Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 4784-4787, October 1978
Biochemistry

Phosphate (oxygen)-water exchange reaction catalyzed by human prostatic acid phosphatase

(^{18}O exchange kinetics/^{31}P nuclear magnetic resonance/gas chromatography-mass spectral analysis)

ROBERT L. VAN ETTEN AND JOHN M. RISLEY

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

Communicated by Myron L. Bender, July 21, 1978

ABSTRACT Conclusive evidence is presented that an acid phosphatase catalyzes phosphate (oxygen)-water exchange. Studies conducted with human prostatic acid phosphatase by two independent methods have established that, despite earlier reports to the contrary, the enzyme catalyzes an exchange reaction between oxygen atoms of phosphate ion and of water. Kinetic data were obtained both by chemical conversion to trimethyl phosphate followed by mass spectroscopy and by a totally independent method involving ^{31}P isotope shift nuclear magnetic resonance spectroscopy. Analysis showed that the enzyme catalyzes the exchange in a random, noncoupled process. If any coupled exchange occurs, it must represent <10% of the total. By mass spectral analysis, catalytic rate constants k_{cat} = 0.14 sec^{-1} (4°) and 1.8 sec^{-1} (37.5°) were obtained. By ^{31}P nuclear magnetic resonance k_{cat} = 1.6 sec^{-1} (31°) was obtained. The energy of activation for the exchange reaction is approximately 13 kcal mol^{-1}. The k_{cat} value for exchange is about 10-fold greater than that observed with *Escherichia coli* alkaline phosphatase

Apparently the only report of a phosphate (oxygen)-water exchange reaction catalyzed by an acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] is that for potato acid phosphatase, and even this seems inconclusive because only one of the two reported experiments is consistent with such an exchange (1). In contrast, it has widely been reported that acid phosphatases (2, 3) and enzymes displaying acid phosphatase-like characteristics (4) do not catalyze an exchange reaction between the oxygen atoms of phosphate and those of water. However, a number of lines of evidence have appeared which lead one to question the validity of this observed nonexchange: transphosphorylation reactions in the presence of alcohols (5, 6) and, particularly, the stoichiometric trapping of a phosphohistidine intermediate after incubation with substrate (7, 8) strongly imply that medium phosphate (oxygen)-water exchange should occur when such enzymes are incubated with inorganic phosphate. The earlier studies (2, 3), which suggested that human prostatic acid phosphatase does not catalyze such an exchange reaction, were done with low or unknown concentrations of impure enzyme at low isotopic enrichments, so it seemed worthwhile to reexamine this system in an effort to resolve the apparent conflict between the early reports and the predictions that result from studies such as our mechanistic investigations. In this communication we describe the kinetics of the phosphate (oxygen)-water exchange catalyzed by human prostatic acid phosphatase.

Two general methods are presently available for following the kinetics of phosphate (oxygen)-water exchange reactions. The first of these involves a chemical conversion of isolated phosphate ion to some volatile derivative, followed by mass spectral analysis. Chemical conversion methods have been used by many authors (2, 9-13), and until recently these were the only methods available. A chemical conversion method frequently requires considerable time and is at best a discontinuous method, making kinetic studies difficult. The recent report of the use of ^{31}P NMR to follow phosphate-phosphate and phosphate (oxygen)-water exchange reactions (14) provides a second approach. Because covalent isotopic substitution of certain nuclei leads to readily detectable shifts in their NMR resonance positions (15, 16), multiple peaks can be observed when, for example, a sample of phosphate ion contains $P^{18}O_4H_2^-$, $P^{16}O^{18}O_3H_2^-$, $P^{16}O_2{}^{18}O_2H_2^-$, $P^{16}O_3{}^{18}OH_2^-$, and/or $P^{16}O_4H_2^-$. Cohn and Hu (14) first clearly described the importance of this fact with respect to studies of phosphate-phosphate and phosphate (oxygen)-water exchange reactions. Very recently, Bock and Cohn (17) have used ^{31}P isotope shift NMR to study exchange catalyzed by *Escherichia coli* alkaline phosphatase, and Trentham and his colleagues (18) have used it to study oxygen exchange catalyzed by myosin. For many systems this approach, which makes possible the nearly continuous kinetic study of the exchange reactions, may well supercede the chemical conversion method.

We have examined the phosphate (oxygen)-water exchange reaction as catalyzed by human prostatic acid phosphatase by both chemical conversion and ^{31}P NMR techniques. Of the chemical conversion methods available, CO_2 equilibration (2), CO_2 synthesis (12), CO synthesis (13), conversion to trimethylsilyl phosphate (9), and conversion to trimethyl phosphate (10, 11), the last was chosen for ease of preparation, accuracy, and the convenience of avoiding natural abundance correction factors necessary when silicon derivatives are used. Data analysis was accomplished by an extension of the treatment given by Eargle *et al.* (9).

MATERIALS AND METHODS

Potassium [^{18}O]phosphate monobasic was synthesized by reaction of PCl_5 with $H_2{}^{18}O$ and isolated as the crystalline potassium salt (19) to yield a product containing 93.7% ^{18}O. Human prostatic acid phosphatase was isolated by affinity chromatography on *N*-(6-aminohexyl)-L-tartramic acid-Sepharose 4B (20). Glass-distilled water was used, and all other reagents were analytical grade.

Exchange Analysis by Mass Spectroscopy. Samples of 3.0 ml were made up to contain 50 mM $KH_2P^{18}O_4$/50 mM sodium acetate buffer (pH 5.0), and the ionic strength was adjusted to 0.15 M with NaCl. For the reaction at 4°, each sample contained 22 units of enzyme activity; at 37.5°, each sample contained 8.6 units of activity. Control samples of 3.0 ml were made up exactly as above; one control sample contained no enzyme and the other contained denatured enzyme at the same concentration. The reaction samples at 4° were incubated for up to 2 weeks and those at 37.5° were incubated for a maximum of 48 hr. It was established independently that the enzyme is stable under these conditions (the enzyme is resistant to dena-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Table 1. Mass spectral data for exchange reaction at 37.5°

Time,	Isotopic phosphate species, %									
hr	P^*_4		P^*_3		P^*_2		P^*_1		P^*_0	
0.0	82.37	—	10.71	—	6.39	—	0.41	—	0.12	—
6.0	76.22	(76.92)	14.54	(15.48)	7.43	(6.84)	1.52	(0.63)	0.28	(0.13)
14.5	68.34	(69.82)	19.67	(21.24)	8.95	(7.83)	2.88	(0.96)	0.16	(0.15)
18.0	62.78	(67.09)	19.73	(23.31)	10.62	(8.33)	5.48	(1.11)	0.10	(0.16)
24.0	64.25	(62.65)	22.79	(26.47)	9.50	(9.31)	2.92	(1.39)	0.55	(0.18)
30.0	56.92	(58.51)	26.02	(29.18)	10.14	(10.40)	4.96	(1.70)	1.96	(0.21)
38.5	56.28	(53.11)	28.99	(32.34)	11.02	(12.10)	2.84	(2.20)	0.87	(0.25)
42.0	51.67	(51.03)	29.70	(33.43)	12.04	(12.83)	4.63	(2.43)	1.96	(0.28)

P^*_4 represents $P^{18}O_4$; P^*_3 is $P^{18}O_3{}^{16}O_1$, etc. Numbers in parentheses are theoretical values calculated for random (noncoupled) exchange.

turation in the presence of these high concentrations of phosphate ion). The reaction was stopped at specific time intervals by removing one of the samples and freezing it in an acetone/Dry Ice bath. The samples were derivatized and analyzed by gas chromatography-mass spectroscopy as described (10, 19).

^{31}P NMR Analysis. Three milliliters of solution containing 58 mM $KH_2P^{18}O_4$, 50 mM acetate, 1 mM sodium EDTA, 1.5 ml of ^2H_2O (99.7 atom % excess), 108 units of enzyme activity, ionic strength 0.15 M with NaCl (pH 5.0) was prepared in a 12-mm tube. The ^{31}P spectra were recorded at 40.5 MHz on a Varian XL-100 spectrometer in the Fourier transform mode. A 45° pulse angle was used, and proton noise was decoupled through 100-Hz square wave modulation. The probe temperature was 31 ± 1°, and each spectrum was recorded with 50 pulses of 7.0 sec aquisition time. Data aquisition was done with a Nicolet 1080 computer. A Dupont 310 curve resolver set for Lorentzian curves was used to resolve the spectra.

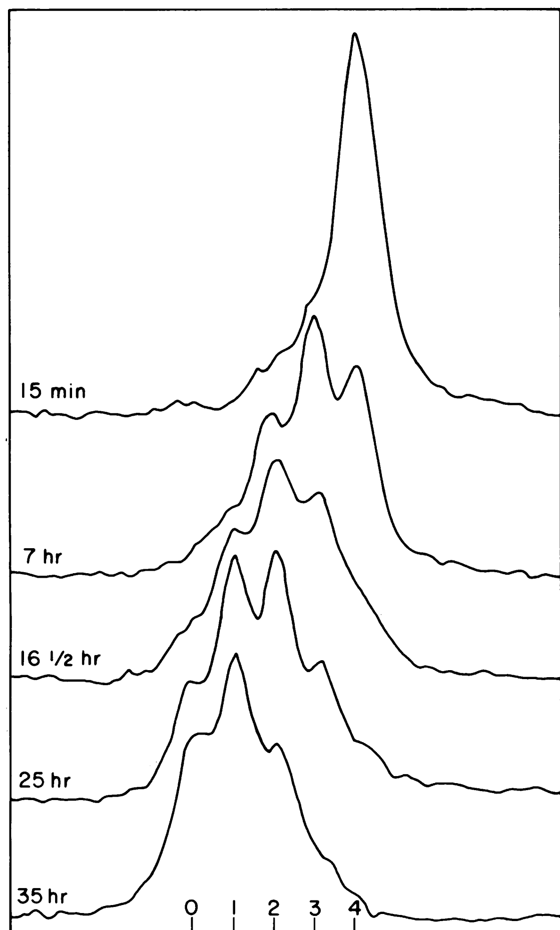

FIG. 1. Representative ^{31}P NMR spectra obtained five times during the course of the phosphate (oxygen)–water exchange reaction catalyzed by human prostatic acid phosphatase. The loss of ^{18}O from highly labeled (93.7% isotopic purity) phosphate ion was followed as a function of time. The positions of the five phosphate species are indicated at the bottom of the figure; these positions were used in resolving the spectra using a Du Pont curve resolver set for Lorentzian curves.

Data Analysis. A previously described (9) mathematical treatment of random noncoupled exchange was modified as follows. We define the total percentage ^{18}O content of a sample at any given time t as $P_T = 100\ \Sigma^4_{n=0} nP^*_n$, where P^*_n are as given by Eargle *et al.* (9). The resulting summation gives $P_T = 100R_1e^{-0.25kt}$; a plot of $\ln P_T$ against t will give a straight line whose slope is $-0.25k$ and whose y intercept is $\ln(100R_1)$. By use of the P^*_n equations, the theoretical curves may be plotted and compared with the experimental data. For purposes of comparison, including the calculation of thermodynamic parameters, the calculated rate constants k can be converted into catalytic rate constants k_{cat} by multiplication of k by the ratio of the number of moles of substrate per moles of enzyme. The specific activity of homogeneous human prostatic acid phosphatase is 290 μmol min^{-1} mg^{-1} when measured with 5 mM p-nitrophenyl phosphate in 0.1 M citrate/sodium citrate, pH 5.0, at 25° (20). The molecular weight of prostatic acid phosphatase is 102,000 (21).

RESULTS AND DISCUSSION

Phosphate (oxygen)–water exchange catalyzed by prostatic acid phosphatase is demonstrated by both the chemical conversion and ^{31}P NMR methods. Table 1 shows the data obtained for the exchange reaction at 37.5°. Solvent water–phosphate (oxygen) exchange in the absence of enzyme could be ignored because at these temperatures and pH values the reaction is so slow as to be beyond the limits of detection (22). The control reactions showed that within experimental error no phosphate (oxygen)–water exchange occurred in the absence of active enzyme. No natural abundance correction factor was applied to the data. Following the mathematical treatment already outlined, the pseudo first-order rate constants were calculated as $k = 0.0025$ hr^{-1} (4°) and $k = 0.114$ hr^{-1} (37.5°), which correspond to $k_{cat} = 0.14$ sec^{-1} (4°) and $k_{cat} = 1.8$ sec^{-1} (37.5°). The values in parentheses in Table 1 are the theoretical values expected for random exchange of each species if exchange occurred at the same rate as that observed for the overall loss of ^{18}O from phosphate ion. The values of k_{cat} were used to calculate an approximate energy of activation of 13 kcal mole^{-1} from the slope of $\ln k_{cat}$ against $1/T$.

Representative spectra taken five times in the course of the phosphate (oxygen)–water exchange reaction as followed by ^{31}P NMR are shown in Fig. 1. The five phosphate species can

be distinguished easily for purposes of curve resolution. For curve resolving, each spectrum was assigned a common reference point, and each phosphate species was assigned a position based on the ^{31}P NMR of an 80:20 mixture of labeled:unlabeled phosphate samples. Successive spectra were fitted by making the necessary changes in the amplitudes of individual peaks, and the amount of each species was calculated as a percentage of the area of the resolved spectrum using the integrator. During curve resolution the positions of the assigned phosphate species changed no more than 0.1 Hz. From the plot of ln P_T against t (29 points, slope -0.0359 hr^{-1}, y intercept 5.95, $r = 0.995$) a value of 0.144 hr^{-1} was calculated for k, and thus k_{cat} (31.0°) = 1.6 sec^{-1}. The value of k was used to calculate the theoretical exchange curves (using as a starting point the mass spectral analysis of the starting labeled phosphate sample) expected for the five isotopic species on the assumption that exchange occurs in a random, noncoupled manner. These curves plus the experimental data points are shown in Fig. 2. Very good agreement between the calculated and experimental data is found; 90% of the experimental points fall within the expected ±5% experimental error and the remainder fall within ±10% of the calculated lines.

To establish further the kinetics of the exchange, we examined a kinetic model in which 1.1 phosphate (oxygen)–water exchanges takes place per reaction (10% "coupling") and the

Table 2. Exchange study results

Temperature, °C	k_{cat}, sec^{-1}	Method of determination
4.0	0.14	Chemical conversion/mass spectrometry
37.5	1.83	Chemical conversion/mass spectrometry
31.0	1.62	^{31}P isotope shift NMR

$E_a \simeq 13.1$ kcal/mol.

kinetic equations for such a coupled exchange were derived (unpublished results). Above the 1.1 exchange level, there were significant deviations between the calculated exchange curves and the experimental data. At the 1.1 exchange level, significant deviations were observed primarily among the three less highly labeled species at long exchange times. Below the 1.1 exchange level, it seems likely that deviations beyond experimental error could not be reliably detected. Although the possibility of low levels of multiple or coupled exchange cannot be eliminated totally, it appears most likely that the phosphate (oxygen)–water exchange catalyzed by human prostatic acid phosphatase is a random noncoupled exhange.

In contrast to previously published indications, we find that human prostatic acid phosphatase does catalyze an exchange reaction between the oxygen atoms of phosphate ion and those of water. Mass spectral analysis of the exchange reaction pro-

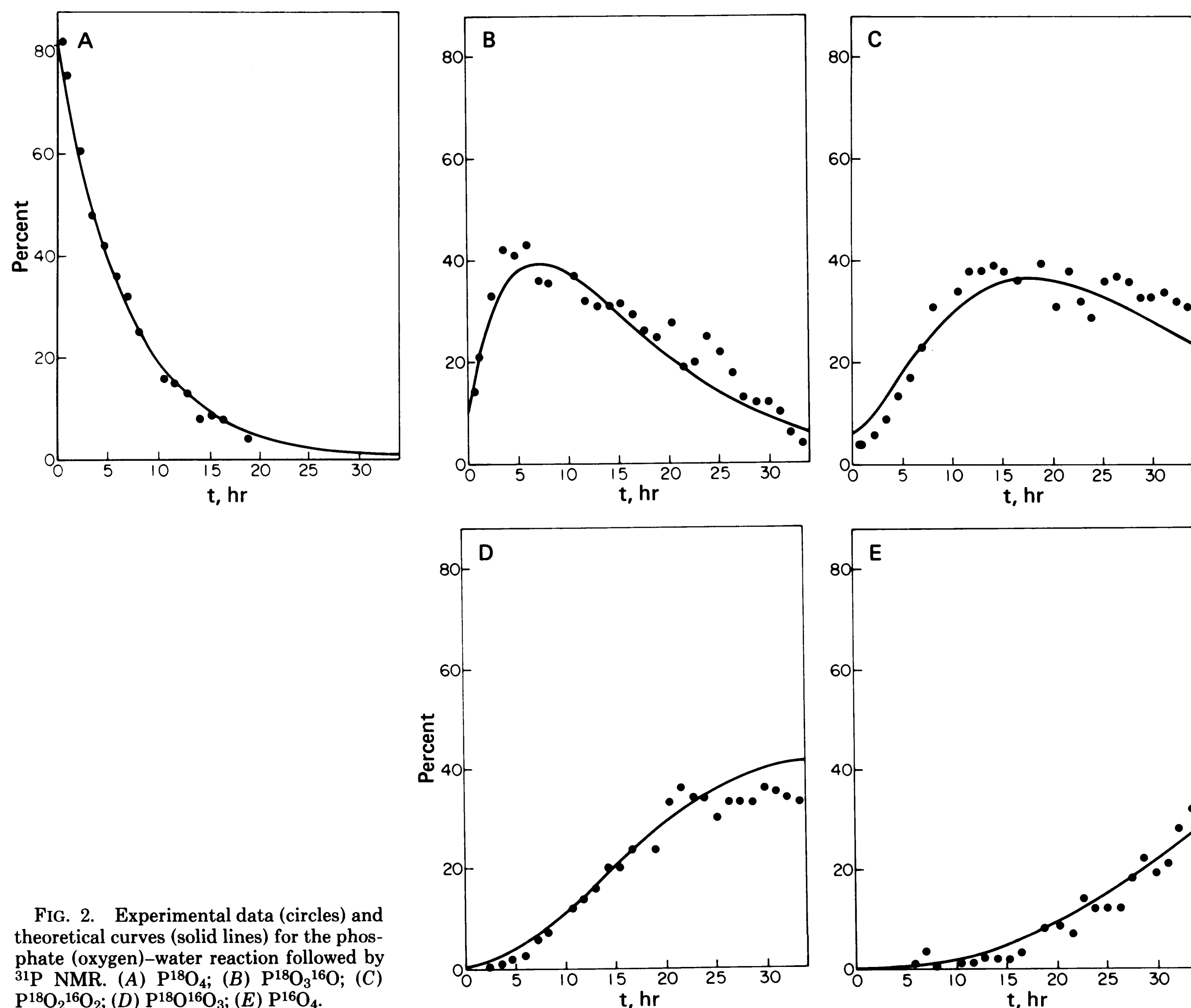

FIG. 2. Experimental data (circles) and theoretical curves (solid lines) for the phosphate (oxygen)–water reaction followed by ^{31}P NMR. (*A*) $P^{18}O_4$; (*B*) $P^{18}O_3{}^{16}O$; (*C*) $P^{18}O_2{}^{16}O_2$; (*D*) $P^{18}O^{16}O_3$; (*E*) $P^{16}O_4$.

vided satisfactory results, and the experimental data and theoretical values show very good agreement (Table 1). The value of k_{cat} measured at 37° is 1.8 sec^{-1}. This number is approximately 10-fold larger than is observed for the metalloenzyme *E. coli* alkaline phosphatase. That enzyme has been known for some years to catalyze such an exchange reaction (9, 17, 23, 24).

The value of ^{31}P NMR for following the course of the exchange reaction is well illustrated in the present experiment. Although we agree that an instrument operating at 145.7 MHz can provide more accurate and more convenient spectra, particularly since peak heights may be used directly for quantitation (14), we find that ^{31}P NMR at 40.5 MHz together with curve resolution can adequately measure the enzyme-catalyzed phosphate (oxygen)–water exchange reaction. Decomposition of the spectra by using the analog curve resolver proved to be relatively rapid and reproducible. A comparison of the data obtained by mass spectra and ^{31}P NMR methods shows good agreement considering that the presence of $^{2}H_2O$ may affect the kinetics of human prostatic acid phosphatase (25) and that EDTA tends to activate the enzyme slightly. Table 2 summarizes the methods and the findings obtained in this study of phosphate (oxygen)–water exchange catalyzed by prostatic acid phosphatase. Although a more detailed comparison of the rates of exchange and of hydrolysis must await careful comparative studies with due attention to temperature, pH, and possible effects of $^{2}H_2O$ on the enzymatic reactions, the present results establish that, at least under these conditions, k_{cat} (hydrolysis) and k_{cat} (exchange) differ by more than two orders of magnitude.

We thank Ms. Karen Gooding and Prof. Richard Caprioli for providing the mass spectral data and Mr. Michael Porubcan for help in obtaining the ^{31}P NMR data. We thank Profs. Stephen Benkovic, George Kenyon, and John Markley for helpful discussions. This work was supported by Grant CA 10585 from the National Cancer Institute of the U.S. Public Health Service.

1. Kelley, J. J. (1959) Dissertation (University of Wisconsin, Madison, WI), pp. 79–84.
2. Cohn, M. (1949) *J. Biol. Chem.* **180**, 771–781.
3. Bunton, C. A., Silver, B. L. & Vernon, C. A. (1957) *Proc. Chem. Soc.* **1957**, 348–349.
4. Christeller, J. T. & Tolbert, N. E. (1978) *J. Biol. Chem.* **253**, 1791–1798.
5. Ostrowski, W. & Barnard, E. A. (1973) *Biochemistry* **12**, 3893–3898.
6. Gallati, H. & Roth, M. (1976) *J. Clin. Chem. Clin. Biochem.* **14**, 581–587.
7. McTigue, J. J. & Van Etten, R. L. (1978) *Biochim. Biophys. Acta*, **523**, 407–421.
8. Van Etten, R. L. & Hickey, M. E. (1977) *Arch. Biochem. Biophys.* **183**, 250–259.
9. Eargle, D. H., Jr., Licko, V. & Kenyon, G. L. (1977) *Anal. Biochem.* **81**, 186–195.
10. Midelfort, C. F. & Rose, I. A. (1976) *J. Biol. Chem.* **251**, 5881–5887.
11. Wimmer, M. J. & Rose, I. A. (1977) *J. Biol. Chem.* **252**, 6769–6775.
12. Boyer, P. D. & Bryan, D. M. (1967) *Methods Enzymol.* **10**, 60–71.
13. Cohn, M. & Drysdale, G. (1955) *J. Biol. Chem.* **216**, 831–846.
14. Cohn, M. & Hu, A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 200–203.
15. Batiz-Hernandez, H. & Bernheim, R. (1967) *Prog. Nucl. Mag. Res. Spect.* **3**, 63–85.
16. Jameson, C. J. (1977) *J. Chem. Phys.* **66**, 4983–4988.
17. Bock, J. & Cohn, M. (1978) *J. Biol. Chem.* **253**, 4082–4085.
18. Webb, M. R., McDonald, G. G. & Trentham, D. R. (1978) *J. Biol. Chem.* **253**, 2908–2911.
19. Risley, J. M. & Van Etten, R. L. J. (1978) *Labeled Compds. Radiopharm.*, in press.
20. Van Etten, R. L. & Saini, M. S. (1978) *Clin. Chem.* **24**, 1525–1530.
21. Derechin, M., Ostrowski, W., Galka, M. & Barnard, E. A. (1971) *Biochim. Biophys. Acta* **250**, 143–154.
22. Bunton, C. A., Llewellyn, D. R., Vernon, C. A. & Welch, V. A. (1961) *J. Chem. Soc.* **1961**, 1636–1640.
23. Schwartz, J. (1963) *Proc. Natl. Acad. Sci. USA* **49**, 871–878.
24. Applebury, M., Johnson, B. & Coleman, J. (1970) *J. Biol. Chem.* **245**, 4968–4975.
25. Van Etten, R. L. & McTigue, J. J. (1977) *Biochim. Biophys. Acta* **484**, 386–397.