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The Determination of Enzyme-Substrate Dissociation Rates by Dynamic Isotope Exchange Enhancement Experiments*

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Sung Chun Kim and Frank M. Raushel‡
From the Departments of Chemistry and Biochemistry, Texas A & M University, College Station, Texas 77843

A new method for the determination of dissociation rates of enzyme-substrate complexes has been developed. The rate of exchange of a labeled product back into the substrate is measured during catalysis of the forward reaction when the forward reaction is kept far from equilibrium by the enzymatic removal of the nonexchanging product. The ratio of the exchange rate and the net rate for product formation is then determined at various concentrations of the exchanging product. A plot of this ratio is a diagnostic indication of the kinetic mechanism and the relative rates of product dissociation from the binary and ternary enzyme complexes. This technique has been applied to the reaction catalyzed by bovine liver argininosuccinate lyase. The ratio for the rate of exchange of fumarate into argininosuccinate and the net rate for product formation was found to increase with the concentration of fumarate but to reach a limit of 3.3. The ratio of rates was half-maximal at 36 mM fumarate. The data have been interpreted to indicate the argininosuccinate lyase has a random kinetic mechanism. The calculated lower limit for the rate of release of arginine from the enzyme-fumarate-arginine complex is 0.35 times as fast as the V_{max} in the reverse direction. The rate of release of arginine from the enzyme-arginine binary complex is 210 times faster than V_{max} in the reverse direction.

Only a few methods have been developed for the determination of the relative rates of ligand dissociation in enzyme-catalyzed reactions. The isotope partitioning technique, for example, was shown by Rose *et al.* (1) to be able to determine the relative rates of release of glucose from the binary and ternary complexes of yeast hexokinase. The isotope partitioning technique has subsequently been applied to reactions catalyzed by fructokinase (2), glutamine synthetase (3), carbamyl-phosphate synthetase (4), malic enzyme (5), and creatine kinase (6) to mention a few. More recently, our laboratory has modified the positional isotope exchange technique of Midelfort and Rose (7) to obtain information about the relative rates of release of arginine and fumarate in the reaction catalyzed by bovine liver argininosuccinate lyase (8).

This paper describes the development of a new technique which is able to determine kinetic mechanisms and rates of ligand dissociation in a single experiment. The present study measures the rate of isotope exchange of a product back into substrate during net catalysis of the forward reaction. The ratio of this exchange rate relative to the rate of net product formation is determined at increasing concentrations of the exchanging product. The results are a diagnostic indication of the relative rates of product release from the binary and ternary enzyme-ligand complexes. This method of dynamic isotope exchange enhancement appears to be more widely applicable than any of the previously developed techniques.

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‡ Recipient of National Institutes of Health Research Career Development Award AM-01366. To whom correspondence should be addressed.

MATERIALS AND METHODS

Argininosuccinate lyase was isolated from bovine liver according to the procedure of Havir *et al.* (9) and Schulze *et al.* (10). Fumarate-2,3-d_2 (98 atom %) was purchased from Merck. All other reagents were acquired from either Sigma or Aldrich.

Preparation of (2S,3S)-Argininosuccinate-2,3-d_2 (II)—(2*S*,3*S*)-Argininosuccinate-2,3-d_2 was made enzymatically by the reverse reaction of argininosuccinate lyase (11). The reaction mixture contained 100 mM phosphate buffer, pH 7.5, 150 mM arginine, 100 mM fumarate-2,3-d_2, and 1.75 units of argininosuccinate lyase in a volume of 50 ml. The course of the reaction was followed by the change in absorbance at 240 nm. The reaction was terminated after 24 h of incubation by the addition of 10 ml of 50% trichloroacetic acid. The labeled argininosuccinate was isolated as the barium salt as described by Ratner *et al.* (11). The overall yield was 62%.

Dynamic Isotope Exchange Enhancement—Argininosuccinate lyase was incubated with 50 mM phosphate buffer, pH 7.5, 10 mM (2*S*,3*S*)-argininosuccinate-2,3-d_2, 420 units of bovine liver arginase, and various amounts of fumarate in a volume of 10 ml. The progress of the reaction was followed by monitoring the increase in the concentration of fumarate at 290 or 300 nm. After the chemical reaction had proceeded to about 40% completion, the reaction was terminated by adding 1.0 ml of trichloroacetic acid. After centrifugation the pH of the solution was raised to pH 7.5 with KOH. The sample was then dried by rotary evaporation. The dried sample was dissolved in 2 ml of D_2O and dried again. This procedure was repeated. The final sample was dissolved in D_2O and analyzed by proton NMR spectroscopy.

Proton Nuclear Magnetic Resonance Measurements—Proton NMR spectra were obtained on a Varian XL-200 spectrometer operating at a frequency of 200 MHz. Typical acquisition parameters were 2600-Hz sweep width, 3.0-s acquisition time, and a 5-μs pulse width.

Enzyme Assays—Enzyme assays and absorbance measurements were made with a Gilford 2600 UV-VIS spectrophotometer and a Hewlett-Packard 7225A plotter. A unit of argininosuccinate lyase is defined as the amount of enzyme needed to catalyze the formation of 1 μmol of fumarate at 25 °C and pH 7.5 at saturating argininosuccinate (12).

Data Analysis—The rate constant for the exchange of labeled product into the substrate was determined from the following equation

$$r(\mathrm{P} \rightarrow \mathrm{A}) = -[\mathrm{P}][\mathrm{A}] \ln(1 - \mathrm{F})/[\mathrm{A} + \mathrm{P}]t \quad (1)$$

where P and A are the average concentrations of the product and substrate undergoing exchange, and F is the fraction of the equilibrium value for the exchange reaction at time *t*.

THEORY

A general mechanism for an enzyme-catalyzed reaction in which one substrate is converted into two products is illustrated in Scheme 1. The partitioning of the ternary enzyme product complex (EPQ) can be determined from the rate in which a labeled product is converted into substrate during the catalysis of the forward reaction by the enzyme. The determination of this exchange rate relative to the net rate for product formation as a function of the initial product concentration is diagnostic for the type of kinetic mechanism and the dissociation rates of products from the ternary and binary complexes. The net chemical reaction is kept far from equilibrium by the continued enzymatic removal of the product that is not being varied. The ratio of the exchange rate relative to net substrate turnover in terms of the rate constants presented in the above model can be easily derived using the theory of net rate constants of Cleland (13).

Ordered Release of P before Q ($k_5 = 0$)—The rate that labeled P* is converted into substrate A during catalysis of the forward reaction is given as

$$r(P^* \rightarrow A) = k_2k_4k_{10}[P^*][EQ]/(k_2k_4 + k_2k_9 + k_3k_9) \quad (2)$$

while the rate of the net forward reaction is given as

$$r(A \rightarrow Q) = k_{11}[EQ] \quad (3)$$

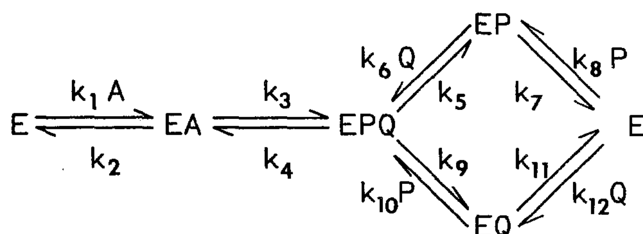
Therefore, the ratio of the exchange rate relative to the net rate for product formation is as follows.

$$(r(P^* \rightarrow A))/(r(A \rightarrow Q)) = k_2k_4k_{10}[P^*]/(k_2k_4 + k_2k_9 + k_3k_9)(k_{11}) \quad (4)$$

A plot of this ratio as a function of the concentration of added P* is illustrated in Fig. 1. The slope of this line is equivalent to $(V_2/E_t)/(K_Pk_{11})$ where $V_2 = V_{max}$ in the reverse reaction, E_t = total enzyme concentration and K_P = Michaelis constant for P. Therefore, the exact ratio of the rate constant for the release of the product Q relative to V_2/E_t can be determined from Equation 5.

$$k_{11}/(V_2/E_t) = 1/(K_P)(\text{slope}) \quad (5)$$

Ordered Release of Q before P ($k_9 = 0$)—If the product Q is required to be released before P then there can be no exchange of labeled P* back into substrate A when Q is removed enzymatically from solution. The exchange rate relative to the net rate for substrate turnover is therefore always zero no matter what the concentration of added P* as illustrated in Fig. 1.



SCHEME 1

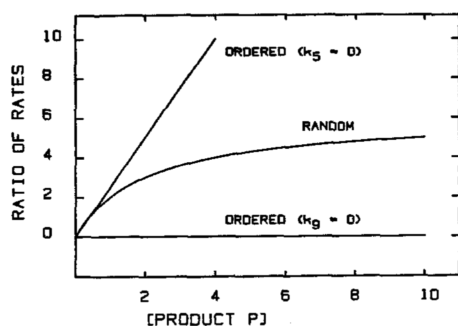


FIG. 1. **A plot of the ratio of the product exchange rate and the rate of net substrate turnover as a function of the concentration of added product inhibitor.** Values are plotted according to Equation 8.

Random Release of P and Q—In a random mechanism the rate from the exchange of labeled P* into A is given by

$$r(P^* \rightarrow A) = [EQ](k'_4k_{10}[P^*])/(k'_4 + k_5 + k_9) \quad (6)$$

where $k'_4 = (k_2k_4)/(k_2 + k_3)$. The rate of the net forward reaction can be expressed as follows.

$$r(A \rightarrow Q) = k_5[EPQ] + k_{11}[EQ] \quad (7)$$

The ratio of the complexes EPQ and EQ is shown in Equation 8.

$$[EQ]/[EPQ] = k_9/(k_{10}[P^*] + k_{11}) \quad (8)$$

Rearrangement of Equation 8 and substitution into Equation 7 gives the rate of the forward reaction.

$$r(A \rightarrow Q) = [EQ](k_5k_{11} + k_5k_{10}[P^*] + k_9k_{11})/k_9 \quad (9)$$

Therefore, the ratio of the exchange rate of P* relative to net product formation is as follows.

$$\frac{r(P^* \rightarrow A)}{r(A \rightarrow Q)} = \frac{k'_4k_9k_{10}[P^*]}{(k'_4 + k_9 + k_5)(k_5k_{11} + k_5k_{10}[P^*] + k_9k_{11})} \quad (10)$$

This ratio increases hyperbolically as a function of P* as illustrated in Fig. 1. A plot of $[r(A \rightarrow Q)]/[r(P^* \rightarrow A)]$ *versus* 1/P* is a straight line. The reciprocal of the intercept at saturating P* is as follows.

$$R(P^* \rightarrow A) = (k'_4k_9)/(k'_4 + k_9 + k_5)(k_5) \quad (11)$$

The slope of this double reciprocal plot is as follows.

$$\frac{K_{P^*}}{R(P^* \rightarrow A)} = \left(\frac{k'_4 + k_9 + k_5}{k'_4k_{10}}\right)\left(\frac{k_5 + k_9}{k_9}\right)(k_{11}) \quad (12)$$

Equation 11 can be rearranged to provide a ratio of k_5 relative to k'_4 as shown below

$$\frac{k_5}{k'_4} = \left(\frac{1}{R(P^* \rightarrow A)} - \frac{k_5}{k_9}\right)\Big/\left(1 + \frac{k_5}{k_9}\right) \quad (13)$$

Since $V_2/E_t = k_2k'_4/(k_2 + k'_4)$ the ratio of k_5 relative to V_2 can be determined by solving for k'_4 and by substitution into Equation 13.

$$\frac{k_5}{(V_2/E_t)} = \left(\frac{1}{R(P^* \rightarrow A)} - \frac{k_5}{k_9}\right)\Big/\left(1 + \frac{k_5}{k_9}\right) + \frac{k_5}{k_2} \quad (14)$$

An expression analogous to Equation 11 can be derived for an experiment in which the exchange of product Q* into substrate A is measured.

$$R(Q^* \rightarrow A) = (k'_4k_5)/(k'_4 + k_9 + k_5)(k_9) \quad (15)$$

The ratio of the maximal exchange rates for the products Q and P thus determines the ratio of k_5 and k_9 as shown below.

$$\left(\frac{R(Q^* \rightarrow A)}{R(P^* \rightarrow A)}\right)^{1/2} = k_5/k_9 \quad (16)$$

The upper limit for the ratio of k_{11} relative to V_2 is given as follows.

$$\frac{k_{11}}{(V_2/E_t)} \leq \frac{K_{P^*}}{(R(P^* \rightarrow A))(K_P)} \quad (17)$$

The above expression can be obtained from Equation 12 and

the value for $K_P/(V_2/E_t)$ is shown below.

$$K_P/(V_2/E_t) = (k'_4 + k_9)/(k_{10}k'_4) \quad (18)$$

The technique of measuring the rate of exchange of labeled product into substrate during the catalysis of the net forward reaction provides both qualitative and quantitative information about the rates of dissociation of products from the binary and ternary enzyme complexes. As illustrated in Fig. 1, a plot of the ratio of the exchange rate relative to net substrate turnover *versus* the concentration of the exchanging product provides a clear distinction between an ordered and random mechanism. Moreover, a numerical analysis of these same plots enables the microscopic rate constants for product dissociation to be obtained in a simple and straightforward manner.

RESULTS AND DISCUSSION

Measurement of Dynamic Isotope Exchange—The rate of exchange of fumarate into argininosuccinate during catalysis of the forward reaction of argininosuccinate lyase was measured by NMR. The reaction was initiated by the addition of enzyme to a solution of (2*S*,3*S*)-argininosuccinate-2,3-d_2 and various amounts of fumarate in the presence of excess arginase. The arginase was added to ensure that the release of arginine from either the binary or ternary enzyme forms was essentially irreversible through the rapid conversion to ornithine and urea. The extent of the net chemical conversion of argininosuccinate into products was determined by monitoring the fate of the protons attached to the carbon adjacent to the guanidino moiety of argininosuccinate (H_d). In Fig. 2*A* the NMR spectrum of a 1:1 mixture of argininosuccinate and ornithine indicated that these protons resonate at 3.12 ppm in argininosuccinate but shift to 2.89 ppm upon conversion to ornithine. Integration of these two resonances thus provides a measure of the extent of conversion of argininosuccinate into products. The rate of fumarate exchange into argininosuccinate was monitored by following the incorporation of protons at positions H_a and H_b of argininosuccinate. As indicated in Fig. 2*B* the argininosuccinate-2,3-d_2 lacks proton signals at 4.08 and 2.38 ppm while H_c appears as a singlet. Therefore, the incorporation of hydrogen into the argininosuccinate pool will result in an increase in the signals at 2.38 and 4.08 ppm in addition to the splitting of H_c at 2.65 ppm.

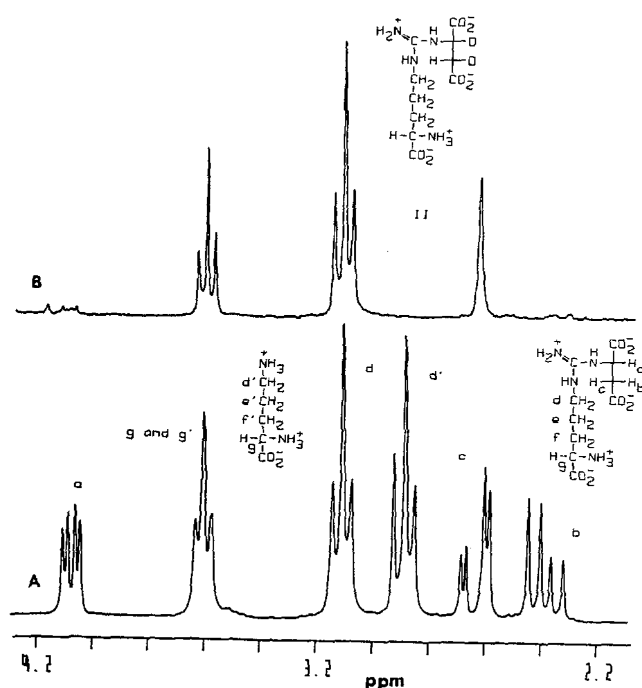


FIG. 2. **Proton NMR spectra of argininosuccinate and ornithine.** *A*, proton NMR spectrum of a 1:1 mixture of argininosuccinate and ornithine. *B*, proton NMR spectrum of (2*S*,3*S*)-argininosuccinate-2,3-d_2.

Argininosuccinase Lyase—Shown in Fig. 3 are the results when the ratio of the exchange and net chemical reaction are plotted as a function of the initial fumarate concentration. Since the ratio of these reaction rates increases with the concentration of fumarate but plateaus at some finite value, the kinetic mechanism for the reaction catalyzed by argininosuccinate lyase must be random. For an ordered kinetic mechanism a linear dependence on the ratio of the reaction rates would have been obtained as a function of the fumarate concentration. This result is consistent with previous conclusions obtained from steady-state (12) and positional isotope exchange experiments (8). The numerical data are tabulated in Table I.

The data in Fig. 3 were fit to the Michaelis-Menten equation using the HYPERO program of Cleland (14) to obtain the maximal ratio of exchange at saturating fumarate, $R(P^* \rightarrow A)$, and the concentration of fumarate that gives the half-maximal ratio of rates. At saturating fumarate, the maximal ratio of rates is 3.3 ± 0.7 while the value for K_{P^*} is 36 ± 13 mM. From Equation 14 it is now possible to obtain a lower limit for the release of arginine from the enzyme-arginine-fumarate complex relative to V_2/E_t. In order to obtain this ratio of rates, it is required that the ratio of k_5 and k_9 be known. The value for k_9/k_5 could be obtained from an analysis of the rate of exchange of labeled arginine back into argininosuccinate as indicated in Equation 16. However, this ratio has previously been obtained from an analysis of the posi-

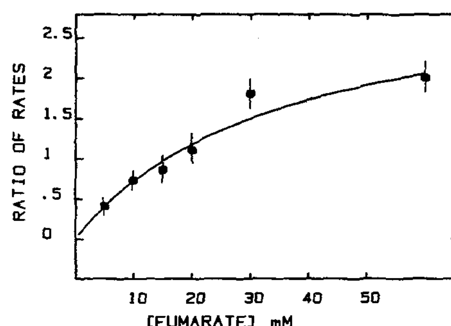


FIG. 3. **Plot of the ratio of the rate of exchange of fumarate into argininosuccinate and the rate of net turnover of argininosuccinate into product as a function of the initial fumarate concentration.** Additional details are given in the text and Table I.

TABLE I

Dynamic isotope exchange reaction catalyzed by argininosuccinate lyase

Fumarate	X[a]	F[b]	$r(P^* \rightarrow A)/r(A \rightarrow Q)$
mM			
5	0.31	0.26	0.40
10	0.24	0.30	0.72
15	0.46	0.47	0.85
20	0.54	0.59	1.1
30	0.44	0.70	1.8
60	0.17	0.35	2.0

[a] Fraction of change of the original argininosuccinate pool at time of quench.

[b] Fraction of equilibrium value for the exchange reaction at time of quench.

tional isotope exchange data (8). The previously determined value for k_9/k_5 is greater than 10. Therefore, the lower limit for the rate of release of arginine from the enzyme-arginine-fumarate complex can be calculated as 0.3 from Equation 14.

The rate constant for the release of arginine from the enzyme-arginine complex can be calculated from Equation 17. These results give a value for $k_{11}/(V_2/E_t)$ of 180 when a Michaelis constant of 0.06 mM for fumarate is used (12). An exact value for $k_{11}/(V_2/E_t)$ can be calculated because k_5 can be eliminated from Equation 12. The results from the dynamic isotope exchange enhancement experiments thus indicate that the release of arginine from the ternary complex is relatively slow while the rate constant for the dissociation of arginine from the binary complex is quite large. These results compare quite favorably with the positional isotope exchange experiments reported earlier (8). In those experiments the lower limit for $k_5/(V_2/E_t)$ was determined to be 0.5. If the experiments reported here are corrected for the small isotope effect on V_{max} due to the dideuteration of argininosuccinate[1] then the relative values are increased to 0.35 and 210 for k_5 and k_{11}, respectively. The agreement is good.

The method of dynamic isotope exchange enhancement reported in this paper is one of only a few techniques presently available that permits the determination of the kinetic mechanism and the limits for the microscopic rate constants for product release. Not only can the order of the release of products be determined unambiguously but the flux through either pathway can be obtained in favorable cases in random mechanisms. This technique should be applicable to any enzyme-catalyzed reaction which is reversible. The only other major requirement is for an analytical method for the continuous removal of the product not undergoing exchange with the substrate. Although the equations in this paper were derived for a Uni Bi reaction, the derivations can be easily expanded to include mechanisms with additional substrates and products. In reactions with more than one substrate and product the DIXE experiment can be completed in both directions to obtain information about the dissociation of all products and substrates from the binary and ternary enzyme complexes.

The information that can be obtained from this type of dynamic isotope exchange experiment is identical to the information that can be obtained from either the isotope partitioning technique of Rose *et al.* (1) or the positional isotope exchange enhancement technique of Raushel and Garrard (8). The major disadvantage of the isotope partitioning experiment is the need for an accurate determination of the enzyme active site concentration and the dissociation constants for the enzyme-ligand complexes. This information is not always easily obtained. However, the isotope partitioning technique can be utilized with reactions that are essentially irreversible whereas the technique developed in this paper requires a reversible reaction. The dynamic isotope exchange enhancement experiment should therefore complement these other techniques for the determination of enzyme-ligand dissociation rates.

The technique of measuring the rate of product exchange into the substrate while the chemical reaction is proceeding was originally developed by Hass and Byrne (15) and has since been applied to a limited number of enzyme-catalyzed reactions (16–18). Hass and Byrne were able to show in the reaction catalyzed by glucose-6-phosphatase that glucose could exchange back into the glucose-6-phosphate but phosphate could not. These results are consistent with the ordered release of glucose before phosphate. However, since the exchange ratio was determined at only a single level of glucose or phosphate the possibility of a partially random mechanism cannot be rigorously ruled out. The lack of exchange of phosphate back into glucose-6-phosphate could be explained by a rapid release of glucose from either the *E*-glucose-phosphate or *E*-glucose complex. The extension and enhancement of this technique as outlined in this paper permits an unambiguous determination of the kinetic mechanism and puts limits on the microscopic rate constants for product release.

[1] The isotope effect on V_{max} when (2*S*,3*S*)-argininosuccinate-2,3-d_2 is used as a substrate has been determined to be 1.15 (S. C. Kim, unpublished observations).

REFERENCES

1. Rose, I. A., O'Connell, E. L., Litwin, S., and Bar Tana, J. (1974) *J. Biol. Chem.* **249,** 5163–5168
2. Raushel, F. M., and Cleland, W. W. (1977) *Biochemistry* **16,** 2176–2181
3. Meek, T. D., Johnson, K. A., and Villafranca, J. J. (1982) *Biochemistry* **21,** 2158–2167
4. Raushel, F. M., and Villafranca, J. J. (1979) *Biochemistry* **18,** 3424–3429
5. Landsperger, W. J., Fodge, D. W., and Harris, G. B. (1978) *J. Biol. Chem.* **253,** 1868–1873
6. Cook, P. F., Kenyon, G. L., and Cleland, W. W. (1981) *Biochemistry* **20,** 1204–1210
7. Midelfort, C. F., and Rose, I. A. (1976) *J. Biol. Chem.* **251,** 5881–5887
8. Raushel, F. M., and Garrard, L. J. (1984) *Biochemistry* **23,** 1791–1795
9. Havir, E. A., Tamir, H., Ratner, S., and Warner, R. C. (1965) *J. Biol. Chem.* **240,** 3079–3088
10. Schulze, I. T., Lusty, C. J., and Ratner, S. (1970) *J. Biol. Chem.* **245,** 4534–4543
11. Ratner, S., Petrack, B., and Rochovansky, O. (1953) *J. Biol. Chem.* **254,** 1859–1868
12. Raushel, F. M., and Nygaard, R. (1983) *Arch. Biochem. Biophys.* **221,** 143–147
13. Cleland, W. W. (1975) *Biochemistry* **14,** 3220–3224
14. Cleland, W. W. (1970) *Methods Enzymol.* **63,** 84–103
15. Hass, L. F., and Byrne, W. L. (1969) *J. Am. Chem. Soc.* **82,** 947–954
16. Cleland, W. W. (1970) in *The Enzymes* (Boyer, P. D., ed) 3rd Ed., Vol. II, pp. 1–65, Academic Press, New York
17. Viola, R. E., Raushel, F. M., Rendina, A. R., and Cleland, W. W. (1982) *Biochemistry* **21,** 1295–1302
18. Kosow, D. P., and Rose, I. A. (1970) *J. Biol. Chem.* **245,** 198–204