Ferrimyoglobin–Fluoride

An Undergraduate Kinetics Experiment

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Recently published experiments on reaction kinetics cover a wide range of innovative ideas. However, in many cases the suggested experiments require elaborate techniques and/or expensive equipment. We propose here an experiment that combines the advantages of simplicity with unusual chemical interest. It concerns the reaction of the protein ferrimyoglobin with fluoride ion, in dilute aqueous solution, using readily available apparatus and the familiar technique of optical absorbance for measurement of concentrations. The experiment is conducted at room temperature, at fixed pH, ionic strength, and protein concentration, but it can be extended to study the effect of varying any of these parameters. The kinetic measurements enable the determination of both forward and reverse rate constants, and hence the equilibrium constant for the reaction under the given conditions. The same data also give the equilibrium concentrations, so that a direct determination of the equilibrium constant can be made. In this manner, one can compare the thermodynamic results of equilibrium measurements with the corresponding quantities that are obtained from a kinetic study of the same well-defined chemical reaction.

Ferrimyoglobin is the iron(III) oxidation state of the hemoprotein myoglobin. Hemoglobin and myoglobin form the oxygen-carrying team in all vertebrates, including humans, where myoglobin stores O_2 in muscle cells and hemoglobin carries O_2 in the blood stream. These were the first globular proteins to have their detailed atomic structures determined (Perutz and Kendrew, Nobel Prize 1962). The hemoglobin molecule is a tetramer; myoglobin is a monomeric polypeptide of 153 amino acids, incorporating a porphyrin ring that strongly chelates one iron atom and neutralizes two of its positive charges. The iron center has a single free coordination site, loosely occupied by H_2O, and carries a net electrostatic charge of +1. Whereas neutral ligands like O_2 and CO bind to (ferro) myoglobin and hemoglobin, in which the iron atom has a net charge of zero, it is anions like OH^-, CN^-, and F^-, which are the ideal ligands for ferrimyoglobin and ferrihemoglobin. Under appropriate and reversible conditions, the ligand-to-metal bonding reaction of fluoride with ferrimyoglobin may be represented as follows:

$$Fe^+(H_2O) + F^- \rightleftharpoons Fe\text{–}F + H_2O \qquad (1)$$

The product Fe–F is ferrimyoglobin fluoride, a high-spin complex. The reaction has a second-order forward rate constant, k_f, and a first-order reverse (back) rate constant, k_b. Three types of rate measurements are possible on this system. By using fluoride at concentrations high enough to carry the reaction to completion, apparent first-order kinetics of the forward reaction can be studied. Conversely, starting with the product, dissociation experiments can be conducted that yield first-order kinetics for the reverse reaction. The proposed experiment here uses moderately high fluoride concentrations that carry the forward reaction to an intermediate equilibrium point, thereby yielding simultaneous measurement of forward and back rates. The experiment was used in a physical chemistry laboratory course at the American University of Beirut and has been tested at Indiana University.

For the reaction in eq 1, at given temperature, pH, and ionic strength, the rate of formation of product is

$$d[Fe\text{–}F]/dt = k_f[Fe^+][F^-] - k_b[Fe\text{–}F] \qquad (2)$$

where, at time t, [Fe] is the molar concentration of the reactant ferrimyoglobin, and [Fe–F] the concentration of product formed; $[F^-]$ is the concentration of fluoride ions (effectively equal to the initial, excess, fluoride salt added). An integrated progress equation can be derived by standard methods.[1] In the present case, considerable simplification is achieved because fluoride is in large excess over ferrimyoglobin, so that

$$\ln([Fe\text{–}F]_e/([Fe\text{–}F]_e - [Fe\text{–}F])) = (k_f[F^-] + k_b) \cdot t \qquad (3)$$

Here, the terms are as already defined, except that [Fe–F] is the variable concentration of product at any time t during the course of the reaction, and $[Fe\text{–}F]_e$ is the final concentration at the point when the reaction attains equilibrium. [Fe–F] and $[Fe\text{–}F]_e$ are conveniently obtained from absorbance measurements since ferrimyoglobin has intense absorption bands in the visible and near ultraviolet. Beer's law applies accurately to these solutions. At a given wavelength and a given initial fluoride concentration, if the absorbance of a certain ferrimyoglobin solution is A_0, the absorbance of fully formed ferrimyoglobin–fluoride is A_{100}, and that of a reaction mixture is A at time t, reaching its equilibrium value A_e, it follows from eq 3 that a plot of the quantity $\ln (A_e - A)$ against time should be linear, with slope equal to $(k_f[F^-] + k_b)$. If the experiment is then repeated at a series of different initial fluoride concentrations, a set of linear slopes is obtained. A plot of these slopes versus fluoride concentrations should also be linear and yields intercept = k_b and slope = k_f. The kinetically derived equilibrium constant would then be obtained from the relation

$$K_{eq} = k_f/k_b \qquad (4)$$

The corresponding equilibrium constant for the reversible reaction is also directly obtained from equilibrium data, since

$$K_{eq} = [Fe\text{–}F]/[Fe] \cdot [F^-] = (1/[F^-]) \cdot (A - A_0)/(A_{100} - A) \qquad (5)$$

where [Fe] and [Fe–F] are the equilibrium molar concentrations of ferrimyoglobin and ferrimyoglobin–fluoride, respectively, and $[F^-]$ is the equilibrium concentration of fluoride ions (equal to the initial, excess, fluoride salt added) at given temperature, pH, and ionic strength. The two determinations of K_{eq} can thus be compared.

Apart from its inherent interest as a protein, myoglobin possesses a number of physicochemical properties that make it particularly suitable for this type of experiment. It is readily available, easy to handle, water soluble, and quite stable. Its iron atom has a single coordination site, and the reaction is therefore well defined (eq 1). Ferrimyoglobin is

[1] Physical chemistry textbooks. See also Ver Ploeg, D. A.; Alberty, R. A. *J. Biol. Chem.* **1968**, *243*, 435.

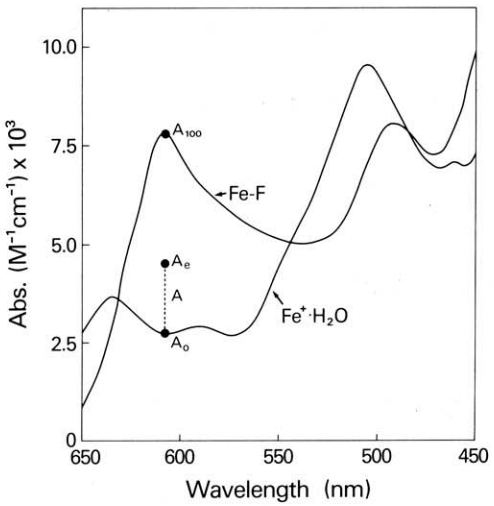

Figure 1. Main part of the absorption spectra, in the visible range, for ferrimyoglobin, $Fe^+(H_2O)$, and ferrimyoglobin–fluoride, Fe–F. Kinetic measurements were made at 608 nm where: A_0 = absorbance of ferrimyoglobin at start of reaction, A = absorbance (variable) during reaction, A_e = absorbance at point of equilibrium attained, and A_{100} = absorbance of fully formed ferrimyoglobin–fluoride product. Details are in the text.

brown (giving cooked meat its characteristic color); it has intense absorption bands that permit the use of very dilute protein solutions, less than 1×10^{-4} M. Consequently, the experiment can be run with a large excess of fluoride over myoglobin, which considerably simplifies the kinetics. Furthermore, kinetic runs take only a few minutes to complete, which makes the work fast and all the more interesting. The experiment can be performed at different levels of sophistication; what we present below is a basic and adaptable procedure for obtaining and analyzing kinetic and equilibrium data.

Experimental

Design

Figure 1 shows the absorption spectrum of ferrimyoglobin and of ferrimyoglobin–fluoride in the visible range, 650 to 450 nm. The fluoride band at 608 nm (molar absorptivity = 7.5×10^3 M^{-1} cm^{-1}) is a suitable wavelength for making absorbance measurements and hence for following the reaction kinetics. However, there is a limit to the pH range over which the experiment can be conveniently carried out. In the first place, the iron-bound H_2O undergoes acid–base dissociation[2] with a pK_a of about 8.9:

$$Fe^+(H_2O) = Fe\text{–}OH + H^+ \qquad (6)$$

and the absorption spectrum of the conjugate base Fe–OH (which was left out of Fig. 1 for clarity) is close to that of the reaction product Fe–F, making it impractical to measure absorbance changes beyond pH 9. The competition between the two equilibria (eqs 1 and 6) also leads to marked decrease in the equilibrium constant for the reaction at high pH, again limiting measurements to pH below 9. Another consideration is the fact that the reaction is catalyzed by H^+, which makes the forward rate too fast for simple kinetic measurements at pH below 7.5.[3] Since the reaction is between oppositely charged ions (eq 1), it can be slowed down by increasing the ionic strength of the solution, for instance by adding the neutral salt KCl. Reasonably slow kinetics are obtained at temperature 18–20 °C, pH 8.0–8.2, ionic strength 0.2–0.4 M, and with protein concentration less than 1×10^{-4} M.

Solutions

Stock aqueous solutions may be prepared ahead of time, using a suitable buffer (such as Tris or borate) of pH about 8 and ionic strength 0.1 M. A suggested plan involves preparing the following: (a) potassium chloride—0.40 M made in buffer, to be used for adjusting ionic strength; (b) potassium fluoride—0.40 M also made in buffer (sodium fluoride has an advantage over the potassium salt in not being deliquescent, but it is limited by lower solubility); (c) ferrimyoglobin—commercial and partially purified protein (usually from sperm whale muscle or horse heart) is reasonably priced; it is best stored frozen. Since the molecular weight is 18,000, about 100 mg of myoglobin may be dissolved in 50 mL of buffer to make a stock solution approximately 1.3×10^{-4} M; this quantity is ample for two groups of students performing the experiment. Before use, the protein solution should be filtered or centrifuged to obtain a clear reddish brown liquid. Absorbance at 608 nm should be adjusted to read about 0.350 (by adding a little more protein or buffer and filtering again if necessary). Since proteins act as surfactants, their solutions tend to froth and sometimes develop insoluble denatured particles; these can be cleaned out by suction with a dropper. The technique provides some experience for students in handling proteins.

Procedure

Students will probably work in pairs. In addition to the stock solutions, they need access to a spectrophotometer (such as a Spec 20 with digital readout). It is assumed that the students will first familiarize themselves with the instrument, setting it at 608 nm, and learning to balance it with blank (buffer) using matched tubes. The success of this experiment depends primarily on the precision and reproducibilty of preparing the reaction mixtures and making absorbance measurements. KCl and myoglobin solutions may be dispensed from burets. The KF solution, which is added to start reaction, can be squirted from a 1-mL syringe or blown from a graduated pipet. Time is read in seconds. A typical experiment runs as follows:

1. Into one of the pre-matched tubes, deliver 3.00 mL myoglobin and 1.50 mL KCl. Check zero absorbance for the blank (in the other matched tube). Then place the reaction tube in the spectrophotometer, and read absorbance. (This may be later converted, by the appropriate dilution factor, into a reading which corresponds to the moment the reaction starts.)
2. Inject 0.50 mL of the KF solution into the reaction mixture, starting the clock simultaneously. A plastic rod may be used to mix the resulting 5.00-mL solution for 2 s and is immediately withdrawn. The lid is then closed, and an absorbance reading is taken at time = 10 s (if possible) and subsequently at 5- or 10-s intervals (depending on the speed) until changes in absorbance become very small or enough readings have been taken.
3. Within 2 or 3 min, equilibrium is attained, and the endpoint reading A_e is taken. The reaction product gives a slightly greenish hue to the solution, which may now be taken out for pH measurement and discarding.
4. A second run is prepared from 3.00 mL of myoglobin and varying KCl and KF, again making a total volume of 5.00 mL. The instrument is checked for zero and blank, and the experiment is repeated. Each kinetic run takes about 5 min to complete. A group of (two) students can complete six runs in an hour. Calculations and graphing are done later. A protocol for making a series of eight solutions is suggested here.

Run	Mb (mL)	KCl (mL)	KF (mL)	$[F^-]$ (mol/L)
1	3.00	1.90	0.10	0.0080
2	3.00	1.80	0.20	0.0160
3	3.00	1.70	0.30	0.0240
4	3.00	1.60	0.40	0.0320
5	3.00	1.50	0.50	0.0400
6	3.00	1.40	0.60	0.0480
7	3.00	1.20	0.80	0.0640
8	3.00	1.10	0.90	0.0720

Treatment of Kinetic Data

Table 1 gives kinetic data from an experiment of eight runs at room temperature, 21 °C, at pH 7.95 and I = 0.24 M.

[2] Hanania, G. I. H.; Irvine, D. H. *J. Chem. Soc. (A)* **1970,** 2389.

Table 1. Kinetic Data at Three Fluoride Concentration Levels[a]

Time	Run 1		Run 4		Run 8	
s	A	$-\ln(A_e - A)$	A	$-\ln(A_e - A)$	A	$-\ln(A_e - A)$
0	0.210	2.937	0.215	2.071	0.216	1.641
10	0.225	3.219	0.265	2.577	0.322	2.430
15	0.231	3.442	0.278	2.765	0.350	2.813
20	0.234	3.507	0.292	3.016	0.371	3.244
25	0.237	3.650	0.303	3.270	0.385	3.689
30	0.241	3.817	0.311	3.507	0.390	3.912
35	0.244	3.963	0.318	3.772	0.398	4.423
40	0.246	4.075	0.323	4.017	0.402	4.828
45	0.249	4.269	0.325	4.135	0.405	
50	0.251	4.423	0.330	4.510		
60	0.254	4.711	0.335			
A_e	0.263		0.341		0.410	

[a] Run 1 (0.0080 M), Run 4 (0.032 M), Run 8 (0.072 M). Room temperature = 21 °C, pH = 7.95, I = 0.24 M, [protein] = 8.0×10^{-5} M. Absorbance A (1.0-cm path) was measured at 608 nm (Fig. 1) at 5-s intervals. A_e is the equilibrium value attained after readings stabilize (3 min). Absorbance at time zero is given by the regression equation and can also be deduced from the original reading before fluoride was added.

Table 2. Summary of Kinetic Data[a]

Run	[F] mol/L	Slope $\ln(A_e - A)/t$
1	0.0080	0.029
2	0.016	0.039
3	0.024	0.042
4	0.032	0.048
5	0.040	0.048
6	0.048	0.056
7	0.064	0.067
8	0.072	0.079

[a] Data are plotted in Figure 1 (insert). Linear slope = k_f = 0.689 $M^{-1}s^{-1}$. Intercept = k_b = 0.0250 s^{-1}.

The data are shown for run 1 (lowest fluoride concentration), run 4 (intermediate), and run 8 (highest). Plots of the quantity $\ln(A_e - A)$ versus time are shown in Figure 1 and are linear as expected. Data from the other runs were treated similarly. It may be noted that the lines in Figure 1 do not extrapolate to the same origin; the reason is that each run has a different fluoride concentration and proceeds to a different endpoint A_e. Each of the eight runs yields a slope that equals the quantity $(k_f[F] + k_b)$ from eq 2. A summary of the kinetic data is given in Table 2.

The resulting eight slopes are now plotted against fluoride concentration, as shown in the inset of Figure 1. As expected, this plot is again linear, its slope and intercept yielding, respectively, the forward and back rate constants: k_f = 0.689 $M^{-1}s^{-1}$; k_b = 0.0250 s^{-1}. Hence, under the given experimental conditions, the reaction in eq 1 has an equilibrium constant $K_{eq} = k_f/k_b$ = 27.6 M^{-1}.

Treatment of Equilibrium Data

Table 3 gives the corresponding equilibrium data for all eight runs of the above experiment. In each case A_0 is the absorbance of the solution at the moment of mixing (obtained by extrapolation of the linear plots in Fig. 2), A_e the observed absorbance at the end of the reaction (equilibrium), and A_{100} the corresponding absorbance of the fully formed ferrimyoglobin–fluoride complex obtained from the relation: $A_{100} = 2.40A_0$ (a ratio deduced from the absorption spectra). The data in Table 3 show that the equilibrium points reached in this experiment cover a range of formation of product from 16% to 60%. The equilibrium constant may now be calculated directly from these data, using eq 5, and yielding K_{eq} values (Table 3, last column) with an average of 24.5 ± 1.5. Lastly, an overall summary of the results is given in Table 4, showing fairly good agreement between the directly measured and the kinetically derived value of the equilibrium constant for the reaction of ferrimyoglobin with fluoride under the conditions of the experiment. The student who carries out these calculations now gets a sense of achievement and, it is hoped, a closer understanding of the relation between kinetics and thermodynamics.

Table 3. Determination of Equilibrium Constant from Equilibrium Data[a]

Run	[F] mol/L	A_0	A_e	A_{100}	Fe–F %	K_{eq} (eq 5)
1	0.0080	0.210	0.263	0.504	18.0	27.4
2	0.016	0.210	0.290	0.504	27.2	23.4
3	0.024	0.212	0.322	0.509	37.0	24.5
4	0.032	0.215	0.341	0.516	41.9	22.5
5	0.040	0.212	0.361	0.509	50.2	25.2
6	0.048	0.214	0.376	0.514	54.0	24.5
7	0.064	0.214	0.394	0.514	60.0	23.4
8	0.072	0.216	0.410	0.518	64.2	24.9

[a] A_0 is obtained from the regression line; A_e is the observed equilibrium value; A_{100} = 2.40 × A_0 (from spectral data). Average K_{eq} = 24.5 (standard deviation = 1.5).

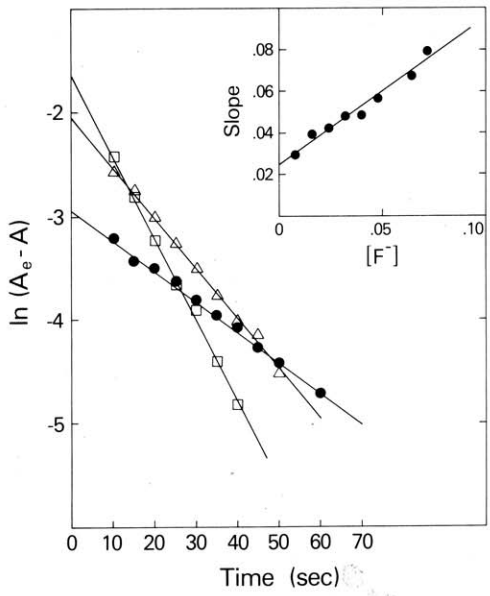

Figure 2. Analytical plots of experimental data from the three runs in Table 1, in which the quantity $\ln(A_0 - A)$ is plotted against time (eq 3 in text). The slopes of these lines are given in Table 2. The inset shows the slopes for all eight runs (also given in Table 2) plotted against fluoride concentration. The best fit line for these points gives a slope and intercept from which the kinetic data in Table 4 are obtained.

Table 4. Comparison of Kinetic and Equilibrium Results (Data from Tables 2 and 3)

Kinetic	k_f = 0.689 $M^{-1}s^{-1}$; k_b = 0.0250 s^{-1} K_{eq} (eq 4) = k_f/k_b = 27.6 M^{-1}
Equilibrium	K_{eq} (eq 5) = 24.5 (+1.5) M^{-1}

Other Considerations

This experiment has been described for an optimized set of conditions of temperature, pH, ionic strength, and protein concentration, which together make the reaction rate convenient to measure by ordinary techniques. Rates and equilibrium constants of reactions can of course be varied by altering experimental conditions. Indeed, the mechanism of the reaction of fluoride with ferrimyoglobin has been extensive-

ly studied and is known to be H^+ catalyzed and to be influenced by heme-linked protonation equilibria on the protein molecule.[2,3] The reaction is known to go much faster at low pH. One can demonstrate the pH effect by having groups of students perform the experiment at different pH values (8.2, 8.0, and 7.8), at constant high ionic strength, I = 0.4 M. However, as eq 6 implies, pH also will have a marked effect on the absorption spectrum of ferrimyoglobin.[2]

Consequently, the ratio of absorbances, A_{100}/A_0 will depend on pH. (It is approximately 2.5 at pH 7.8, 2.4 at pH 8.0, 2.3 at pH 8.2). This variability must be taken into account in making the equilibrium calculations of Table 3.

The reaction of fluoride with ferrimyoglobin becomes faster at lower ionic strength, as would be expected for ionic reactions involving oppositely charged species. The ionic strength effect can be demonstrated by having groups of students perform the experiment at I = 0.4, 0.2, and 0.1 M, maintaining constant pH 8.2. In addition, temperature is a parameter that needs to be considered. At pH 8, the forward rate constant has an activation energy of about 12 kcal/mol, and $\Delta H°$ for the overall equilibrium is −2.5 kcal/mol. These quantities are moderate and kinetic variations which result from temperature fluctuations may be assumed to fall within the limits of experimental error.

Finally, it must be recognized that, as with other proteins, the myoglobin of various animal species may show differences in composition and structure, and hence in reactivity. Significant differences may be observed in experimental data obtained from the reaction of fluoride with, say, myoglobin of horse heart muscle and myoglobin of sperm whale skeletal muscle. This fact is itself of some interest because it underscores the relation between structure and reactivity in protein chemistry.

[3] George, P.; Hanania, G. I. H. *Nature* **1954,** *174,* 33; *Far. Soc. Disc.* **1955,** *20,* 216.

A Useful Method for Obtaining Crystals from Viscous Oils

Every organic chemist is well aware of the significance of isolation of crystalline reaction products. It is very difficult to obtain pure compounds from viscous mixtures. Crystals are readily purified by recrystallization[1], and purity of the products is monitored by melting point constancy or spectral properties such as NMR and/or IR.

Simple and reduced pressure distillations are usually the first choice for liquid products, whereas crystallization is usually the first choice for solid products. Trituration works occasionally with viscous oils to separate solids from reaction residues. Chromatography is a universal separation technique in almost all cases. A drawback is that it generally takes a longer time than other simple techniques, so that some investigators usually try everything else and leave chromatography as a last choice.

In most organic chemistry lab textbooks it is stated that when doing a crystallization, flasks should be capped while waiting for crystals to form[2]. This process might take a long time. Extensive details about crystallization have been discussed in specialized books[3]. However, in some cases the crude materials are soluble in many common solvents. It is hard to find a good one for crystallization. Mixed-solvent systems are often be used in these circumstances. A similar method called liquid diffusion crystallization has been successfully applied to growing crystals for X-ray crystallography studies. This method might be useful for handling organic reaction mixtures[4]. Another method involves dissolving solids in small amounts of volatile solvents in different flasks or small test tubes that are left uncapped[5]. The solvents are allowed to evaporate gradually. If evaporation is slow enough, crystals might be formed. Common solvents can be used, such as methanol, ethanol, diethyl ether, light petroleum ether, etc.

In this short communication I report a serendipitously discovered easy and convenient method for isolation of solid products from oily reaction mixtures. The above-mentioned method of slowly evaporating solvents was tried in the present study with oily reaction residues, which did not crystallize on standing for some time. It worked for the first three different experiments tried. After completion of the reactions and workup followed by removal of the reaction solvents, the oily residues were divided into several small Erlenmeyer flasks. The residues in each flask were mixed in about 1:1 proportions with some common volatile solvents. The flasks were left open. After standing crystals were found in some flasks. Once crystals are obtained, they can be used for "seeding" other reaction mixtures. The types of the compounds studied so far were an alcohol and two ketones.

The reason why this method works is not yet fully understood. It is known that for crystallization, conditions have to be created to induce formation of crystal nuclei. In oily reaction residues molecular tumbling is decreased and therefore not favorable for formation of nuclei. In solution the viscosity is reduced and molecular motion is increased. If the concentration reaches a critical point, a "nucleus" might form and crystal formation could take place followed by precipitation of crystals gradually. Solvation is another factor to be considered here. Residual organic solvents are very difficult to remove completely even under reduced pressure. Addition of a second solvent might compete with the reaction solvent in the solvent–solute interaction. If the product has a different solubility in the new solvent, it is more likely to come out as a solid or a precipitate.

The method proposed seems to work very well in the isolation of solid products from oily reaction mixtures. It is relatively fast and convenient especially for handling products on a large scale. It offers advantages to beginners in organic chemistry research, because isolation of crystals could be frustrating.

[1] Vogel, Arthur. *Textbook of Practical Organic Chemistry,* 4th ed.; Longman: London, 1978; Chapter 1, Section 20, p 105.
[2] Gilman, J. J., Ed. *The Art and Science of Growing Crystals*; Wiley: New York, 1963; Chapters 8 and 11.
[3] Mullin, J. W. *Crystallization*; Butterworth: London, 1961.
[4] Orvig, C. *J. Chem. Educ.* **1985,** *62,* 84.
[5] Sulb, S. L. *J. Chem. Educ.* **1985,** *62,* 81.

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