermediate LRC is unstable as judged by the values of k_{-3} and k_4. It is of special interest that the value of k_{-3} is much larger than that of k_4. With the estimated values of the constants, it is expected that a very large part (ca. 95% under the experimental condition employed) of the once formed intermediate is subject to the reverse reaction to reproduce the intact holoenzyme. Indeed, as noticed in Figures 5 and 6A, the kinetic curve of cAMP binding by the holoenzyme exhibits a very retarded development of cAMP binding after a relatively fast initiation, which is in contrast with the kinetic curve by the regulatory subunit (Figure 4). Employing the values of k_3 and k_{-3} given above, the time necessary to reach the equilibrium, $\mathrm{RC} + \mathrm{L} \rightleftarrows \mathrm{LRC}$, is estimated to be within 4 s with about 9% of the initial amount of RC in the ternary complex under the experimental conditions used (4×10^{-8} M cAMP, 0 °C). Further, if the reverse reaction, $\mathrm{LR} + \mathrm{C} \rightarrow \mathrm{LRC}$, can be ignored, the calculated time needed for 90% activation of the enzyme is as long as 6 min under the experimental conditions. It is expected, however, that the activation of enzyme proceeds much faster in actual cells in which the temperature is much higher.

The significant difference between the cAMP binding curve of the holoenzyme and that of the regulatory subunit (Figure 2) can clearly be explained by the above mechanism of enzyme activation with the values of kinetic constants given above. An interesting feature of the difference is that significant cAMP binding is not elicited in the case of holoenzyme until the nucleotide concentration exceeds about 10^{-9} M, while the half-maximal binding is attained at the same ligand concentration in the case of the regulatory subunit. This implies that, in spite of the similarity between the values of k_1 and k_3, the enzyme activation will not take place to an appreciable extent due to the occurrence of the reverse reaction, $\mathrm{LRC} \rightarrow \mathrm{L} + \mathrm{RC}$, with the relatively large value of k_{-3}. If this interpretation applies to the situation in actual cells, the cells would be protected from unnecessary consumption of the enzyme due to small fluctuations of the cellular cAMP level until the nucleotide concentration dramatically increased.

A Hill plot of cAMP binding by the holenzyme resulted in a curved line in which only the middle portion approximated a straight line with a slope much less than unity (0.6–0.8), whereas the slope of the upper portion increased sharply as the binding approached saturation. Such a profile would seemingly imply involvement of a mixed cooperativity (Cornish-Bowden and Koshland, 1975), but, since the holoenzyme used in the present study is a heterodimer (RC), the cooperativity concept is not applicable. Regarding this, the concept of retrocooperativity has been proposed (Swillen and Dumont, 1976) where the released catalytic subunit induces dissociation of cAMP–regulatory subunit complex and thus counteracts itself as its concentration increases, giving an apparent negative cooperative feature in the Hill plot. Our results shown above, however, demonstrated that such a complexed binding profile can be explained by allowing a ternary intermediate. The use of diagnostic Hill plots has been proposed to recognize the mode of activation of cAMP-dependent heterodimeric (RC) and heterotetrameric (R_2C_2) protein kinases (Ogez and Segel, 1976). As for the heterodimeric enzyme, the authors examined four possibilities (mechanisms 7, 10, and 18 and the concurrence of mechanisms 10 and 18 according to our designation). A Hill plot of our data seems to be compatible with their diagnostic Hill plots for mechanism 10 but apparently incompatible with those for mechanism 18 and for the concurrence of mechanisms 10 and 18, but mechanism 7 remains ambiguous. As a whole, the curves presented in the diagnostic Hill plots appear to be so close to one another that recognition of one valid model among others seems to be difficult. One reason may be that the concurrent reversible reaction, $\mathrm{RL} \rightleftarrows \mathrm{R} + \mathrm{L}$, was not taken into consideration by the authors. This reaction seems to have a significant effect on the overall equilibrium states.

In comparison with other organisms, *D. melanogaster* protein kinase seems to be peculiar in having a relatively small regulatory subunit, while the size of catalytic subunit resembles those from other sources (Tao et al., 1970; Gill and Grarren, 1971; Labrier et al., 1971; Corbin et al., 1972a,b; Beavo et al., 1974a; Erlichman et al., 1973). The yeast protein kinase (Takai et al., 1974) appears to resemble the insect enzyme in the molecular weights of the holoenzyme and the subunits. In addition to this, the insect protein kinase appears to occur as a heterodimer (RC) while it is a heterotetramer (R_2C_2) in some other tissues (Beavo et al., 1975; Rosen and Erlichman, 1975; Hofmann et al., 1975). Nevertheless, the apparent K_d (2.7×10^{-8} M) and K_m (5.0×10^{-8} M) of the insect enzyme for cAMP resemble those reported for enzymes from other sources such as mammalian (Walsh et al., 1971; Garren et al., 1971; Erlichman et al., 1973), insect (Tsuzuki and Newburgh, 1974), and yeast (Takai et al., 1974). Since the embryos were accumulated in a −80 °C freezer before their use, the possibility cannot be ruled out that an original tetramer might have been degraded to dimers. If this is the case, we have found no evidence for the presence of $(\mathrm{cAMP\text{–}R})_2$ complex. Although our insect protein kinase is different from those of other

sources, our results may apply to the fundamental mode of activation of the cAMP-dependent protein kinase of various organisms.

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References

Ashby, C. D., and Walsh, D. A. (1973), *J. Biol. Chem. 248,* 1255–1261.

Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1974a), *Methods Enzymol. 38,* 299–308.

Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1974b), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3580–3583.

Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975), *Adv. Cyclic Nucleotide Res. 5,* 241–251.

Boeynaemes, J. M., and Dumont, J. E. (1977), *Mol. Cell. Endocrinol. 7,* 275–295.

Bray, G. A. (1960), *Anal. Biochem. 1,* 279–285.

Brostrom, C. O., Corbin, J. D., King, C. A., and Krebs, E. G. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2444–2447.

Corbin, J. D., Bromstrom, C. O., Alexander, R. L., and Krebs, E. G. (1972), *J. Biol. Chem. 247,* 3736–3743.

Corbin, J. D., and Krebs, E. G. (1969), *Biochem. Biophys. Res. Commun. 36,* 328–336.

Corbin, J. D., Soderling, T. R., and Park, C. R. (1973), *J. Biol. Chem. 248,* 1813–1821.

Cornish-Bowden, A., and Koshland, D. E., Jr. (1975), *J. Mol. Biol. 95,* 201–212.

Demaille, J. G., Peters, K. A., and Fischer, E. H. (1977), *Biochemistry 16,* 3080–3086.

Donnelly, T. E., Jr., Kuo, J. F., Miyamoto, E., and Greengard, P. (1973), *J. Biol. Chem. 248,* 199–203.

Erlichman, J., Rubin, C. S., and Rosen, O. M. (1973), *J. Biol. Chem. 248,* 7607–7609.

Erlichman, J., Rosenfeld, R., and Rosen, O. M. (1974), *J. Biol. Chem.,* 5000–5003.

Garren, L. D., Gill, G. N., and Walton, G. M. (1971), *Ann. N.Y. Acad. Sci. 185,* 210–226.

Gill, G. N., and Garren, L. D. (1970), *Biochem. Biophys. Res. Commun. 39,* 335–343.

Gill, G. N., and Garren, L. D. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 786–790.

Gilman, A. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 305–312.

Glynn, I. M., and Chapell, J. B. (1964), *Biochem. J. 90,* 147–149.

Haddox, M. K., Newton, N. E., Hartle, D. K., and Goldberg, N. D. (1972), *Biochem. Biophys. Res. Commun. 47,* 653–661.

Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975), *J. Biol. Chem. 250,* 7795–7801.

Krebs, E. G. (1972), *Curr. Top. Cell. Regul. 5,* 99–133.

Labrie, F., Lemaire, S., and Courte, C. (1971), *J. Biol. Chem. 246,* 7293–7302.

Langan, T. A. (1968), *Science 162,* 579–580.

Langan, T. A. (1973), *Adv. Cyclic Nucleotide Res. 3,* 99–153.

Maeno, H., Reyes, P. L., Veda, T., Rudolph, S. A., and Greengard, P. (1974), *Arch. Biochem. Biophys. 164,* 551–559.

Miyamoto, E., Petzold, G. L., Harris, J. S., and Greengard, P. (1971), *Biochem. Biophys. Res. Commun. 44,* 305–312.

Ogez, J. R., and Segel, I. H. (1976), *J. Biol. Chem. 251,* 4551–4556.

Rangel-Aldao, R., and Rosen, O. M. (1976), *J. Biol. Chem. 251,* 3375–3380.

Reiman, E. M., Walsh, D. A., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1986–1995.

Rosen, O. M., and Erlichman, J. (1975), *J. Biol. Chem. 250,* 7788–7794.

Sanborn, B. M., Bhalla, R. C., and Korenman, S. G. (1973), *J. Biol. Chem. 248,* 3593–3600.

Swillens, S., and Dumont, J. E. (1976), *J. Mol. Med. 1,* 273–288.

Swillens, S., van Cauter, E., and Dumont, J. E. (1974), *Biochim. Biophys. Acta 364,* 250–259.

Szmigielski, A., Guidotti, A., and Costa, E. (1977), *J. Biol. Chem. 252,* 3848–3853.

Takai, Y., Yamamura, H., and Nishizuka, Y. (1974), *J. Biol. Chem. 249,* 530–535.

Tao, M. (1972), *Biochem. Biophys. Res. Commun. 46,* 56–61.

Tao, M., Salas, M. L., and Lipmann, F. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 408–414.

Tsuzuki, J., and Kiger, J. A., Jr. (1974), *Prep. Biochem. 4,* 283–294.

Tsuzuki, J., and Kiger, J. A., Jr. (1975), *Biochim. Biophys. Acta 393,* 225–235.

Tsuzuki, J., and Newburgh, R. W. (1974), *Biochim. Biophys. Acta 354,* 101–106.

Ueland, P. M., and Døsekeland, S. O. (1977), *J. Biol. Chem. 252,* 677–686.

Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1968–1976.

Yuh, K. C. M., and Tao, M. (1974), *Biochemistry 13,* 5220–5226.