Correcting for Incomplete Saturation and Off-Resonance Effects in Multiple-Site Saturation-Transfer Kinetic Measurements

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The effects of incomplete saturation and off-resonance irradiation on nuclear magnetic resonance saturation-transfer measurements of three-site chemical-exchange rates are discussed. A new method that uses double-saturation measurements is compared with two published methods, one that uses single-saturation measurements and one that uses a single-saturation measurement and a double-saturation measurement. Several formulas are compared for measuring the exchange rate constant k_{DE} for exchange from a detected spin D to an exchanging spin E in the presence of exchange from spin D to a competing spin C. For each method, formulas are derived with corrections for incomplete saturation or off-resonance effects, with both corrections, and with neither correction. Exact formulas are available for three exchanging sites with incomplete saturation if there are no off-resonance effects. Off-resonance corrections are imperfect even with complete saturation. © 2000 Academic Press

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INTRODUCTION

One way to measure rates of exchange between two chemicals or sites is the NMR technique of saturation transfer (*1–15*). In a conventional two-site saturation-transfer experiment the intensity of a detected spin D is measured with and without saturation of an exchanging spin E. Calculation of k_{DE}, the rate constant for exchange from spin D to spin E, also requires measurement of either the effective spin–lattice relaxation rate constant of spin D, $R_{1eff} = 1/T_{1eff}$, while spin E is saturated (*3–11*), or the R_1 of spin D in the absence of chemical exchange (*11–16*). Formulas for calculating k_{DE} in a two-site system in the presence of incomplete saturation and off-resonance effects of the radiofrequency (RF) irradiation ("spillover") have been compared recently (*11*).

If another competing spin C exchanges with spin D, a more complex measurement is required. It is convenient to differentiate the two published methods by the number of saturating RF frequencies during each stage of the measurement. In the "1-2" method, introduced by Forsén and Hoffman (*2*) and modified

slightly by Ugurbil (*3*), k_{DE} is calculated by measuring the intensity of spins D and E with no irradiation (Fig. 1a) and while spin C is saturated (Fig. 1b), and spin D intensity while both spins C and E are saturated (Fig. 1c) (*2–5*). The R_{1eff} of spin D also is measured while spins C and E are saturated. The "1-1" method, introduced by Perrin and Johnston, uses the same R_{1eff} measurement, and k_{DE} and k_{DC} are calculated by first saturating spin C (Fig. 2b), then saturating spin E in a separate measurement (Fig. 2d) (*1*).

Off-resonance effects originally were assumed to be negligible (*2, 6*). Later attempts to correct for off-resonance effects involved a control irradiation, the same distance from peak D but in the opposite direction (Figs. 1b, 2c, and 2e) (*1, 4, 5*). Inclusion of direct off-resonance effects does not lead to an exact formula for k_{DE}, because the steady-state longitudinal magnetization of spin D, M_{zD}, depends on transverse spin E magnetization, M_{xE} and M_{yE}, as well as M_{zE} (*10, 11*). Furthermore, the return of longitudinal magnetization, M_z, to steady state is no longer purely monoexponential (*10, 17–19*). Under certain conditions the recovery has a large exponential component, which can be considered the R_{1eff} for calculating k_{DE} (*10*).

Some published formulas for calculating k_{DE} attempt to correct for off-resonance effects on signal intensities (*1, 3–5, 9–13*) and on T_{1eff} (*11, 12, 20*). Incomplete saturation of spin E can cause errors in k_{DE} calculations in a two-site system (*10, 11, 13*), and presumably incomplete saturation of spin E in a three-site system would cause similar errors. Although spin C is not always completely saturated (*5*), the effects of incomplete saturation of spin C in a three-site system have not been reported.

Extension of the formulas for two-site exchange to a three-site system is not straightforward. With a single RF irradiation all magnetization components eventually reach a constant steady state in the RF rotating frame of reference, even with chemical exchange (*10*). With two RF irradiations M_z continues to oscillate about an average value in any reference frame. Therefore the intensities and relaxation rate constants must be
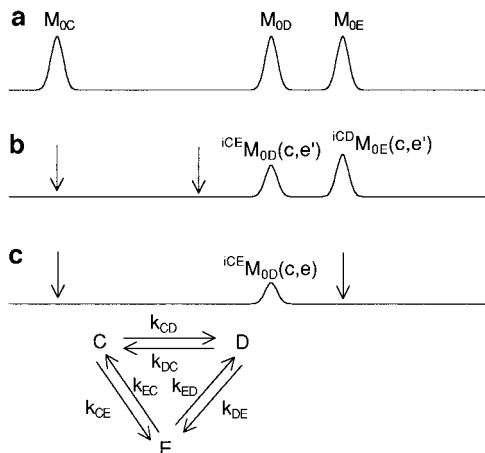
FIG. 1. A typical 1-2 saturation-transfer experiment to measure k_{DE} in a three-site system. (a) Equilibrium magnetization with no RF irradiation. (b) Saturation of spin C and the control for spin E relative to spin D. (c) Saturation of spins C and E. The three-site system is shown below (c).

determined by numerical estimation or approximate formulas rather than by exact analytic formulas.

The present work has several purposes. (1) It presents a new "2-2-2" method for measuring exchange rate constants in a three-site system from three double-saturation measurements. (2) It modifies published formulas to correct for incomplete saturation and off-resonance effects. (3) It compares the accuracy of the 1-2, 1-1, and 2-2-2 methods in a three-site ^{31}P spin system with off-resonance effects. All calculations are based on the Bloch equations (21) modified for chemical exchange (10, 11, 22).

NOTATION

The notation is necessarily detailed to include single and double saturations, control irradiations, off-resonance effects, incomplete saturation, and three different experimental methods (Table 1). However, the formulas are straightforward and are easily incorporated into a spread sheet or other computer program.

The relaxation rate constants and magnetization levels will include a subscript C, D, or E to indicate the relevant spin. A preceding superscript C, D, or E will indicate exchange with spin C, D, or E. Off-resonance effects will be indicated by a preceding superscript "i". Direct irradiation of spin C, D, or E will be indicated by (c), (d), or (e), respectively. For example, $^{iCE}M_{0D}(c, e)$ is the average steady-state M_z of spin D while spins C and E are irradiated with off-resonance effects included, and $^{i}R_{1D}$ is the $R_{1\text{eff}}$ of spin D with off-resonance irradiation and no exchange. Longitudinal magnetization normalized to equilibrium magnetization will be represented by $C_z = M_{zC}/M_{0C}$, $D_z = M_{zD}/M_{0D}$, and $E_z = M_{zE}/M_{0E}$.

Irradiation of a "control" position for spin E (the same distance from the observed spin, but in the opposite direction,

see Figs. 1b, 2c, and 2e) will be indicated by (e'), with similar notation for spin C or D controls. If spin C is 300 Hz downfield from spin D and spin E is 100 Hz upfield from spin D (Fig. 2), $^{iCE}M_{0D}(c')$ indicates irradiation 300 Hz upfield from spin D (Fig. 2c) and $^{iCD}M_{0E}(c')$ indicates irradiation 400 Hz upfield from spin E. When the control position is not relative to the observed spin, the reference for the control will be indicated. For example, with irradiation 100 Hz downfield from spin D, the three intensities are $^{iDE}M_{0C}(e' \text{ rel } D)$, $^{iCE}M_{0D}(e')$, and $^{iCD}M_{0E}(e' \text{ rel } D)$ (Fig. 2e). With irradiation of two control positions or one spin and one control position, the control is always relative to the spin not mentioned in parentheses. For example, in $^{iDE}M_{0C}(c, e')$ the e' means irradiation 100 Hz downfield from spin D, not 400 Hz downfield from spin C.

Formulas for calculating k_{DE} involve measuring the reduction in intensity of one or more signals upon saturation of one or more spins. Each signal decrease is measured relative to a reference intensity, $M_0(\text{ref})$. Signal intensities usually are measured as ratios of intensities, with the reference intensity and the reduced intensity both normalized to the same value $M_0(\text{norm})$. These signal changes will be represented in the 1-1 and 2-2-2 methods as

$$\Delta D(d \text{ rel } C) = [M_{0D}(\text{ref}) - {}^{iCE}M_{0D}(d)]/M_{0D}(\text{norm})$$

[1]

$$\Delta D(c, e) = [M_{0D}(\text{ref}) - {}^{iCE}M_{0D}(c, e)]/M_{0D}(\text{norm}).$$

[2]

With no off-resonance correction $M_{0D}(\text{ref}) = M_{0D}(\text{norm}) = M_{0D}$. Corrections for off-resonance effects use $M_{0D}(\text{ref}) =$
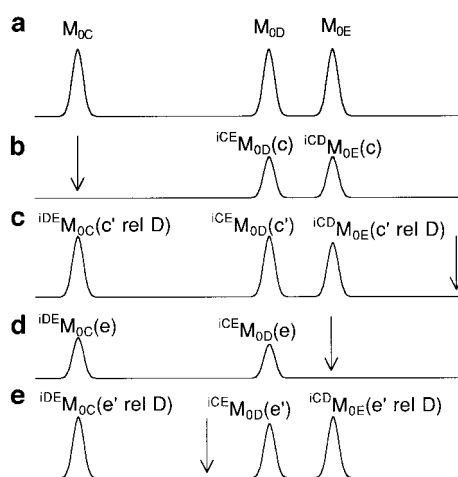

FIG. 2. A typical 1-1 saturation-transfer experiment to measure k_{DE} and k_{DC} in a three-site system. (a) Equilibrium magnetization with no RF irradiation. (b) Saturation of spin C. (c) Irradiation of a control for spin C relative to spin D. (d) Saturation of spin E. (e) Irradiation of a control for spin E relative to spin D.

TABLE 1
Notation for Magnetization in the Presence of Chemical Exchange and Off-Resonance Effects

Symbol	Meaning
Preceding superscipts	
i	Includes effects of off-resonance irradiation
CE	Chemical exchange with spins C and E, with constant spin C magnetization and spin E magnetization
Magnetization symbols	
M_{0C}, M_{0D}, M_{0E}	Equilibrium magnetization with no irradiation
$^{CE}M_{0D}$	$M_{zD}(\text{ss})^a$ with chemical exchange with spins C and E, Eq. [10]
$^{i}M_{0D}$	$M_{zD}(\text{ss})^a$ with off-resonance irradiation and no exchange
(c, e)	Spins C and E are irradiated (saturated)
(c', e')	Irradiation of the control positions for spins C and E
$^{iCE}M_{0D}(e)$	$M_{zD}(\text{ss})^a$ while spins C and E are saturated, including off-resonance effects
Magnetization ratios	
C_z, D_z, E_z	M_{zC}/M_{0C}, M_{zD}/M_{0D}, M_{zE}/M_{0E}
$^{iCE}D_z(c, e')$	$^{iCE}M_{0D}(c, e')/M_{0D}$
$^{iCE}D_z(c, e)$	$^{iCE}M_{0D}(c, e)/M_{0D}$
$^{iCD}E_z(c, e')$	$^{iCD}M_{0E}(c, e')/M_{0E}$
$^{iCD}E_z(c, e)$	$^{iCD}M_{0E}(c, e)/M_{0E}$
$\Delta D(c, e)$	Fractional decrease in $M_{zD}(\text{ss})^a$ when spins C and E are irradiated, Eq. [2]
Relaxation rate constants	
$^{i}R_{1D}$	$R_{1D,\text{eff}}$ with off-resonance effects, Eq. [12]
$^{iCE}R_{1D}$	$R_{1D,\text{eff}}$ with chemical exchange and off-resonance effects, Eq. [15]

^{a} Here, $M_{zD}(\text{ss})$ is steady-state longitudinal magnetization of spin D.

$^{iCE}M_{0D}(d' \text{ rel } C)$ in Eq. [1] and $M_{0D}(\text{ref}) = {}^{iCE}M_{0D}(c', e')$ in Eq. [2]. This notation does not apply to the 1-2 method, where the reference intensity is measured during irradiation of one spin, which must be indicated. Theoretical derivations usually lead to a correction that we will call "type A," normalizing to equilibrium magnetization, $M_{0D}(\text{norm}) = M_{0D}$ (4, 5, 11). Some studies have used a correction that we will call "type B," normalizing to the magnetization with control irradiation, $M_{0D}(\text{norm}) = M_{0D}(\text{ref})$ (1, 11).

Some three-site formulas have expressed the formula for k_{ED} in terms of $^{CE}R_{1D}(M_{0D}/M_{0E})$ (1, 2). This is not consistent with two-site formulas (4–15) and another three-site formula (3–5), where $^{CE}R_{1D}$ is used to calculate k_{DE}. Since $k_{DE}M_{0D} = k_{ED}M_{0E}$, the three-site formulas can be modified to express k_{DE} in terms of $^{CE}R_{1D}$. This modification has been applied to the formulas from (1, 2) for consistency between the different methods.

BLOCH EQUATIONS WITH CHEMICAL EXCHANGE

The Bloch equations can be modified to include chemical exchange between spin D and spins C and E (10, 11, 22) and two RF fields. The two RF fields have strengths $\omega_1 = \gamma B_1$ and $\omega_2 = \gamma B_2$ and frequencies ω_{rf1} and ω_{rf2}, and the frequency offsets from spin D are $\omega_A = \omega_0(D) - \omega_{\text{rf1}}$ and $\omega_B = \omega_0(D) - \omega_{\text{rf2}}$. With this notation the Bloch equations for spin D in the B_1 rotating frame become

$$dM_{zD}/dt = -\{\omega_1 + \omega_2\cos[(\omega_A - \omega_B)t]\}M_{yD}$$
$$- \omega_2\sin[(\omega_A - \omega_B)t]M_{xD} + R_{1D}(M_{0D} - M_{zD})$$
$$- (k_{DC} + k_{DE})M_{zD} + k_{CD}M_{zC} + k_{ED}M_{zE} \quad [3]$$

$$dM_{xD}/dt = \omega_A M_{yD} + \omega_2\sin[(\omega_A - \omega_B)t]M_{zD}$$
$$- R_{2D}M_{xD} - (k_{DC} + k_{DE})M_{xD}$$
$$+ k_{CD}M_{xC} + k_{ED}M_{xE} \quad [4]$$

$$dM_{yD}/dt = -\omega_A M_{xD} + \{\omega_1 + \omega_2\cos[(\omega_A - \omega_B)t]\}M_{zD}$$
$$- R_{2D}M_{yD} - (k_{DC} + k_{DE})M_{yD}$$
$$+ k_{CD}M_{yC} + k_{ED}M_{yE}. \quad [5]$$

Similar equations can be written for spins C and E. At equilibrium the flux into each site must equal the flux out of that site, so

$$k_{CD}M_{0C} = k_{DC}M_{0D} \quad [6]$$

$$k_{ED}M_{0E} = k_{DE}M_{0D} \quad [7]$$

$$k_{CE}M_{0C} = k_{EC}M_{0E}. \quad [8]$$

With these substitutions Eq. [3] can be rearranged to

$$dM_{zD}/dt = -\{\omega_1 + \omega_2\cos[(\omega_A - \omega_B)t]\}M_{yD}$$
$$- \omega_2\sin[(\omega_A - \omega_B)t]M_{xD}$$
$$+ {}^{CE}R_{1D}({}^{CE}M_{0D} - M_{zD}), \quad [9]$$

where

$$^{CE}M_{0D} = M_{0D}(R_{1D} + k_{DC}C_z + k_{DE}E_z)/^{CE}R_{1D} \qquad [10]$$

$$^{CE}R_{1D} = R_{1D} + k_{DC} + k_{DE}. \qquad [11]$$

Equation [10] agrees with Eq. [3] of (1) if $C_z = 0$ or $E_z = 0$.

When a single RF field of magnitude $\omega_1 = \gamma B_1$ is applied to a spin system for a long time, the transition to a new steady state for each magnetization component M_x, M_y, and M_z usually has an exponential term modified by decaying oscillations (10, 17–19, 23). The oscillations eventually disappear and M_x, M_y, and M_z reach constant values in a reference frame rotating at the B_1 frequency (10, 17–19, 21), even with chemical exchange or cross-relaxation between spins (10). The steady state exists because $\mathbf{B}_{eff}$, the vector sum of $\mathbf{B}_1$ and the off-resonance component of the static field $\mathbf{B}_0$, is constant in the B_1 rotating frame. The magnetization is aligned near the effective field $\mathbf{B}_{eff}$, so that the small force exerted by $\mathbf{B}_{eff}$ balances T_1 and T_2 relaxation. In other reference frames the z component of $\mathbf{B}_{eff}$ is unchanged and the angle between $\mathbf{B}_{eff}$ and $\mathbf{B}_0$ is constant, so M_z reaches the same steady state. However, the x and y components of $\mathbf{B}_{eff}$ oscillate, and M_x and M_y oscillate about average values and never reach single steady-state values.

With two RF fields the magnitude of $\mathbf{B}_{eff}$ and the angle between $\mathbf{B}_{eff}$ and $\mathbf{B}_0$ oscillate in any reference frame. As a result, not only do M_x and M_y continue to oscillate without reaching single steady-state values, M_z also continues to oscillate about an average value. To understand this, consider the steady-state condition with one RF field. When a second RF field is added, this new $\mathbf{B}_2$ field rotates in the B_1 reference frame. With this rotating $\mathbf{B}_2$ field, the net $\mathbf{B}_{eff}$ becomes time-dependent in any reference frame. Both the magnitude B_{eff} and the angle between $\mathbf{B}_{eff}$ and $\mathbf{B}_0$ oscillate with a frequency $|\omega_A - \omega_B| = |\omega_{rf2} - \omega_{rf1}|$ in any reference frame. As a result M_z oscillates with this same frequency in any reference frame.

With chemical exchange the oscillation frequency of the exchanging spin generally is different from the frequency of the observed spin, further complicating the oscillation pattern. Although there does not appear to be an exact analytic formula for the average steady-state magnetizations and oscillation magnitudes with two RF fields and chemical exchange, numerical simulations can yield very accurate results. The numerical simulations reported here were performed with Mathematica version 3.0 on a Macintosh computer. Magnetization recovery curves were calculated at 500 evenly spaced points over 30 s ($>7 \ T_1$), and the average of the last 50 points over multiple cycles was considered the steady-state value. Relaxation rate constants determined from a three-parameter nonlinear least-squares fit of the calculated values were within 1% of values calculated with the formula

$$^iR_1 \approx R_1 + (R_2 - R_1)\{[\,\omega_1^2/(\omega_A^2 + \omega_1^2)\,]$$
$$+ [\,\omega_2^2/(\omega_B^2 + \omega_2^2)\,]\}, \qquad [12]$$

which was used for all calculations of exchange rate constants. With one RF field Eq. [12] reduces to a previously published approximation based on an analytic solution of the Bloch equations (10).

One way to approximate the off-resonance effects with a single RF field is to ignore the oscillations induced by the RF field, and to consider the exponential relaxation rate constant $^iR_{1D}$ and the steady-state magnetization $^iM_{0D}$ in the absence of exchange (10, 21), then add chemical exchange. When this approach is extended to two RF fields with $^iR_{1D}$ of Eq. [12], the ω_1 and ω_2 terms of Eqs. [3] and [9] are incorporated into $^iR_{1D}$ and $^iM_{0D}$ so that

$$dM_{zD}/dt = \ ^{iCE}R_{1D}(^{iCE}M_{0D} - M_{zD}), \qquad [13]$$

where

$$^{iCE}M_{0D} = [\,^iM_{0D}{}^iR_{1D} + M_{0D}(k_{DC}C_z + k_{DE}E_z)]/^{iCE}R_{1D} \qquad [14]$$

$$^{iCE}R_{1D} = \ ^iR_{1D} + k_{DC} + k_{DE}. \qquad [15]$$

Equation [14] can be rewritten

$$^{iCE}D_z{}^{iCE}R_{1D} = \ ^iD_z{}^iR_{1D} + k_{DC}C_z + k_{DE}E_z. \qquad [16]$$

FORMULAS FOR CALCULATING k_{DE}

Formulas for the 1-2, 1-1, and 2-2-2 methods can be derived from Eq. [16]. Calculation of exchange rate constants requires measurement of a longitudinal relaxation rate constant. The 1-2, 1-1, and 2-2-2 methods use $^{iCE}R_{1D}$, and the 2-2-2 method also uses $^{iDE}R_{1C}$ and $^{iCD}R_{1E}$. Relaxation rate constants $^iR_{1C}$, $^iR_{1D}$, and $^iR_{1E}$ were calculated with the approximation of Eq. [12], and $^{iDE}R_{1C}$, $^{iCE}R_{1D}$, and $^{iCD}R_{1E}$ were calculated from Eq. [15] or equivalent equations for spins C and E.

1-2 Method

The 1-2 method begins by saturating spin C and irradiating the control position for spin E to measure $^{iCE}D_z(c, e')$ and $^{iCD}E_z(c, e')$ (Fig. 1b) (2–5). Then, while keeping spin C saturated, spin E also is saturated and $^{iCE}D_z(c, e)$ is measured along with $^{iCE}R_{1D}$ (Fig. 1c). If Eq. [16] with spins C and E saturated is subtracted from Eq. [16] with irradiation of spin C and the control position for spin E,

$$^{iCE}R_{1D}[\,^{iCE}D_z(c, e') - \ ^{iCE}D_z(c, e)]$$
$$= k_{DC}[\,^{iDE}C_z(c, e') - \ ^{iDE}C_z(c, e)]$$
$$+ k_{DE}[\,^{iCD}E_z(c, e') - \ ^{iCD}E_z(c, e)]. \qquad [17]$$

If spin C magnetization is constant in the two spectra, ${}^{iDE}C_z(c, e') = {}^{iDE}C_z(c, e)$ and

$$k_{DE} = {}^{iCE}R_{1D}[{}^{iCE}D_z(c, e') - {}^{iCE}D_z(c, e)]/$$
$$[{}^{iCD}E_z(c, e') - {}^{iCD}E_z(c, e)]. \qquad [18]$$

If ${}^{iCD}E_z(c, e) = 0$, Eq. [18] simplifies to a previously published formula that assumes complete saturation of both spins C and E (3):

$$k_{DE} = {}^{iCE}R_{1D}[{}^{iCE}D_z(c, e') - {}^{iCE}D_z(c, e)]/{}^{iCD}E_z(c, e'). \qquad [19]$$

Equation [19] can be compared to Eq. [7] of (2):

$$k_{ED} = {}^{iCE}R_{1D}(M_{0D}/M_{0E})[{}^{iCE}D_z(c) - {}^{iCE}D_z(c, e)]/{}^{iCD}E_z(c). \qquad [20]$$

Equation [20] can be converted to Eq. [19] by applying Eq. [7] and including a control irradiation. Thus the method of (3) is essentially that of (2) with control irradiation and with k_{DE} measured using ${}^{iCE}R_{1D}$ rather than ${}^{iCD}R_{1E}$.

1-1 Method

A fundamentally different approach was introduced by Perrin and Johnston, who assumed complete saturation, used a type B off-resonance correction, and expressed k_{ED} in terms of ${}^{iCE}R_{1D}$ (1). They saturated both spins C and E to measure ${}^{iCE}R_{1D}$, but they measured signal intensity changes with only one spin saturated. Application of Eq. [16] to measurements with control irradiation and saturating irradiation yields, with incomplete saturation,

$$k_{DE} = {}^{iCE}R_{1D}\left\{\frac{\Delta D(e)\Delta C(c \text{ rel } D) - \Delta C(e \text{ rel } D)\Delta D(c)}{\Delta E(e \text{ rel } D)\Delta C(c \text{ rel } D)} \atop {} - \Delta C(e \text{ rel } D)\Delta E(c \text{ rel } D)\right\}.$$
$$[21]$$

The Δ notation was explained in Eqs. [1] and [2]. The Δ formulas derived by this method would have a type A off-resonance correction, with $M_0(\text{norm}) = M_0$ (see Eq. [16]), and a type B correction can be calculated for comparison. Notice that measurement of k_{DE} (and k_{DC}) requires irradiation of spin C and spin E in separate experiments. With a type B off-resonance correction and complete saturation, $\Delta C(c \text{ rel } D) = \Delta E(e \text{ rel } D) = 1$, and Eq. [21] becomes Eq. [5] of (1), modified to express k_{DE} in terms of ${}^{iCE}R_{1D}$:

$$k_{DE} = {}^{iCE}R_{1D}\left\{\left[1 - \frac{M_{0D}(e)}{M_{0D}(e')}\right] - \left[1 - \frac{M_{0C}(e)}{M_{0C}(e' \text{ rel } D)}\right]\right.$$
$$\times \left[1 - \frac{M_{0D}(c)}{M_{0D}(c')}\right] \bigg/ \left[1 - \frac{M_{0C}(e)}{M_{0C}(e' \text{ rel } D)}\right]$$
$$\left.\times \left[1 - \frac{M_{0E}(c)}{M_{0E}(c' \text{ rel } D)}\right]\right\}. \qquad [22]$$

2-2-2 Method

The 2-2-2 method uses signal intensities with two spins saturated. This results in three separate signal intensity measurements, which can be acquired during measurements of the three $R_{1\text{eff}}$s. Three exchange rate constants are determined independently, and the others are determined by Eqs. [6]–[8]. Formulas for calculating the exchange rate constants can be derived by applying Eq. [16] to each spin with control irradiations and with two spins saturated, yielding equations similar to Eq. [17]:

$${}^{iDE}R_{1C}\Delta C(d, e) = k_{CD}\Delta D(d, e) + k_{CE}\Delta E(d, e) \qquad [23]$$
$${}^{iCE}R_{1D}\Delta D(c, e) = k_{DC}\Delta C(c, e) + k_{DE}\Delta E(c, e) \qquad [24]$$
$${}^{iCD}R_{1E}\Delta E(c, d) = k_{EC}\Delta C(c, d) + k_{ED}\Delta D(c, d). \qquad [25]$$

After applying Eqs. [6]–[8] to replace k_{DC}, k_{ED}, and k_{CE} in these equations, the resulting formula for k_{DE} is

$$k_{DE} = [{}^{iCE}R_{1D}\Delta D(c, e)\Delta C(c, d)\Delta D(d, e)$$
$$+ {}^{iCD}R_{1E}\Delta E(c, d)\Delta C(c, e)\Delta E(d, e)$$
$$\times (M_{0E}/M_{0D}) - {}^{iDE}R_{1C}\Delta C(d, e)$$
$$\times \Delta C(c, e)\Delta C(c, d)(M_{0C}/M_{0D})]/$$
$$[\Delta C(c, d)\Delta D(d, e)\Delta E(c, e)$$
$$+ \Delta C(c, e)\Delta D(c, d)\Delta E(d, e)]. \qquad [26]$$

The Δ formulas derived by this method would have a type A off-resonance correction, as with the 1-1 method in Eq. [21]. Formulas for the other rate constants can be derived by exchanging the spin labels, and by applying Eqs. [6]–[8] when appropriate.

Extension to Four Sites

Consider a cyclic four-site system where spins C and E exchange with a fourth spin F, with no direct exchange between spins D and F or between spins C and E. The 1-1 and 1-2 methods can be applied exactly as in the three-site case to calculate k_{DC} and k_{DE}, even with direct C–E exchange. Contrary to previous assertions (3), the 1-1 method (1) does not "require the measurement of four relaxation rate constants (one for each exchange site), 12 fractional reductions in intensity, and a simultaneous solution of 16 equations," and the method

TABLE 2
Relaxation and Intensity Parameters for the ^{31}P Spin System

Spin	Chemical	M_0	T_1 (s)	T_2 (s)
C	PCr	4	4	0.2
D	ATP$_\gamma$	2	1.25	0.05
E	P$_i$	1	2.5	0.08

of (*2*) does not require solving a pair of equations to obtain k_{DE} and k_{ED} together. The 2-2-2 method requires measurement of each spin's intensity while its exchange partners are saturated. Thus, it is sufficient to saturate spins C and E to observe D and F, and then saturate spins D and F to observe C and E.

SAMPLE CALCULATIONS

To compare the accuracy of the different methods for calculating k_{DE} with off-resonance effects, consider the three-site ^{31}P spin system with exchange of phosphate groups between phosphocreatine (PCr, spin C), the γ-phosphate of adenosine triphosphate (ATP$_\gamma$, spin D), and inorganic phosphate (P$_i$, spin E) (*3–5, 7–9, 13–15*):

$$\text{PCr} \leftrightarrow \text{ATP}_\gamma \leftrightarrow \text{P}_i.$$

We will assume a field strength of 1.5 T, that of a typical clinical magnetic resonance imaging scanner. Because there is no direct exchange between PCr and P$_i$, the PCr-to-ATP$_\gamma$ and P$_i$-to-ATP$_\gamma$ rates can be measured with a conventional two-site saturation-transfer measurement by saturating ATP$_\gamma$ (*4, 5, 7–11, 13–16*), and corrections for incomplete saturation and off-resonance effects have been suggested (*11, 13*). Measurement of the reverse reactions, from ATP$_\gamma$ to PCr and P$_i$, requires a three-site saturation-transfer measurement (*3–5*). The simulated data assume typical T_1 and T_2 times in rat brain (*24*). Those measured relaxation times were not corrected for chemical exchange, which can cause significant errors (*16*), and they were at 4.7 T rather than 1.5 T, but they provide reasonable values to compare the published and proposed formulas for k_{DE} calculations. Table 2 shows the relaxation times and relative intensities. In addition $k_{CD} = k_{ED} = 0.5$ s^{-1}, PCr is 400 rad/s (~64 Hz or 2.5 PPM at 1.5 T) downfield from ATP$_\gamma$ and 800 rad/s (~128 Hz or 5 PPM at 1.5 T) upfield from P$_i$.

Four RF strengths were considered: $\omega_1 = 5$ rad/s for significantly incomplete saturation; $\omega_1 = 15$ rad/s for slightly incomplete saturation with small off-resonance effects; $\omega_1 = 40$ rad/s for moderate off-resonance effects; and $\omega_1 = 120$ rad/s for large off-resonance effects. The $R_{1\text{eff}}$ values and the relative signal intensities with irradiation of two spins or two control positions with these ω_1 values are summarized in Table 3. The amount of off-resonance effects can be seen in the intensities with control irradiations, and the completeness of saturation can be seen in the residual intensity when a spin is irradiated.

Rate constants were calculated by the 1-2, 1-1, and 2-2-2 methods with corrections for either incomplete saturation or off-resonance effects (type A or type B correction), with both corrections, and with no correction. The percentage of errors (rounded to the nearest integer) in the calculated rate constants for exchange between ATP$_\gamma$ and P$_i$ are summarized in Tables 4 and 5, and the creatine kinase-catalyzed exchange between PCr and ATP$_\gamma$ will be included in the discussion. The k_{EC} (which is actually zero) calculated by each method is shown in Table 6. The calculated k_{CE} generally was about one-fourth of the calculated k_{EC}. With the 2-2-2 method the percentage of errors in k_{DE} and k_{ED} are equal and k_{CE} errors differ from k_{EC} errors by the factor M_{0E}/M_{0C} (see Eq. [8]).

For comparison k_{ED} was calculated by the two-site method with different corrections. The following equations from (*11*) were used: Eq. [17] for no corrections, Eq. [20] for incomplete saturation, Eq. [34] for type A off-resonance, Eq. [35] for type B off-resonance, Eq. [27] for incomplete saturation plus type A off-resonance, and Eq. [29] for incomplete saturation plus type B off-resonance.

Several conclusions can be drawn from Tables 4–6 and the creatine kinase calculations. (1) The best results were obtained with corrections for both incomplete saturation and off-resonance effects. (2) None of the three-site methods (1-2, 1-1, and 2-2-2) was consistently better than the others. (3) The 2-site method was better than the three-site methods for calculating k_{ED}, mostly because of reduced off-resonance effects, which cannot be corrected completely. (4) Type A off-resonance corrections were better than type B corrections with the 2-2-2 method and with the creatine kinase reactions. Type B correc-

TABLE 3
Signal Intensities and Effective Relaxation Rate Constants for the ^{31}P Spin System with Chemical Exchange[a]

Calculated value	ω_1 (s^{-1})			
	5	15	40	120
$^{iD}C_z(d', e')$	0.9954	0.9607	0.7761	0.2772
$^{iD}C_z(d, e)$	0.6422	0.3916	0.3135	0.1779
$^{iD}C_z(c, d)$	0.0774	0.0069	0.0010	0.0013
$^{iD}C_z(c, e)$	0.0907	0.0107	0.0016	0.0009
$^{iCE}D_z(c', e')$	0.9969	0.9724	0.8321	0.3527
$^{iCE}D_z(c, e)$	0.4610	0.3931	0.3515	0.1957
$^{iCE}D_z(c, d)$	0.3198	0.0732	0.0117	0.0022
$^{iCE}D_z(d, e)$	0.4648	0.0950	0.0145	0.0014
$^{iD}E_z(c', d')$	0.9990	0.9908	0.9379	0.6209
$^{iD}E_z(c, d)$	0.6216	0.4815	0.4285	0.3046
$^{iD}E_z(c, e)$	0.2240	0.0326	0.0048	0.0006
$^{iD}E_z(d, e)$	0.2240	0.0246	0.0033	0.0004
$^{iD}R_{1C}$	0.7509	0.7583	0.8089	1.2467
$^{iCE}R_{1D}$	2.0533	2.0800	2.2614	3.8254
$^{iD}R_{1E}$	0.9007	0.9061	0.9436	1.2861

[a] Signal intensities as a fraction of equilibrium intensities were calculated by numerical simulation with two spins irradiated or with two control irradiations. Effective relaxation rate constants were calculated with Eqs. [12] and [15].

TABLE 4
Percentage of Error in Measuring $k_{DE} = 0.25$ s^{-1}
(from ATP$_\gamma$ to P$_i$) with Different Formulas

Method	ω₁ (s⁻¹)			
	5	15	40	120
No corrections				
1-2[a]	−27	−3	4	17
1-1	−27	−2	11	69
2-2-2	48	15	25	195
Correction for incomplete saturation				
1-2	5	1	4	17
1-1	0	2	12	69
2-2-2	0	3	23	195
Off-resonance correction type A				
1-2[a]	−27	−4	−3	−39
1-1	−27	−2	13	137
2-2-2	48	12	1	−2
Off-resonance correction type B				
1-2	−27	−3	5	0
1-1[a]	−27	−2	11	111
2-2-2	48	15	20	127
Corrections for incomplete saturation and type A off-resonance				
1-2	5	0	−2	−39
1-1	0	2	14	137
2-2-2	0	0	−1	−3
Corrections for incomplete saturation and type B off-resonance				
1-2	5	1	5	0
1-1	0	2	12	112
2-2-2	0	3	17	127

[a] Previously published formulas.

tions were better when exchange between ATP$_\gamma$ and P$_i$ was measured with the 1-1 method. (5) With corrections for incomplete saturation and off-resonance effects, the 2-2-2 method was similar to or better than the other three-site methods. (6) When a rate constant is zero, all three methods may calculate significant nonzero rates, depending on the corrections being used (Table 6).

DISCUSSION

Saturation transfer can be used to measure exchange rate constants in a wide variety of reactions (*7, 9*), including two-site systems with spins D and E and three-site systems with spins C, D, and E. Previously published formulas include Eq. [20] for the 1-2 method with no corrections (*2*), Eq. [19] for the 1-2 method with type A off-resonance correction (*3*), and Eq. [22] for the 1-1 method with type B off-resonance correction (*1*).

In the linear three-site system considered here, with no direct exchange between spins C and E, saturation of spin D allowed calculation of k_{CD} and k_{ED} by the two-site formulas. These two-site measurements always were similar to or better than the three-site methods. One application of saturation transfer

experiments is to look for direct exchange between two sites. In such cases a small amount of direct exchange may be calculated even if it does not exist (Table 6), so such measurements must be interpreted with caution.

In a few cases it may be possible to saturate spins C and E almost completely without significantly reducing the intensity of spin D, especially with narrow peaks and large chemical shift differences (*11*). In many cases, especially with broad peaks (short T_2) or small chemical shift differences, either saturation will be incomplete or there will be significant off-resonance effects on spin D. Most previous attempts to correct for off-resonance effects in two-site (*4, 5, 7, 9, 13, 15*) and three-site measurements (*1, 3–5*) have neglected the decrease in T_{1eff} (*10, 12, 20*) and the nonexponential recovery of longitudinal magnetization (*10, 17–19, 23*) caused by the RF irradiation. Formulas to correct for incomplete saturation and off-resonance effects in a two-site system have been compared (*11*).

All three methods for three-site calculations (1-2, 1-1, and 2-2-2) can give accurate results when corrected for off-reso-

TABLE 5
Percentage of Error in Measuring $k_{ED} = 0.5$ s^{-1}
(from P$_i$ to ATP$_\gamma$) with Different Formulas

Method	ω₁ (s⁻¹)			
	5	15	40	120
No corrections				
2-site	−51	−10	1	25
1-2[a]	−61	−15	1	38
1-1	−61	−15	−1	3
2-2-2	48	15	25	195
Correction for incomplete saturation				
2-site	0	0	3	25
1-2	0	1	4	39
1-1	0	0	2	3
2-2-2	0	3	23	195
Off-resonance correction type A				
2-site	−51	−10	−2	−1
1-2[a]	−61	−16	−4	−13
1-1	−61	−15	1	45
2-2-2	48	12	1	−2
Off-resonance correction type B				
2-site	−51	−10	−1	9
1-2	−61	−15	0	15
1-1[a]	−61	−15	2	38
2-2-2	48	15	20	127
Corrections for incomplete saturation and type A off-resonance				
2-site	0	0	0	−1
1-2	0	0	−1	−12
1-1	0	1	4	45
2-2-2	0	0	−1	−3
Corrections for incomplete saturation and type B off-resonance				
2-site	0	0	1	9
1-2	0	0	3	16
1-1	0	1	4	38
2-2-2	0	3	17	127

[a] Previously published formulas.

TABLE 6
Values of k_{EC} (P_i to PCr) Calculated with Different Formulas[a]

Method	ω_1 (s^{-1})			
	5	15	40	120
No corrections				
1-2[b]	0.14	0.05	0.05	0.54
1-1	0.14	0.04	0.04	0.34
2-2-2	−0.40	−0.10	−0.09	−0.58
Correction for incomplete saturation				
1-2	0.16	0.05	0.05	0.54
1-1	0.00	0.00	0.03	0.34
2-2-2	0.00	−0.01	−0.07	−0.58
Off-resonance correction type A				
1-2[b]	0.14	0.04	0.00	−0.04
1-1	0.14	0.04	0.00	−0.12
2-2-2	−0.40	−0.10	−0.01	0.01
Off-resonance correction type B				
1-2	0.14	0.04	0.00	−0.05
1-1[b]	0.14	0.04	0.01	0.00
2-2-2	−0.40	−0.11	−0.09	−0.48
Corrections for incomplete saturation and type A off-resonance				
1-2	0.16	0.04	0.00	−0.04
1-1	0.00	0.00	−0.01	−0.13
2-2-2	0.00	0.00	0.00	0.01
Corrections for incomplete saturation and type B off-resonance				
1-2	0.16	0.04	0.00	−0.05
1-1	0.00	0.00	0.00	0.00
2-2-2	0.00	−0.01	−0.07	−0.48

[a] Units are s^{-1}. The actual value is $k_{EC} = 0$.
[b] Previously published formulas.

nance effects and incomplete saturation, although extreme off-resonance effects may cause significant errors. The slight errors in the 1-2 method with low ω_1 values probably are due to small changes in C_z during the two measurements. For example, compare $C_z(c, d)$ and $C_z(c, e)$ in Table 2. It should be emphasized that these simulations did not consider such factors as spin–spin coupling, frequency spread caused by poor shimming, B_1 inhomogeneity, noisy data, changes in the sample over time, difficulties in T_1 measurements (see below), and the possible irradiation of a severely broadened peak such as adenosine diphosphate (ADP) in biological systems. Although poor shimming can be approximated by changing the T_2 to reflect the total linewidth, B_1 inhomogeneity has more complex effects (23). The choice of method will depend on these factors and on the time available and the information desired (see below).

The most accurate formulas contain an off-resonance correction and a correction for incomplete saturation. The preferred off-resonance correction, type A or type B, depends on the exchange being studied, linewidths, and whether transverse magnetization is transferred (11). In many cases a small amount of residual magnetization may not be detected in the noise, in which case only the off-resonance correction is applied. The results with $\omega_1 = 15$ and 40 rad/s correspond to

common experimental conditions, with some incomplete saturation and significant (but not drastic) off-resonance effects. With these ω_1 values all three methods had similar accuracy (Tables 4–6).

Each of the three methods considered here has advantages in specific applications. To measure all six rate constants, the 2-2-2 method is fastest and the 1-1 method is slightly slower than the 1-2 method (Table 7). To measure a single rate constant, the 2-2-2 method is the slowest and the 1-1 method is still slightly slower than the 1-2 method. The relative accuracies may change under different conditions. For example, in measurements at low RF power without the correction for incomplete saturation, the 2-2-2 method had the worst accuracy for k_{DE} (Table 4) and the best accuracy for k_{ED} (Table 5). The 2-2-2 method has two disadvantages compared to the 1-1 and 1-2 methods. First, it does not allow a separate measurement of k_{ED} to confirm the measurement of k_{DE}. Second, exchange of spin C or E with other unknown spins will interfere with the calculation of all rate constants.

All three methods can be understood in terms of Eq. [16], which states that D_z changes linearly with C_z and E_z. With the 1-2 method, spin C magnetization and off-resonance effects are assumed to be constant, so the change in spin D intensity is directly proportional to the change in spin E intensity (Eq. [18]). In contrast, the 1-1 and 2-2-2 methods require solving two or three simultaneous equations because spin C magnetization is not constant. This results in more complex formulas, which also give more information (two or six rate constants instead of only one). Actually, the 1-2 method indirectly gives

TABLE 7
Number of Measurements Needed for Each Method in a Cyclic Three-Site System

Method	Relaxation	Double saturation	Other intensity measurements	
			With controls	Without controls
Calculation of k_{DE}				
1-2	1	1	1	1
1-1	1	0	4	2
2-2-2	3	3	3	0
Calculation of all six rate constants				
1-2	3	3	6	3
1-1	3	0	9	3
2-2-2	3	3	3	0

Note. The number of each type of measurement needed to measure rate constants by the different methods is shown for a cyclic three-site system, where each site exchanges directly with the other two sites (see Fig. 1). Measurement of equilibrium magnetizations, needed for each method, are not shown. The relaxation measurements (column 2) require saturation of the other two spins, and the double-saturation intensities (column 3) can be acquired as part of the relaxation data. The number of other intensity measurements (column 4) includes single-saturation measurements and control irradiations, and it may be reduced if off-resonance effects are negligible (column 5).

k_{DC} along with k_{DE}. With no off-resonance effects the sum $k_{DC} + k_{DE}$ can be determined from $^{iCE}R_{1D}$ and the total intensity drop when spins C and E are both saturated (Eq. [10]) (8), and subtracting k_{DE} from this sum yields k_{DC}. This calculation assumes that the three-site model is correct and does not include all the control data for off-resonance effects, so it may be more accurate to measure k_{DC} directly.

The $R_{1\mathrm{eff}}$ used in these calculations may not equal the measured value under all conditions. Although there are several ways to measure T_1 (25), two methods commonly used for saturation-transfer studies are progressive saturation ($4, 5, 13, 14$) and following the decrease in intensity when saturation commences ($8, 15$). With incomplete saturation the magnetizations of spins C and E are not constant in a progressive saturation experiment. In simulations this caused a decrease in the measured $R_{1\mathrm{eff}}$. On the other hand, strong off-resonance effects can cause oscillations when there is a large initial magnetization (10), and inversion-recovery or measuring the decrease in intensity can lead to erroneous results.

The calculations used here are based on the assumption that phase coherence is transferred during chemical exchange, so that M_x and M_y are transferred between sites. It is plausible that phase coherence could be lost during an enzyme-catalyzed reaction. In these cases the signal intensities would be slightly different, and this could increase or decrease the errors in measuring rate constants, as shown for the two-site case (11). The results also will be slightly different if saturation is achieved by a train of RF pulses instead of a continuous RF irradiation ($4, 12$). If T_2 is longer, more M_x and M_y are transferred from an irradiated spin, and the relative accuracies of type A and B off-resonance corrections may change (11).

These methods also can be applied to situations where spin C undergoes cross-relaxation with spins D and E instead of chemical exchange. Such cross-relaxation may have interfered with attempts to measure rotation about the C–N bond in *N,N*-dimethylacetamide (26). Cross-relaxation and chemical exchange have identical effects on M_z exchange, but M_x and M_y do not exchange during cross-relaxation (10). The off-resonance effects will be slightly different with cross-relaxation than with chemical exchange, so the errors with strong RF fields will be slightly different (11).

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