Kinetics of Gonadotropin Binding by Receptors of the Rat Testis. Analysis by a Nonlinear Curve-Fitting Method†

Jean-Marie Ketelslegers,‡ Gary D. Knott,§ and Kevin J. Catt*

ABSTRACT: The kinetics of the reaction between human chorionic gonadotropin (hCG) and specific gonadotropin receptors in the rat testis were determined at 24 and 37°, over a wide range of hormone concentrations. Hormone concentrations were corrected for the binding activity of the [^{125}I]hCG tracer preparations. Analysis of the experimental data was performed with an interactive nonlinear curve fitting program, based upon the second-order chemical kinetic differential equation. The mean values for the association rate constant (k_1) were 4.7×10^7 M^{-1} min^{-1} at 24°, and 11.0×10^7 M^{-1} min^{-1} at 37°. At both temperatures, the values of k_1 were independent of hormone concentration. Initial dissociation rates were consistent with first-order kinetics, with dissociation rate constant (k_2) of 1.7×10^{-3} and 4.6×10^{-3} min^{-1} at 24 and 37°, respectively. When studied over longer periods at 24°, the dissociation process appeared to be multiexponential. The kinetics of degradation of [^{125}I]hCG and receptors were determined at both temperatures, and a mathematical model was developed by modification of the second-order chemical kinetic differential equation to take these factors into account. The application of such a model to hCG kinetic binding data demonstrated that reactant degradation had little significant effect on the derivation of the association rate constant (k_1), but caused significant overestimation of the dissociation rate constant (k_2) values derived from association experiments. The model was also applied by computer simulation to a theoretical analysis of the effects of degradation of free hormone and receptor sites upon kinetic and steady-state binding data. By this method, the initial velocities of hormone binding were shown to be less affected by degradation than the steady-state levels of hormone-receptor complex. Also, reactant degradation in simulated steady-state experiments caused an underestimate of the apparent equilibrium association constant, but had relatively less effect on the determination of binding site concentration.

Gonadotropin binding sites with high affinity and specificity for luteinizing hormone[1] and human chorionic gonadotropin have been demonstrated in the Leydig cells of the rat testis (Catt et al., 1971, 1972b, DeKretser et al., 1969, 1971). It has also been shown that unbound [^{125}I]hCG undergoes significant degradation during incubation with the decapsulated rat testis at 37°, while the hormone bound to receptors sites retains full biological activity (Dufau et al., 1972a). Hormone degradation has also been described in other receptor binding systems (Freychet et al., 1972; Glossmann et al., 1974; Lee and Ryan, 1973; Marx et al., 1973; Pohl et al., 1972). The effect of such hormone degradation has usually been neglected during calculation of the rate constants which define hormone-receptor interaction in various target tissues, though it is clearly a potentially important factor in the determination of such parameters.

In order to derive the rate constants for the reaction between hCG and its specific binding sites in the rat testis, we have analyzed the kinetics of this process under various experimental conditions. The kinetics of degradation of both hCG and receptor sites have also been studied. The various kinetic parameters were computed from the experimental data by applying an interactive nonlinear curve fitting program. This method had the advantages of permitting the use of more complex models, and of performing simultaneous fits to multiple sets of data. In particular, a model was developed which enabled the computation of association rate constants by taking hormone and receptor degradation or inactivation into account during application of the differential equation for the kinetics of the second-order reaction. Such a model was also applied to an extensive evaluation by computer simulation of the influence of hormone and receptor degradation on the binding function.

Materials and Methods

(A) Experimental Procedures

Gonadotropin Preparations. Highly purified hCG, with biological activity of 10,000–11,000 IU (2nd International Standard hCG)/mg, as measured by the ventral prostate weight assay (McArthur, 1952), by steroidogenesis in the isolated rat testis (Dufau et al., 1971), and by radioligand–receptor assay (Catt et al., 1972a), was provided by Dr. R. E. Canfield. Partially purified hCG (Pregnyl-Organon) was employed to provide an excess of unlabeled hCG in the incubation media, when necessary for the determination of nonspecific binding, or for the study of the dissociation rate of the hormone from its binding sites.

Preparation and Characterization of [^{125}I]hCG. Biologically active [^{125}I]hCG was prepared by a modification of the chloramine-T method (Catt et al., 1971; Dufau et al., 1972a) with purification on an agarose-concanavalin A column (Dufau et al., 1972b). The specific activity of each la-

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[1] Abbreviations used are: hLH, human luteinizing hormone; hCG, human chorionic gonadotropin; PBS, 0.01 *M* phosphate buffer (pH 7.4) with 150 m*M* NaCl and 2.5 m*M* $CaCl_2$; RLA, radioligand-receptor assay; RIA, radioimmunoassay.

beled hormone preparation determined by self-displacement during radioimmunoassay in antibody-coated tubes (Catt, 1969), against standards of the hormone employed for labeling, ranged from 10 to 25 Ci/g. The specific activities of four different batches of [^{125}I]hCG were also determined by self-displacement in the radioligand–receptor assay (RLA) (see below) and compared to the values obtained in the radioimmunoassay (RIA). In both the RIA and RLA, the self-displacement curves of [^{125}I]hCG were parallel to the binding–inhibition curves obtained with unlabeled hCG. The mean ratio between the results obtained by the two methods (RLA/RIA) was 3.0 ± 0.5 (SE, n = 4); this factor was used to correct the specific activities of the earlier preparations evaluated only by RIA.

The maximum binding activity of the labeled hormone was measured by incubation of 10,000–20,000 cpm of [^{125}I]hCG with an excess of receptor sites (20,000-g fraction of disrupted interstitial cells, or 1500-g fraction of testes homogenates (Catt et al., 1971, 1972a,b)). The incubations were performed at 24° for 18 hr in 0.01 M phosphate buffer (pH 7.4) containing 150 mM NaCl and 2.5 mM $CaCl_2$ (PBS), and 0.1% of bovine serum albumin. The final incubation volume was 600 μl. The mean value obtained was 50 ± 7% (SD, n = 7).

Rat Testis Homogenate Preparations. All kinetic experiments were performed with the 1500-g fraction of adult rat testes homogenate (Catt et al., 1972b). The 1500-g sediments (0.5 g/testis) were resuspended in a volume of cold PBS equivalent to 10–20 ml/testis (0.8–2.5 mg of protein/ml by the method of Lowry et al. (1951)) and neomycin sulfate was added to the suspension to achieve a final concentration of 0.01% in all incubation mixtures.

Determination of Specifically Bound hCG. Particle-bound and free hormones were separated by filtration of the incubation mixtures after dilution of the 1-ml samples with 3 ml of ice-cold PBS containing 0.1% bovine γ-globulin. Filtrations were performed through 2.4-cm Whatman GF/C glass fiber filters, or through Millipore nitrocellulose HAWP (0.45 μ) filters previously soaked in PBS containing 5% bovine serum albumin. After filtration of each sample, the filter was washed twice with 3 ml of ice-cold PBS containing 0.1% bovine serum albumin. In equilibrium experiments, bound and free hormone were also separated by centrifugation (Catt et al., 1972b). The radioactivity of the filters or pellets was determined in a γ spectrometer with ^{125}I counting efficiency of about 45%. Each sample was counted for sufficient time to give a counting error of less than 3%.

For each experimental point, the nonspecific binding was determined in the presence of an excess of unlabeled hCG (100 IU/ml), and did not exceed 1–2% of the total radioactivity. The specifically bound radioactivity was calculated by subtracting the nonspecifically bound from the total bound radioactivity.

Measurement of Hormone Degradation during Incubation. [^{125}I]hCG (500,000 cpm/ml, approximately 200 pM) (25 ml) diluted in PBS containing 0.1% bovine γ-globulin was incubated for 6 hr, with 25 ml of testis homogenate (1 testis/20 ml) under continuous shaking, at 4, 24, and 37°. In control experiments, [^{125}I]hCG was incubated under the same conditions in buffer alone. At zero time (i.e., immediately after the [^{125}I]hCG was mixed with the homogenate), and at succeeding time intervals, 6-ml samples were withdrawn from the incubation media and centrifuged at 1500g for 15 min at 4°. The supernatant solutions were stored in ice, and 0.25 ml of each of the fractions (40,000–50,000 cpm) were subsequently evaluated for their capacity to bind to freshly prepared rat testis homogenate during a second incubation. Due to the uptake of [^{125}I]hCG by the testis homogenate during the first incubation, the total radioactivity of these aliquots decreased as a function of the duration of the first incubation. The second incubation was performed at 24°, for 22 hr with 0.5 ml of fresh homogenate (1 testis/20 ml) and 0.25 ml of PBS containing 0.1% bovine γ-globulin; bound and free hormone were separated by centrifugation. Under the experimental conditions selected (i.e., low hormone concentrations relative to the concentration of binding sites), the concentrations of hormone–receptor complex at steady state were linearly related to the total hormone concentrations. Therefore, the decrease in the ratio of specific binding to total radioactivity $(B/T)_t$, expressed as a function of the preincubation time (t), was considered to be directly proportional to the hormone degradation. For the computation of the B/T ratios, the "nonbindable" fraction present in the original tracer was subtracted from the total radioactivity of each aliquot. Labeled and unlabeled hCG were assumed to undergo equal inactivation. In addition, it was assumed that reactant degradation was constant during the second incubations, and affected equally the fresh hormone and the hormone preincubated with the homogenate. In order to validate the latter assumption, another series of experiments were performed, in which the hormone degradation occurring at 37° was evaluated by short term second incubations (30 min at 37°). Under these conditions, the effects of reactant degradation were considered to be negligible. The data were analyzed on the basis that under the experimental conditions selected, the initial binding velocities were linearly related to hormone concentrations.

Measurement of Receptor Degradation during Incubation. Testis homogenate (1 testis/20 ml) (0.5 ml) and 0.4 ml of PBS with 0.1% of bovine γ-globulin were incubated for different periods at 4, 24, and 37°. At the end of the preincubation, 50,000 cpm of [^{125}I]hCG in 0.1 ml of PBS containing 0.1% bovine γ-globulin was added to each tube to achieve a hormone concentration of approximately 20 pM, and a second incubation was performed at 24° for 22 hr; bound and free hormones were separated by centrifugation. Under the experimental conditions selected, the concentrations of hormone–receptor complex after incubation of a constant amount of [^{125}I]hCG with serial dilutions of testis homogenate were linearly related to the binding site concentrations. Therefore, the decrease in the ratio of specific binding to total radioactivity $(B/T)_t$, expressed as a function of the preincubation time (t), was considered to be directly proportional to receptor degradation. It was assumed that reactant degradation was constant during the second incubation, and affected equally the fresh and preincubated samples. The validity of this assumption was tested by another series of experiments, in which the second incubations were carried out at 37° for 10 min, with 250,000 cpm of [^{125}I]hCG (approximately 100 pM). Under these conditions, the effects of reactant degradation were considered to be negligible. The data were analyzed on the basis that initial binding velocities under these experimental conditions were linearly related to receptor concentrations.

Kinetic Association Experiments. Hormone solutions were prepared in PBS containing 0.1% bovine γ-globulin; various ratios of [^{125}I]hCG and unlabeled hCG were employed to obtain a wide range of hormone concentrations. After equilibration at 24 or 37°, equal volumes of the reac-

tants (hormone solution and testis homogenate) were mixed and incubated with continuous shaking at 150 cpm as individual aliquots of 1 ml, or as bulk suspensions of 40 ml. At successive intervals, 1-ml samples were filtered as described above to determine the amount of hCG specifically bound to the receptor at each time point.

Kinetic Dissociation Experiments. The dissociation rate of hCG from its binding sites was studied after the addition of an excess of cold hormone to testis homogenate preincubated with [^{125}I]hCG. The preincubations were performed as bulk suspensions (20–40 ml) containing equal amounts of homogenate (1 testis/20 ml), and of [^{125}I]hCG in PBS with 0.1% of bovine γ-globulin (200,000–300,000 cpm/ml). Corresponding suspensions containing 100 IU of hCG/ml were similarly preincubated and subsequently treated in order to determine the nonspecifically bound radioactivity at each time point of the dissociation experiment. After 3–6 hr, the incubation mixtures were centrifuged at 1500*g* for 20 min, at 4°, washed once with ice-cold PBS, and resuspended in 20–40 ml of PBS containing 0.1% of bovine γ-globulin; 100–200-μl aliquots of these mixtures were dispensed in 10 × 75 glass tubes. After equilibration at 24 or 37°, 100 IU of hCG in a volume of 10–20 μl was added to each tube and the specifically bound hormone, at successive time intervals, was determined by filtration.

Equilibrium Studies. Aliquots of testis homogenate (0.5 ml) were incubated with 50,000–100,000 cpm of [^{125}I]hCG and increasing amounts of unlabeled hCG in a total volume of 1 ml for 18 hr at 24°. Binding–inhibition data were converted to saturation curves or Scatchard plots, with corrections for maximum binding activity of the labeled hormone as described below. Identical saturation curves and Scatchard plots were obtained by incubating increasing concentrations of [^{125}I]hCG with constant amounts of homogenate, under the same conditions. These and previous studies of [^{125}I]hCG indicated that the labeled hormone retains full biological activity (Dufau et al., 1972a) and behaves identically with unlabeled hCG during binding studies.

Calculation of the Total, Bound, and Free Hormone Concentrations. The specific activity of [^{125}I]hCG preparations, as determined in the radioligand–receptor assay, represented a measure of the amount of radioactivity corresponding to a given quantity of biologically active hormone; this value was used to calculate the total concentrations of intact [^{125}I]hCG. Maximum bindability experiments showed that an average of 50% of the tracer radioactivity corresponded to [^{125}I]hCG capable of reacting with the receptors. The value for the specific activity used to calculate the concentrations of bound hormone was corrected accordingly, since the radioactivity specifically bound to testicular tissue was believed to represent only intact hCG. The free hormone concentrations were obtained by subtracting the bound from the total hormone concentrations. The specific activities of the labeled hormone preparations were expressed in terms of a particular hCG preparation; therefore, calculation of the molar concentrations of hCG was corrected for the biological activity of this preparation to the expected potency of 15,000 IU/mg for fully active hCG. All calculations of hCG molar concentrations were based on a molecular weight of 38,000, as previously described (Dufau et al., 1973).

(B) Mathematical Models

The hormone–receptor interaction was treated as a reversible bimolecular reaction

$$P + Q \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B$$

where P = concentration of free hCG; Q = concentration of free receptor; B = concentration of hCG–receptor complex; k_1 = association rate constant (M^{-1} min^{-1}); k_2 = dissociation rate constant (min^{-1}).

The corresponding differential equation describing second-order chemical kinetics was used to analyze the experimental data, namely

$$dB(t)/dt = k_1P(t)Q(t) - k_2B(t) \qquad B(0) = 0 \quad (1)$$

where $B(t)$ = concentration of hormone–receptor complex at time t; $B(0)$ = concentration of hormone–receptor complex at time 0; $P(t)$ = concentration of free hCG at time t; $Q(t)$ = concentration of free receptor at time t; t = time (minutes).

When no hormone or receptor degradation occurs, $P(t)$ and $Q(t)$ of (1) can respectively be expressed as: $P(t) = P_0 - B(t)$ and $Q(t) = Q_0 - B(t)$, where P_0 = initial concentration of free hCG, or total hCG concentration and Q_0 = initial concentration of free receptor, or total receptor concentration.

The dissociation experiments were analyzed with two different models: a single negative exponential model, corresponding to first-order kinetics, and a double negative exponential model.

The first model is expressed as

$$B_t = B_0e^{-k_2t} \quad (2)$$

where B_t = concentration of hormone–receptor complex at time t and B_0 = concentration of hormone–receptor complex measured at the moment when the association process is interrupted.

The double exponential model is represented by the function

$$B_t = B_1e^{-k_2't} + B_2e^{-k_2''t} \quad (3)$$

where k_2' and k_2'' represent the dissociation rates of the two different components of B, and B_1 and B_2 their respective initial concentrations.

To compensate for the influence of hormone and receptor degradation on the values of k_1 and k_2 derived from kinetic data, we used an elaborated model which was based on the following assumptions. (1) Only the free hormone and free receptor were subjected to degradation. This was based on previous studies which demonstrated that the hormone bound to receptor sites was protected from degradation (Dufau et al., 1972a). (2) The hormone–receptor complex was stable. (3) Hormone and receptor degradation occurred following first-order or pseudo-first-order kinetics. This was validated by experimental evidence (see Results and Figures 1 and 2).

The hormone degradation was expressed by

$$P \xrightarrow{k_3} P'$$

where P = concentration of free hormone; P′ = concentration of "degraded hormone"; k_3 = rate constant of the hormone degradation process (min^{-1}).

The rate of change of P′ is expressed by

$$dP'(t)/dt = k_3P(t) \qquad P'(0) = 0 \quad (4)$$

were, $P'(t)$ = concentration of degraded hormone, at time t; $P'(0)$ = concentration of degraded hormone at time 0; $P(t)$ = concentration of intact free hormone at time t.

The values of k_3 were determined from hormone degra-

dation experiments, using as the model

$$dp(t)/dt = -k_3 p(t) \qquad p(0) = 100 \qquad (5)$$

where $p(t)$ = concentration of hormone, time t, as determined during hormone degradation experiments; $p(0)$ = concentration of hormone, at time 0, in the hormone degradation experiments; since hormone degradation was expressed in percent of the initial hormone concentration, $p(0) = 100$.

The loss of binding sites occurring during incubation was formulated as

$$Q \xrightarrow{k_4} Q'$$

where Q = free receptor concentration; Q′ = degraded receptor concentration; k_4 = rate constant of the receptor degradation process (min^{-1}).

The rate of change of Q′ is expressed by

$$dQ'(t)/dt = k_4 Q(t) \qquad Q'(0) = 0 \qquad (6)$$

where $Q'(t)$ = concentration of degraded receptor at time t; $Q'(0)$ = concentration of degraded receptor at time 0; $Q(t)$ = concentration of intact free receptor at time t.

The values of k_4 were determined from receptor degradation data, using as the model

$$dq(t)/dt = -k_4 q(t) \qquad q(0) = 100 \qquad (7)$$

where $Q'(t)$ = concentration of degraded receptor at time mined during receptor degradation experiments; $q(0)$ = concentration of receptor, at time 0, in the receptor degradation experiments; since receptor degradation was expressed in percent of the initial receptor concentration, $q(0) = 100$.

With the assumptions defined above, the rate of change of the free hormone and free receptor during an association kinetic experiment are expressed as the resultant of two phenomena: (1) the degradation of the free hormone and free receptor; (2) the formation and dissociation of the hormone–receptor complex.

This was written as

$$dP(t)/dt = -dP'(t)/dt - dB(t)/dt \qquad P(0) = P_0 \qquad (8)$$

$$dQ(t)/dt = -dQ'(t)/dt - dB(t)/dt \qquad Q(0) = Q_0 \qquad (9)$$

Equations 8 and 9 were used to define the terms $P(t)$ and $Q(t)$ in (1), and the set of eq 1, 4, 6, 8, and 9 provided an adequate model for analysis of association data, taking in account the serial degradation of the free hormone and free receptor occurring during incubation. The k_1 and k_2 parameters were adjusted to provide the best fit of this model with association experiments, the k_3 and k_4 parameters being held constant at values determined independently from the analysis of hormone and receptor degradation data. Curve fitting was also performed simultaneously to association and hormone and receptor degradation experiments: this was done to obtain the best values of the unknown parameters of the different models applying simultaneously to the corresponding experimental data. In order to evaluate more extensively the influence of free hormone and free receptor degradation on the binding function, theoretical kinetic and saturation curves were computed, assigning different values to k_3 and k_4, the k_1 and k_2 parameters being held constant.

The equilibrium data were analyzed by the Scatchard model (Scatchard, 1949) or by an equation relating the bound hormone to the total hormone concentrations. Defining equilibrium as the state at which the rate of change of the hormone–receptor complex approaches zero, and assuming no degradation, eq 1 can be expressed as

$$k_1(P_0 - B(t_e))(Q_0 - B(t_e)) - k_2 B(t_e) = 0 \qquad (10)$$

where t_e is the time required to reach equilibrium conditions and $B(t_e)$ the concentration of hormone–receptor complex at equilibrium. Solving for $B(t_e)$, the function (11), relating bound ($B(t_e)$) to total (P_0) hormone concentrations at equilibrium, was obtained, where K_a is the association equilibrium constant (k_1/k_2), and Q_0 the total receptor concentration.

$$B(t_e) = 0.5[(P_0 + Q_0 + 1/K_a) - \sqrt{(P_0 + Q_0 + 1/K_a)^2 - 4P_0Q_0}] \qquad (11)$$

The interactive computer program (MLAB) with differential equation solving abilities (Knott and Reece, 1972; Knott and Shrager, 1972) was used to perform all curve fitting and calculations. This program, running on a PDP-10 time-sharing computer, also provided the original graphic output of the illustrations which accompany this report. All curve fitting was performed with the assumption that the variance was homogeneous during time-course experiments.

Results

I. Degradation of [^{125}I]*hCG.* Progressive degradation of [^{125}I]hCG was observed during incubation with rat testis homogenate, the process being more rapid at 37° than at 24° (Figure 1). No detectable hormone degradation was observed at 4°, and no significant loss of binding activity could be detected when the tracer was incubated for 6 hr at 4, 24, or 37° in buffer alone. When studied over a period of 6 hr, hormone degradation was consistent with first- or pseudo-first-order kinetics, the fit of the data with a single negative exponential model being satisfactory. At 24°, the k_3 value was of 0.8×10^{-3} min^{-1} ($t_{1/2}$ = 14.4 hr). At 37°, the k_3 value was of 1.4×10^{-3} min^{-1} ($t_{1/2}$ = 8.2 hr); a similar result was obtained when hormone inactivation occurring at that temperature was measured by short-term reincubation experiments ($k_3 = 1.2 \times 10^{-3}$ min^{-1}; $t_{1/2}$ = 9.3 hr).

II. Degradation of Gonadotropin Binding Sites. The specific gonadotropin binding sites of the testis homogenate were degraded more rapidly at 37° than at 24° (Figure 2). No significant receptor degradation could be demonstrated in testis homogenates kept at 4° for 24 hr. The time course of the degradation of free binding sites was consistent with first- or pseudo-first-order kinetics, a good fit of the data with a single negative exponential model being obtained. At 24°, the k_4 value was of 0.3×10^{-3} min^{-1} ($t_{1/2}$ = 36.4 hr); at 37°, the k_4 value was of 4.6×10^{-3} min^{-1} ($t_{1/2}$ = 2.5 hr). Similar results were derived from short-term second incubation studies: at 24°, $k_4 = 0.3 \times 10^{-3}$ min^{-1} ($t_{1/2}$ = 36.4 hr); at 37°, $k_4 = 3.7 \times 10^{-3}$ min^{-1} ($t_{1/2}$ = 3.1 hr).

III. Association Experiments. The initial binding velocities of hCG to specific receptors in the testis homogenate were functions of the hormone concentrations at 24 and 37° (Figure 3). Values for k_1 and k_2 were determined by curve fitting of association experiments performed at both temperatures, for incubation periods ranging from 30 to 45 min. Equation 1 was used as the model, without corrections for reactant degradation. In all instances, a good fit of the experimental data with the computed curves was obtained. At 24°, for a range of hCG concentrations varying from 1 to 456 p*M*, and for a receptor concentration range of 14 to 63 p*M*, the mean values for k_1 and k_2 were respectively $4.7 \pm 0.3 \times 10^7$ M^{-1} min^{-1} (SE, n = 8) and $6.2 \pm 1.9 \times 10^{-3}$

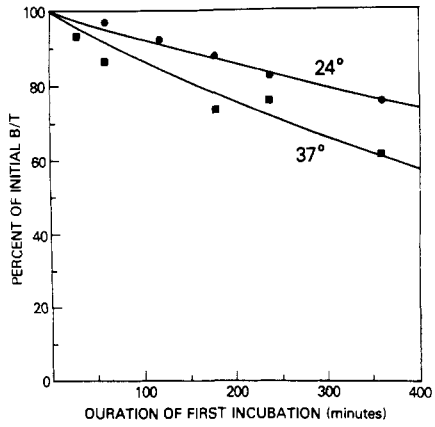

FIGURE 1: Time course of the [^{125}I]hCG degradation occurring during incubation with testis homogenate at 24 and 37°. Degradation was measured by second incubations performed at 24° for 22 hr (see Materials and Methods). The bound (B) over total (T) ratios, obtained in the second incubations with [^{125}I]hCG preincubated for various periods, were expressed as a percentage of the initial B/T ratio determined with fresh tracer. Each point is the mean of triplicate determinations. The solid lines represent the fits of a single negative exponential model (eq 5) with the data.

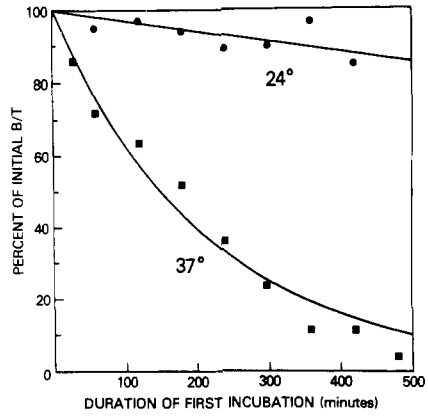

FIGURE 2: Time course of the receptor degradation occurring during incubation of rat testis homogenate at 24 and 37°. Degradation was measured by second incubations performed at 24° for 22 hr (see Material and Methods). The bound (B) over total (T) hormone ratios, obtained in the second incubations with homogenates subjected to various preincubation periods, were expressed as a percentage of the initial B/T ratio determined with fresh homogenate. Each point is the mean of triplicate determinations. The solid lines represent the fits of a single negative exponential model (eq 7) with the data.

min^{-1} (SE, n = 6). In two cases, when the hormone concentrations were low compared to the receptor concentrations, the best fit of the data was obtained with k_2 values lower than 10^{-11} min^{-1}. This indicated that under these conditions of reactant concentrations and short incubation periods, the reaction appeared to be almost irreversible, i.e., that the contribution of the dissociation process was practically undetectable. When such reactions were allowed to proceed for longer periods, a k_2 value in the range of 6.0×10^{-3} min^{-1} was observed. At 37°, for hCG and receptor concentrations ranging respectively from 12 to 907 pM and 14 to 19 pM, the mean value for k_1 was of $11.0 \pm 1.2 \times 10^7$ M^{-1} min^{-1} (SE, n = 5). The mean value for k_2 derived from these association experiments was $15.0 \pm 3.3 \times 10^{-3}$ min^{-1} (SE, n = 5). At 24° as well as at 37°, no relation between the association rate constants and the initial reactant concentrations was apparent. The mean values of k_2 derived by curve fitting from association data were 2–3 times higher than those obtained from direct dissociation experiments. Therefore, the k_1 values were also calculated for all association experiments using eq 1, without compensation for degradation, and assigning to k_2 the value derived from dissociation experiments (1.7×10^{-3} min^{-1} at 24° and 4.6×10^{-3} min^{-1} at 37°). The values of k_1 so obtained were only slightly smaller: $4.4 \pm 0.3 \times 10^7$ M^{-1} min^{-1} (SE, n = 8) at 24° and $9.5 \pm 1.5 \times 10^7$ M^{-1} min^{-1} (SE, n = 5) at 37°. The fit of the theoretical curves to the association data was still satisfactory, and k_1 remained independent from the hormone concentrations.

When the association rate of [^{125}I]hCG to testis homogenate was followed at 37° over longer periods, the concentration of hormone-receptor complex declined with time after an initial steady state had been reached (Figure 4). This association profile is typical of systems in which degradation occurs at a significant rate, and could be fitted using eq 1 in conjunction with eq 4, 6, 8, and 9, as described in Mathematical Models. The fit obtained with this more complex model was significantly better than when degradation was not taken into account. Table I shows the influence of hormone and receptor degradation on the derivation of k_1 and k_2 from association experiments. The values of k_2 were significantly lower when the fit was corrected for reactant degradation; the derivation of k_1 appeared to be only slightly affected by the degradation occurring in the system. The values of k_3 and k_4 obtained by simultaneous curve fitting of association, hormone, and receptor degradation data were not significantly different from the values obtained by analyzing the degradation of both reactants independently.

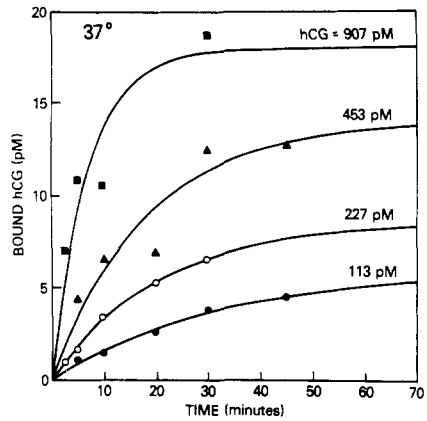

FIGURE 3: Effect of the initial concentration of hCG on the binding velocity of the hormone with its receptor in the rat testis homogenate at 37°. The incubations were carried out in individual glass tubes, in total volumes of 1 ml, containing 0.5 ml of testis homogenate (1 testis/20 ml), and 0.5 ml of the hCG solutions. The binding capacity of the incubation mixture was 19 pM. The initial HCG concentrations were as indicated on the figure. Each point is the average of duplicate determinations. The solid lines represent the best fits of the data, with eq 1, used without corrections for the reactant degradation.

The influence of hormone and receptor degradation on the binding reaction has been evaluated more extensively by computer simulation. Association curves have been generated in the presence of increasing rates of free receptor degradation (k_4), assuming no hormone degradation (Figure 5). It was clearly apparent that receptor degradation had a much greater effect on the concentration of hormone-receptor complex at steady state than on the initial velocities of the binding. Similar results were obtained when association curves were computed with increasing rates of free hormone degradation and without receptor loss. Computer sim-

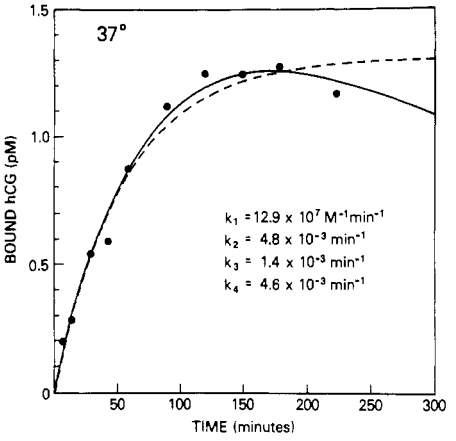

FIGURE 4: Time course of the binding of [^{125}I]hCG by its receptors in the rat testis at 37°, fitted with and without corrections for hormone and receptor degradation. Experimental conditions were as described in the legend of Figure 3. The binding capacity of the incubation mixture was 14 pM; the hCG concentration was of 12 pM. The solid line represents the fit of eq 1 in conjunction with eq 4, 6, 8, and 9 to the association data; simultaneously k_3 and k_4 were adjusted to obtain the best fit of (5) and (7) with hormone and receptor degradation data, respectively. The dotted line represents the fit of (1) to the association data without taking degradation into account.

ulations also showed that the rates of appearance of degraded receptor were inversely related to the initial hormone concentrations. These results exemplified one of the assumptions on which the model was based, i.e., that the receptor was protected from degradation when occupied by the hormone.

IV. Dissociation Experiments. Dissociation of hCG from its binding sites was a relatively slow process, and occurred more rapidly at 37° than at 24°. These findings were consistent with previous observations on dissociation of the testicular hormone-receptor complex in vitro (Catt et al., 1972b). The temperature dependence of dissociation has also been observed in the ovarian particulate LH receptor (Lee and Ryan, 1973). Initial dissociation velocities were consistent with first-order kinetics (eq 2), the dissociation rate constants, k_2, being 1.7×10^{-3} and 4.6×10^{-3} min^{-1} at 24 and 37°, respectively. At 24° dissociation was studied over longer periods, and the best fits of these data were obtained with the double negative exponential model (eq 3) (Figure 6); the dissociation rate constant of the first component (k_2') was $2.1 \pm 0.5 \times 10^{-3}$ min^{-1} (SE, $n = 3$); the determination of k_2'' was relatively imprecise, and was usually of the order of 10^{-12}–10^{-14} min^{-1}. A similar biexponential dissociation with a second slow component was occasionally observed at 37°. The relative concentrations of both components varied among homogenate preparations.

V. Analysis of Saturation Curves. The values for K_a and Q_0 obtained by analyzing equilibrium data with the Scatchard model or eq 11 were in good agreement. The mean value for K_a, at 24°, was of $4.0 \pm 2.7 \times 10^{10}$ M^{-1} (SD, $n = 16$). This value was close to the k_1/k_2 ratio observed at 24° (2.6×10^{10} M^{-1}). The mean binding capacity of the homogenates for hCG corresponded to $1.0 \pm 0.4 \times 10^{-12}$ mol/g of testis (SD, $n = 14$). The effects of hormone and receptor degradation on saturation curves were studied by computer simulation. As shown in Figure 7, the apparent binding capacity of a given receptor preparation was less affected by free receptor degradation than the apparent K_a of the binding reaction. The values of K_a and Q_0 corresponding to the Scatchard plots shown in Figure 7 were computed

Table I: Effect of Hormone and Receptor Degradation on the Derivation of k_1 and k_2 from Association Experiments.[a]

	Without Correction[b] for Degradation		With Correction[c] for Degradation	
	24°	37°	24°	37°
k_1 ($10^7 M^{-1}$ min^{-1})	4.7	14.0	4.6	12.9
k_2 (10^{-3} min^{-1})	3.1	14.4	1.8	4.8
k_3 (10^{-3} min^{-1})	0.0	0.0	0.8	1.4
k_4 (10^{-3} min^{-1})	0.0	0.0	0.3	4.6

[a] The duration of the incubations was of 225 min. Concentration of hCG = $12 \times 10^{-12} M$; concentration of binding sites = $14 \times 10^{-12} M$. [b] Association experiments were fitted using (1) without corrections for degradation. [c] k_1, k_2, k_3, and k_4 were adjusted by simultaneous fitting of association, hormone, and receptor degradation data with their corresponding models, as described in Mathematical Models.

by curve fitting of the simulated data, using the Scatchard model (Scatchard, 1949). The values obtained were respectively for K_a and Q_0: 2.0×10^{10} M^{-1} and 15.0×10^{-12} M ($k_4 = 0$); 1.4×10^{10} M^{-1} and 14.9×10^{-12} M ($k_4 = 1.0 \times 10^{-3}$ min^{-1}); 0.9×10^{10} M^{-1} and 14.9×10^{-12} M ($k_4 = 4.0 \times 10^{-3}$ min^{-1}); 0.4×10^{10} M^{-1} and 14.8×10^{-12} M ($k_4 = 16.0 \times 10^{-3}$ min^{-1}). The same results were derived by curve fitting of the simulated saturation curves presented in Figure 7, using eq 11 as the model. Degradation of the free hormonal ligand was observed to produce the same type of effects on simulated saturation curves.

Discussion

The use of an interactive nonlinear curve fitting program has enabled us to derive valid and precise values for the rate constants of the reaction between hCG and the gonadotropin binding sites of the rat testis. Using the MLAB computation program, we were able to employ more complex and presumably more correct models without encountering a concomitant increase in the difficulty of computation. This enabled an extensive evaluation of the influence of hormone and receptor degradation on the derivation of rate constants from kinetic experimental data.

For optimal determination of the binding constants of the interaction between peptide hormones and their receptors, it is essential to characterize the labeled ligand in terms of retention of biological activity and specific activity. Because gonadotropins employed for iodination are not usually of maximum attainable biological activity, the labeled preparations commonly contain a proportion of inactive labeled molecules. Damage of glycoprotein molecules during the labeling procedure can also lead to the formation of inactive labeled material. This fraction can be determined by measuring the proportion of labeled hormone bound by an excess of receptor sites (Midgley et al., 1974). In the present study, this factor was always taken into account for computations of the hormone concentrations. If binding data are to be employed for quantitative analysis, determination of specific activity of labeled tracer hormone should be performed by quantitation in terms of the unlabeled gonadotropin in a suitable assay system. Measurements of tracer mass can be performed by self-displacement in a radioimmunoassay or radioligand-receptor assay (Catt et al., 1972a; Lee and Ryan, 1973). In this study, a systematic discrepancy between the values obtained by both methods was observed. This was attributable to the presence, in the [^{125}I]hCG preparations, of labeled material which had lost

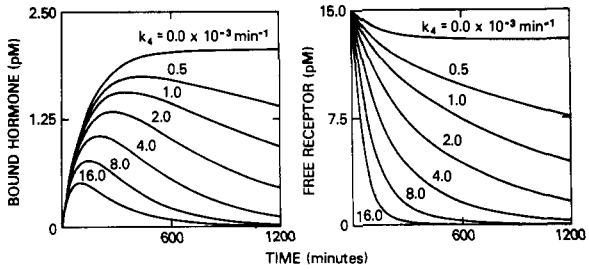

FIGURE 5: Computer simulation of the effects of various rates of free receptor degradation on association curves and on the rate of change of free receptor concentrations. The model is represented by eq 1, in conjunction with eq 4, 6, 8, and 9. The different k_4 values varying from 0.0 to 16.0 × 10^{-3} min^{-1} were as indicated in the figure; k_1, k_2, and k_3 were held constant at the values of 10^{-4} (pM^{-1} min^{-1}), 5 × 10^{-3} (min^{-1}), and 0; the values of 10 (pM) and 15 (pM) were assigned to P_0 and Q_0, respectively.

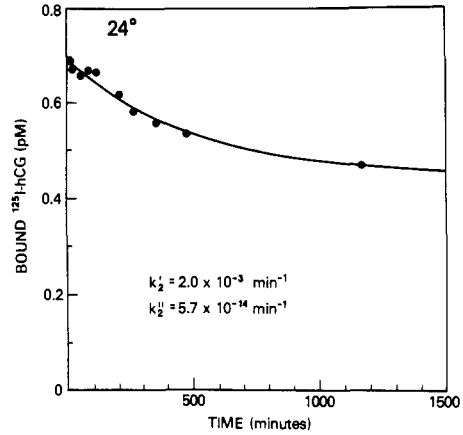

FIGURE 6: Time course of the dissociation of hCG from its receptors in the rat testis at 24°, studied over a period of 22 hr. Each point represents the mean of duplicate determinations. The solid line represents the fit of eq 3 to the data; k_2', k_2'', B_1, and B_2 were adjusted by the curve-fitting process. B_1 = 0.25 pM; B_2 = 0.44 pM.

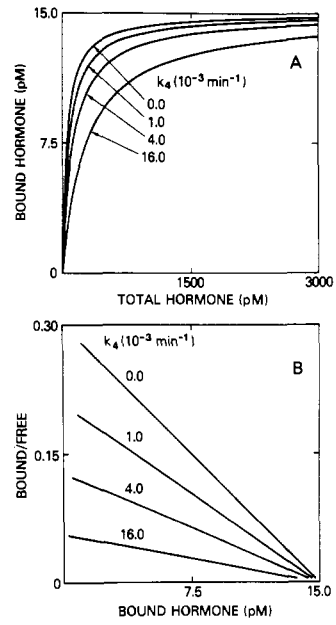

FIGURE 7: (A) Effect of increasing rates of free receptor degradation on the shape of computer simulated saturation curves. Equation 1 in conjunction with eq 4, 6, 8, and 9 was used as the model. The values of k_1, k_2, and Q_0 were 10^{-4} (pM^{-1} min^{-1}), 5 × 10^{-3} (min^{-1}), and 15 (pM), respectively. k_3 was set to zero and the values for k_4 were as indicated in the figure. For each value of k_4, the bound hormone concentrations (B(t)) were computed for increasing values of P_0, at a time (t), long enough to obtain equilibrium or steady steate at the lowest concentration of P_0 (5 pM). (B) The same data, expressed as Scatchard plots. The free hormone concentrations were computed by subtracting the bound from the total hormone concentration. The negligible effect of degradation upon receptor binding capacity under these conditions is due to the stability of the hormone–receptor complex, which is rapidly formed during incubation with the high gonadotropin concentrations employed during saturation studies.

the capacity to react with testis binding sites, but had retained immunological reactivity. Therefore, the tracer mass evaluated in the radioligand–receptor assay was used for all calculations of hormone concentrations.

The interaction of hCG with its binding sites was treated as a single, reversible bimolecular reaction. The equilibrium studies previously performed at 24° (Catt et al., 1972b) provided evidence for apparent homogeneity of the binding sites in the rat testis homogenate. These observations were confirmed and extended by the present study, which has validated the use of the second-order chemical kinetic equation for the analysis of the kinetic data obtained under these conditions. A good fit of the association data with the second-order chemical kinetic equation was obtained at 24 and 37°, over relatively short incubation times and over a wide range of hCG concentrations. The mean values of k_1 and k_2 were higher at 37° than at 24°, confirming the temperature dependence of the binding rate of gonadotropins to their target organ (Catt et al., 1972b). At both temperatures, no relation between k_1 and the hormone concentration was observed. This is an essential criterion for the validity of the application of the second-order kinetic model. Such independence of the association rate constant from hormone concentration has also been shown for the binding of hCG to the particulate receptor of the rat ovary (Lee and Ryan, 1973). Analysis of the initial dissociation velocities produced values for k_2 which were two to three times smaller than those derived from association data. This discrepancy was partially corrected by taking into account the hormone and receptor degradation occurring during the association experiments. When the association rate constants were determined with the k_2 parameter held constant at the values derived from dissociation experiments, slightly lower values for k_1 were obtained and the fit of the initial association velocities was only slightly affected. Thus, although reliable and precise association rate constants can be derived from short-term association data, the determination of dissociation rate constants by curve fitting of these association experiments appeared to be relatively inaccurate.

The values of k_1 derived from our association data were in good agreement with previous observations concerning the binding of hCG to the rat testis (Catt et al., 1972b). In contrast, the value of k_1 at 24° derived from our study was approximately 2.5–3 times lower than the association rate constant reported for the binding of ovine LH to bovine corpus luteum plasma membranes at 23° (Gospodarowicz, 1973) and roughly seven times lower than the values reported for the binding of hCG to a 2000g subcellular fraction of pseudopregnant rat ovaries (Lee and Ryan, 1973). Also the slow dissociation rate of the hCG–receptor complex in the rat testis contrasts with the rapid dissociation of ovine LH from bovine corpus luteum plasma membranes (Gospodarowicz, 1973). However, Lee and Ryan (1973) observed a relatively slow biphasic dissociation process of hCG from the rat ovaries. Differences in the degree of purity of the receptor preparations and the nature or the sta-

bility of the labeled hormones could account for the discrepancies between values published by different laboratories. Differences in the ionic strength of the incubation buffers have also been shown to influence the binding of LH to the ovarian receptor (Lee and Ryan, 1972). It is also possible that the intrinsic rate constants of gonadotropin receptors in the testis and ovary may differ slightly, though the equilibrium association constants are similar in each tissue (Tsuruhara et al., 1972; Dufau et al., 1974).

When studied over long periods of time, the dissociation process did not follow simple first-order kinetics. A better fit was obtained with a double exponential model, one of the components having an extremely slow dissociation rate constant. Whether the prolonged dissociation component is related to a secondary interaction between bound [^{125}I]hCG and the particulate receptor has not yet been determined. The length of the preincubation time could influence the dissociation curves, since at 24°, no significant rapid component was apparent when the preincubations were performed overnight (Catt et al., 1972b). The rapid dissociation component was also observed to be reduced in the particulate LH receptors of the rat ovary when preincubation with tracer hormone was prolonged (Lee and Ryan, 1973). The two dissociation components postulated in eq 3 are not interconvertible, and this assumption is implicit in our analysis of the dissociation curves. However, the possibility of some degree of interconversion between B_1 and B_2 cannot be excluded since the properties of both components seem to be affected by the duration of the preincubation. At the present time, the physiological meaning of this phenomenon remains unclear.

Hormone and/or receptor degradation have been shown to occur in many hormone–receptor systems (Freychet et al., 1972; Glossmann et al., 1974; Lee and Ryan, 1973; Marx et al., 1973; Pohl et al., 1972). Our data demonstrated that hCG and its receptors were degraded in a time- and temperature-dependent manner during incubation. No systematic study of the nature of the degraded hormone was performed. Since the degradation process of hCG is not fully understood the terms "hormone degradation" or "hormone inactivation" have been used throughout this paper in a general sense; they do not necessarily imply a proteolytic cleavage of the hormone as has been shown in the case of insulin degradation (Freychet et al., 1972). Indeed, the inactivation process of LH occurring during incubation with particulate receptor preparations of the ovary was not followed by the appearance of hormonal fragments or by a corresponding loss in the immunoreactivity of the molecule (Lee and Ryan, 1973). The same kind of qualification probably also applies to the term "receptor degradation". Enzymatic degradation of receptor sites could account for the loss of binding occurring in testis homogenates during incubation. Spontaneous solubilization or release of the receptor from the particulate preparation could also lead to reduction of hormone binding to the homogenate.

Since hormone and receptor degradation occurred during incubation, uncorrected calculations of k_1 and k_2 from association experiments could be subject to a systematic error from this source. When studied over long periods of time at 37°, association curves appeared to be affected by degradation of the interacting species. The more complex model, derived from the differential equation for second-order chemical kinetics, was shown to give satisfactory fits of these data. It also permitted the demonstration that reactant degradation in the hCG binding system had relatively little effect upon the derivation of k_1, but caused systematic overestimates of the k_2 parameter derived from association experiments. That model has been based on the assumption that the hormone–receptor complex is stable, a factor which should be experimentally validated before the model is applied to other systems. Computer-simulated association curves showed that the initial velocities were less affected by degradation than the levels of bound hormone at steady state. This result was obtained for reactant degradation rates as much as four to five times more rapid than those observed in the hCG binding system at 37°. Determination of the binding capacity of testis homogenates could be affected by degradation. However, simulations of the effects of degradation on saturation curves showed that such error was negligible under our experimental conditions, in which binding sites were protected from degradation by rapid formation of the hormone–receptor complex during incubation with increasing concentrations of gonadotropin. The analysis of the kinetics of labile systems by the present method has also proven useful for the modeling of similar effects in the angiotensin II receptor of the adrenal cortex (Catt et al., 1975) and represents an approach of general value for more precise analysis of the relatively complex systems exemplified by hormone–receptor interaction.

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^{1}H Nuclear Magnetic Resonance Study of Restricted Internal Rotation of N^6,N^6-Dimethyladenine in Aqueous Solution†

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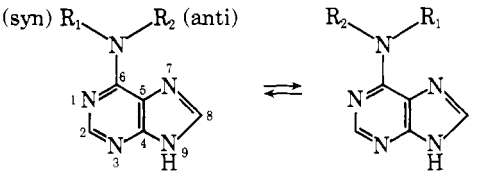
ABSTRACT: Kinetics of internal rotation about the C(6)–N(6) bond of N^6,N^6-dimethyladenine (M_2^6A) was investigated by ^{1}H nuclear magnetic resonance line-shape analysis of the methyl resonances (220 MHz). Rates of rotation were determined for M_2^6A deuterated at N(1) and for neutral M_2^6A. Activation parameters for monodeuterated M_2^6A at 22° are E_a = 13.8 kcal/mol, log A = 12.6, $\Delta G^\ddagger$ = 14.9 kcal/mol, $\Delta H^\ddagger$ = 13.2 kcal/mol, $\Delta S^\ddagger$ = −5.8 eu; for neutral M_2^6A: E_a = 15.5 kcal/mol, log A = 14.9, $\Delta G^\ddagger$ = 12.6 kcal/mol, $\Delta H^\ddagger$ = 14.9 kcal/mol, $\Delta S^\ddagger$ = 7.8 eu. Vertical stacking of bases interferes with internal rotation of the dimethylamino group.

Methylated nucleic acid bases play important roles in determining the activity and conformational properties of biomolecules of which they are components. The defense mechanism of certain strains of *Escherichia coli* against bacteriophage infection involves N(6) methylation of strategically located adenines of the bacterial DNA by modification methylases; by means of homospecific restriction endonucleases which do not hydrolyze the methylated DNA, bacteria preferentially degrade the phage DNA (Brockes et al., 1974; Marinus and Morris, 1974; for reviews see Arber, 1974 and Meselson et al., 1969). Modified nucleosides are located adjacent to the anticodon in certain types of tRNA: e.g., N^6-methyladenosine in *E. coli* valine tRNA (Saneyoshi et al., 1969). The exact reason for the presence of these minor components at this position is not certain, but it has been suggested that they may contribute to precise codon–anticodon pairing by altering the conformation of the anticodon loop (see review by Nishimura, 1972). N^6-substituted adenines display a wide range of biological and pharmacological activity: for example, various adenine derivatives, including N^6-methyladenosine and N^6,N^6-dimethyladenosine, inhibit tRNA methylation (Wainfan and Landsberg, 1973); N^6-phenyladenosine potentiates the effects of 6-mercaptopurine against leukemia L1210 (Grindley et al., 1973); and adenine derivatives inhibit accumulation of adenosine cyclic 3′,5′-monophosphate in fat cells (Fain, 1973). To understand the role of N^6-substituted adenines at the molecular level, we have initiated a program directed toward investigating the solution properties of the monomeric units.

As a consequence of the partial double bond character of the exocyclic C–N bond, the amino substituents of N(6)-methylated adenines are coplanar with the purine ring. One



of the resulting rotational isomers has R_1 syn to N(1); the other has R_2 syn to N(1). For monosubstituted adenines (R_2 = H) X-ray (McMullan and Sundaralingam, 1971; Bugg and Thewalt, 1972; Thewalt and Bugg, 1972; Sternglanz and Bugg, 1973a,b; Parthasarathy et al., 1974) and ^{1}H nuclear magnetic resonance (NMR) studies in nonaqueous solvents (Engel and von Hippel, 1974) indicate the preferred orientation for the alkyl substituent is syn to N(1). This orientation would block normal Watson–Crick base pairing if retained under physiological conditions. Engel and von Hippel (1974) demonstrated that Watson–Crick pairing was, in fact, blocked in a mixture of N^6-methyl-N^9-ethyladenine and 1-cyclohexyluracil; Hoogsteen pairing was favored. Ikeda et al. (1970) demonstrated that the 1:1 complex of poly(N^6-methyladenylic acid) with poly(U) is markedly destabilized by the presence of the methyl groups, even though, in this case, Watson–Crick base pairing occurs.

The kinetics and thermodynamics of syn–anti rotational isomerism of cytosine derivatives (Becker et al., 1965; Martin and Reese, 1967; Shoup et al., 1967, 1971, 1972; Engel

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