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THE KINETICS OF CORTISOL DISSOCIATION FROM PURIFIED HUMAN CORTICOSTEROID-BINDING GLOBULIN: STUDIES IN A FLOWING SYSTEM*

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SUMMARY

We have used a flowing system to determine the rate at which cortisol dissociates from purified human corticosteroid-binding globulin. Rate constants obtained at 4, 10 and 18°C yielded a linear Arrhenius plot, allowing calculation of the activation energy for the dissociation, 21 Kcal/mole. Extrapolation of the Arrhenius plot to 37°C showed a half-time for dissociation of 5·2 sec.

INTRODUCTION

HUMAN plasma contains an alpha globulin, corticosteroid-binding globulin (CBG), which binds cortisol and several other C21 steroids with high affinity [1–3]. The interaction between cortisol and CBG at equilibrium has been studied extensively while data on the kinetics of this interaction are limited to a single study by Dixon[4] done with plasma rather than pure CBG. If the availability of unbound cortisol in plasma is an important determinant of the entry of the hormone into certain cells, then it is important that the rate of dissociation of the complex be known with some accuracy. We have used purified human CBG in a flowing system in order to determine accurately the rate at which cortisol dissociates from its binding site on CBG.

MATERIALS AND METHODS

General

[1,2-^{3}H]-Cortisol, specific activity 44 Ci/mmole (New England Nuclear Corp) was purified by partition chromatography on celite prior to use. Radioactivity was determined in a liquid scintillation spectrometer in a dioxane-based phosphor [5] at an efficiency of 23% for samples containing 2 ml of buffer or water and 10 ml of the scintillation solution. Human CBG was prepared and its purity and activity determined as previously described[6]. Radioinert cortisol (Steraloids, Inc.) was recrystallized prior to use.

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Instrumentation

The flow rate dialysis cell was purchased from Bel Art and is basically the same one used by Colowick and Womack[7] with two small but important changes. Figure 1 is a diagrammatic representation of the instrumentation. The changes we have introduced are the glass tubing on the opening to the upper chamber which allows the entire cell to be immersed in a constant temperature bath and the coil of tubing interspersed between the peristaltic pump and the entrance to the lower chamber. The coil is necessary as we found that passage of buffer through the pump increased its temperature thus creating a temperature gradient between the two chambers of the cell. Details concerning the cell are given in the legend to Fig. 1.

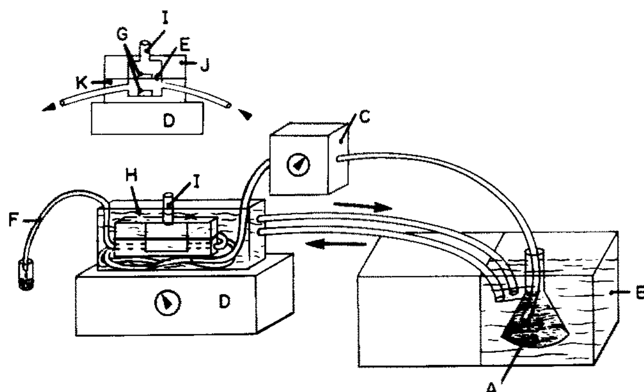

Fig. 1. Instrumentation for flow rate dialysis. (A) Buffer reservoir; (B) Constant temperature bath; (C) Peristaltic pump; (D) Stirring motor; (E) Dialysis membrane – made from 5/8 in. dia., 0·0008 in. wall thickness Visking dialysis tubing; (F) Outflow from lower chamber; (G) Magnetic stirring bars; (H) Second constant temperature bath. Used because it allows the dialysis cell to be more easily accessible than if placed directly in (B); (I) Port for sample addition; (J) Upper chamber; (K) Lower chamber. The inset in the upper left hand corner is provided to give a more detailed view of the dialysis cell. The diameter of the membrane between the two chambers is 1·9 cm and the volume of the lower chamber, corrected for the volume displaced by the magnetic stirring bar is 2·35 ml.

Theory

As discussed by Colowick and Womack[7], they developed this apparatus for use in the measurement of association constants in short periods of time. We have found that kinetic measurements can be made by using data obtained when the system is not at steady state.

The reaction we wish to consider is the dissociation of cortisol from its binding site on CBG.

$$[P]+[F] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [PF] \tag{1}$$

where: $[P]$ = concentration of free binding sites on CBG; $[F]$ = concentration of free cortisol; and $[PF]$ = the concentration of bound cortisol or occupied sites on CBG. In order to measure k_2, [^{3}H]-cortisol (F^*) is added to CBG and equilibrium allowed to occur. At this time radioinert cortisol (F), in large molar excess compared to the total concentration of CBG, is added to the reaction mixture.

F will then occupy initially free binding sites on P and in addition bind to sites freed by the dissociation of F^*. Whereas prior to the addition of F the velocity of the binding reaction equalled that of the dissociation reaction,

$$k_1[P][F^*] = k_2[PF^*]; \quad (2)$$

after the addition of F, $[P]$ is markedly reduced so that:

$$k_2[PF^*] > k_1[P][F^*]. \quad (3)$$

If the inequality, (3), is sufficiently great (for large enough addition of F) the system will approximate an irreversible reaction. The first order decay of $[PF^*]$ will then be given by:

$$\ln[PF^*] = \ln[PF^*]_0 - k_2 t \quad (4)$$

where: t = time and the subscript '0' refers to t = zero time. Since we actually measure $[F^*]$ and obtain $[PF^*]$ by subtraction it is important that the loss, by dialysis, of F^* from the upper chamber be minimal, i.e.

$$[PF^*]_0 + [F^*]_0 = [PF^*] + [F^*] \quad (5)$$

be satisfied. In the course of an experiment dialysis of F^* out of the upper chamber does take place but the fraction removed is so small that equation (5) is valid. Concentrations in equations (4) and (5) refer to the upper chamber i.e. $[U_{F^*}]$ or $[U_{PF^*}]$. These are not measured directly but must be inferred from concentration vs time measurements of F^* in the lower chamber, $[L_{F^*}]$. $[L_{F^*}]$ changes with time under the influence of two factors: influx of F^* from the upper chamber via dialysis; and removal of F^* from the lower chamber by pumping. This relationship is expressed by:

$$\frac{d[L_{F^*}]}{dt} = \frac{1}{V}(D[U_{F^*}] - \alpha[L_{F^*}]) \quad (6)$$

where: V = volume of the lower chamber; α = flow rate through the lower chamber; and D is a constant which reflects the rate of passage of free cortisol through the membrane. As has been suggested[7], it must be shown that D is independent of the mass of cortisol in the upper chamber. Furthermore, prior to the addition of radioinert cortisol, a steady state of the system must be shown by demonstrating that $[F^*]$ in the lower chamber is constant, i.e. that $(d[L_{F^*}]/dt) = 0$. After addition of radioinert cortisol to the upper chamber the inequality[3] obtains and the buildup of $[U_{F^*}]$ is reflected in increasing $[L_{F^*}]$ (Fig. 2). An adequate approximation of $d[L_{F^*}]/dt$ can be obtained by considering the difference in $[L_{F^*}]$ in two consecutive time periods, i.e. $\Delta[L_{F^*}]/\Delta t$. The value of $\alpha[L_{F^*}]$, representing the rate of depletion of F^* from the lower chamber, can be calculated using the mean value of $[L_{F^*}]$ for the same two time periods. Thus each two consecutive time periods allow an approximate calculation of $d[L_{F^*}]/dt$ and $\alpha[L_{F^*}]$ and hence of $[U_{F^*}]$. The membrane constant, D, can be determined from steady state measurements in which the upper chamber contains buffer and F^*

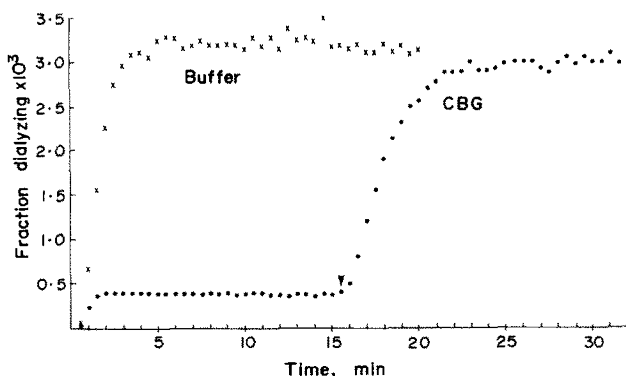

Fig. 2. Flow rate dialysis at 10°C. Two separate experiments are shown on the same set of coordinates. Fraction dialyzing $= [L_{F^*}]/[U_{F^*}]$. (×××××) = Buffer plus $1{\cdot}24 \times 10^6$ cpm in the upper chamber at time = 0. (......) = 134 μg ($2{\cdot}6 \times 10^{-9}$ moles) CBG plus $1{\cdot}22 \times 10^6$ c.p.m. in the upper chamber at time = 0. At the end of 15 min, as indicated by the vertical arrow, 19 μg ($5{\cdot}24 \times 10^{-8}$ moles) of cortisol was added to the upper chamber in a volume of 100 μl in both experiments.

but no CBG by use of

$$D = \alpha \frac{[L_{F^*}]}{[U_{F^*}]}. \tag{7}$$

It should be noted that the procedure of Colowick and Womack[7] differs from that described here in that they integrate equation (6) on the assumption that free ligand in the upper chamber does not vary with time (instantaneous chemical equilibration in the upper chamber). If, as in the present case, $[U_{F^*}]$ varies with time, integration of equation (6) is not possible and the differential form must be used.

EXPERIMENTAL

The buffer reservoir (A, Fig. 1) is charged with 0·05M sodium phosphate buffer, pH 7·4 and allowed to come to the desired temperature. The peristaltic pump is started and the flow rate adjusted to precisely 4·0 ml/min. At this time the upper chamber is charged with 1·5 ml of buffer (control experiments) or 1·5 ml of CBG (134 μg, $2{\cdot}6 \times 10^{-9}$ moles). The CBG used in all the experiments was stored in the lyophilized state and solubilized in phosphate buffer before use. It contained 0·78 moles of cortisol/mole CBG. At time 0 approximately 10^6 cpm of ^{3}H-cortisol ($4{\cdot}4 \times 10^{-11}$ moles) contained in 20 μl of buffer is added to the upper chamber. At this time, collection of the effluent from the lower chamber is started. Fractions are taken every 30 sec (2 ml). When steady state is reached, as indicated by the same counting rate in 20–25 successive fractions, $5{\cdot}24 \times 10^{-8}$ moles of radioinert cortisol in 100 μl of phosphate buffer are added to the upper chamber. The addition consumes 2–3 sec. At the termination of the experiment an aliquot of the upper chamber is taken for counting as a check on the amount of radioactivity added at time 0. Since the entire effluent from the lower chamber is collected and counted this allows precise calculation of the concentration of tritium in the upper chamber during any 30 sec period.

RESULTS

Figure 2 illustrates results from two typical flow rate experiments at 10°C. The data are normalized for slightly different additions of [^{3}H]-cortisol in the two experiments by plotting the fraction of cortisol, i.e. [c.p.m. in lower chamber]/[c.p.m. in upper chamber], appearing per unit time rather than the absolute number of counts. This form also renders constant the steady state values which would otherwise slope downward when there is significant depletion of F^* from the upper chamber as in buffer runs at high temperatures. A further advantage of this form of presentation is that it compensates for the small volume increases (20 μl for the [^{3}H]-cortisol addition and 100 μl for the radioinert cortisol addition) which occur during the course of the experiment. Note that there is no change in the rate of dialysis of [^{3}H]-cortisol after addition of carrier cortisol when only buffer is present in the upper chamber; hence the rate of transport of [^{3}H]-cortisol across the membrane depends only on the concentration of free [^{3}H]-cortisol in the upper chamber and not on the total concentration of cortisol. The close approach of the two curves reflects the almost quantitative displacement of [^{3}H]-cortisol from its binding sites by the excess of radioinert steroid. This type of experiment was done at each of the temperatures at which rate constants were determined.

Figure 3 shows the marked temperature effects on D (equations (6) and (7)).

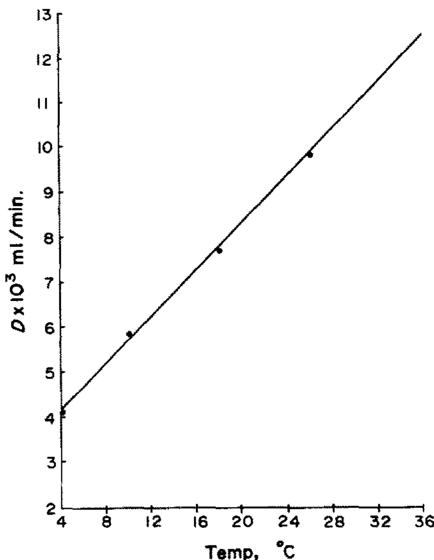

Fig. 3. The effect of temperature on the rate of dialysis of [^{3}H]-cortisol across the dialysis membrane. D is the membrane constant as discussed in the text (equations (6) and (7)).

Figure 4 illustrates that (PF^*) decays in a first order fashion and allows the calculation of k_2. Not illustrated are the data obtained at 26 and 37°C as k_2 was too rapid to allow meaningful analysis of the data. The calculated rate constants are shown in Table 1.

The rate constants can be used to construct an Arrhenius plot and this is shown in Fig. 5. Extrapolation of this data to 22 and 37°C is the source of the k_2's at those temperatures shown in Table 1. The activation energy derived from this plot is 21 Kcal/mole.

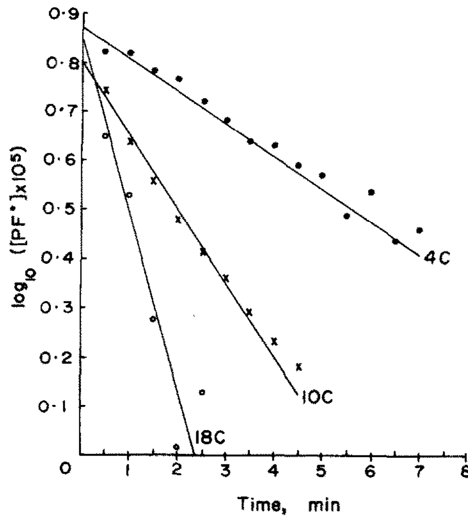

Fig. 4. Dissociation of [^{3}H]-cortisol (F^*) from CBG at three different temperatures. [PF^*] is the concentration of the CBG-cortisol complex.

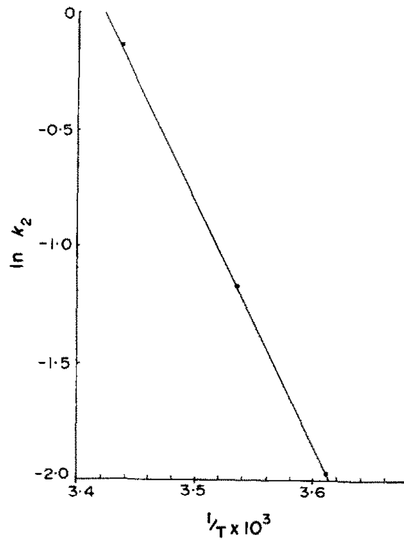

Fig. 5. Arrhenius plot of the dissociation rates (k_2) observed at 4, 10 and 18°C. The straight line was fitted by the method of least squares.

DISCUSSION

We describe in this communication a system which is useful for the determination of dissociation rate constants of dialyzable ligands from non-dialyzable macromolecules. Accurate control of temperature can be attained in conjunction with continuous monitoring of unbound ligand, without disturbing the reaction which is taking place. As can be seen from Fig. 4, the system is capable of ascertaining rate constants with half-lives greater than about 30 sec.

The dissociation of cortisol from CBG has been systematically examined by

Table 1. Rate constants for the dissociation of the CBG-cortisol complex*

	4°C	10°C	18°C	22°C	37°C
k_2 (min^{-1}) (this study)	0·14	0·31	0·87	1·4	8·0
k_2 (min^{-1}) (Dixon)	0·027	—	—	0·75	4·2
t 1/2 (min) (this study)	5·0	2·2	0·80	0·49	0·087
t 1/2 (min) (Dixon)	26	—	—	0·93	0·17

*The rate constants at 4, 10 and 18°C were determined experimentally (Fig. 4) while those at 22 and 37°C were obtained by application of the Arrhenius equation (Fig. 5). The data labeled "Dixon" are from Ref. [4].

only one other investigator[4] and his results (see Table 1) are not in agreement with ours. The possible sources of error which might arise from the method he uses to measure the kinetics of dissociation have been discussed by Dixon himself[4]. There is, however, an important non-methodological difference; we have used purified CBG while he used plasma. It is known that cortisol exists in plasma in three states: that which is bound to CBG, 77%; that which is bound to albumin, 15%; and that which is free, 8%[8]. The percentages represent the situation at 37°C and are not precise since they depend on the concentrations of albumin and CBG in the plasma being studied. Since the association constant for CBG is markedly temperature dependent while that for albumin is not, the differing distribution of cortisol at different temperatures complicates the interpretation of kinetic data obtained from unfractionated plasma. We believe that the data presented in this communication represent the true rate constants for the dissociation of cortisol from pure CBG. The linear Arrhenius plot (Fig. 5) lends credence to this contention.

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