Deme, D., de Billy, G., & Chatagner, F. (1972) *Biochimie 54*, 1089-1092.
Dunathan, H. C. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol. 5*, 79-134.
Fischer, E. H., & Krebs, E. G. (1959) *Abstracts of Papers*, 136th National Meeting of the American Chemical Society, Atlantic City, NJ, American Chemical Society, Washington, D.C.
Ford, G. C., Eichele, G., & Jansonius, J. N. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2559-2563.
Gehring, H., Rando, R. R., & Christen, P. (1977) *Biochemistry 16*, 4832-4836.
Gray, W. R. (1972) *Methods Enzymol. 25*, 121-138.
Greenberg, D. M. (1962) *Methods Enzymol. 5*, 936-942.
Heyl, D., Harris, S. A., & Folkers, K. (1948) *J. Am. Chem. Soc. 70*, 3429-3431.
Hirs, C. H. W., Moore, S., & Stein, W. H. (1954) *J. Am. Chem. Soc. 76*, 6063-6065.
Hunkapillar, M. W., & Hood, L. E. (1978) *Biochemistry 17*, 2124-2133.
John, R., Bossa, F., Barra, D., & Fasella, P. (1973) *Biochem. Soc. Trans. 1*, 862-864.
Lai, C. Y. (1977) *Methods Enzymol. 47*, 240.
Martinez-Carrion, M., Slebe, J. C., & Gonzalez, M. (1979) *J. Biol. Chem. 254*, 3160-3162.
Matsuo, Y., & Greenberg, D. M. (1958) *J. Biol. Chem. 230*, 545-560.
Morino, Y., & Okamoto, M. (1973) *Biochem. Biophys. Res. Commun. 50*, 1061-1067.
Morino, Y., & Tanase, S. (1978) *J. Biol. Chem. 253*, 252-256.
Mushahwar, I. K., & Koeppe, R. E. (1973) *J. Biol. Chem. 248*, 7407-7411.
Peterson, D. L., & Martinez-Carrion, M. (1970) *J. Biol. Chem. 245*, 806-813.
Posner, B. I., & Flavin, M. (1972) *J. Biol. Chem. 247*, 6412-6419.
Silverman, R. B., & Abeles, R. H. (1976) *Biochemistry 15*, 4718-4723.
Silverman, R. B., & Abeles, R. H. (1977) *Biochemistry 16*, 5515-5520.
Tanase, S., Kojima, H., & Morino, Y. (1979) *Biochemistry 18*, 3002-3007.
Washtien, W., & Abeles, R. H. (1977) *Biochemistry 16*, 2485-2491.
Yamasaki, M., Tanase, S., & Morino, Y. (1975) *Biochem. Biophys. Res. Commun. 65*, 652-657.

Nitrogen-15 Nuclear Magnetic Resonance Investigation of Nitrite Reductase–Substrate Interaction†

Russell Timkovich* and Margaret S. Cork

ABSTRACT: Nitrogen-15 nuclear magnetic resonance (^{15}N NMR) spectroscopy at 30.4 MHz was employed to determine the interaction of the substrate nitrite (97.2% enriched) with bacterial nitrite reductase, denoted cytochrome cd_1, from *Pseudomonas aeruginosa*. The addition of ferric enzyme to nitrite did not alter the chemical shift of the bulk nitrite resonance, nor was it possible to observe a new resonance from a hypothetical bound form. However, the spin–lattice relaxation time (T_1) was lowered from 13.2 to 2.7 s, and the spin–spin relaxation time (T_2) was halved. Values of T_1 were measured by progressive saturation and values of T_2 by line widths. Control experiments involving ferric cytochrome *c* and metmyoglobin demonstrated that the perturbations did not arise from the bulk paramagnetic properties of the protein solutions. Variable enzyme/substrate ratios were measured to assess the strength of interaction. The most reasonable model consistent with the data proposes a weak association between nitrite and ferric reductase with a value of 1.3 M^{-1} for the association constant.

The dissimilatory nitrite reductase in many denitrifying bacteria is a cytochrome called cytochrome cd_1. The enzyme in native form is a dimer composed of two subunits of ca. 60 000 daltons with each subunit containing one heme *c* and one heme d_1 as prosthetic groups (Kuronen et al., 1975). In the catalytic cycle the enzyme alternates between oxidized and reduced states as it accepts electrons from a donor, usually a bacterial cytochrome *c*, and transfers them to nitrite, reducing it predominantly to nitric oxide (Wharton & Wintraub, 1980). In such a system, a pertinent question to ask is whether the substrate NO_2^- first binds to oxidized enzyme, which then is reduced by a donor, or whether the enzyme is first reduced and then interacts with substrate. Visible spectroscopy has been extensively used to demonstrate that reduced enzyme is fully capable of an interaction with nitrite that includes the transfer of reducing equivalents (Yamanaka & Okunuki, 1963; Silvestrini et al., 1979). However, the binding constant to the reduced form has not been measured, and any attempts would be ambiguous because of enzyme turnover. The available data do not rule out significant binding to the oxidized form where such an event may not be evident with light spectroscopy. Some electron paramagnetic resonance (EPR) data have been suggestive of an interaction with the oxidized form (Muhoberac & Wharton, 1980).

Nuclear magnetic resonance (NMR) spectroscopy of the ^{15}N nucleus has not been widely employed in biological studies because of low natural abundance (0.38%) and a relative

† From the Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616. *Received February 5, 1982.* Supported by Grants GM-23869 and GM-26071-0251 from the National Institutes of Health.

0006-2960/82/0421-3794$01.25/0

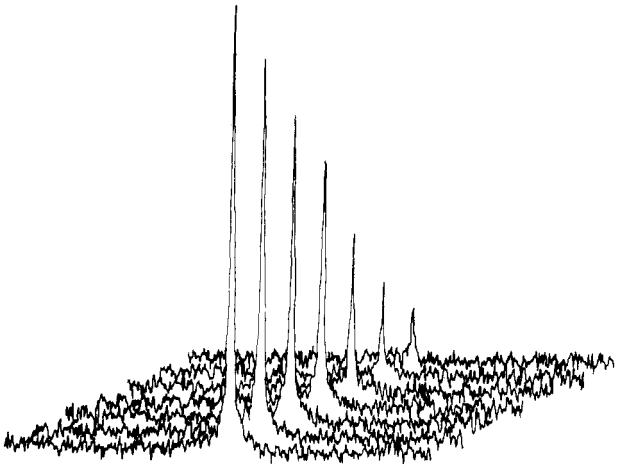

FIGURE 1: Spin–lattice relaxation data by progressive saturation in a typical experiment. Spectra shown were for 100 mM $^{15}NO_2^-$ plus 2 mM ferric cytochrome *c*. Delay values τ for the sequence $(90–\gamma–90–\gamma)_n$ ranged from 1.3 to 75 s. Data acquisition, 1000 transients total for each spectrum, occurred during the τ delay after steady-state magnetization was obtained. Signal to noise was poorer for lower nitrite concentrations, and the number of transients was increased to 4000. Total spectral width is 400 Hz.

insensitivity (0.001 compared to ^1H). The purpose of the present report is to demonstrate that these limitations are surmountable in the study of denitrification enzymes and that ^{15}N spectroscopy may be used to provide mechanistic evidence in such systems.

Materials and Methods

Cytochrome cd_1 was isolated and purified from *Pseudomonas aeruginosa* (ATCC 19429) by the method of Parr et al. (1976). Although the enzyme samples used in the NMR work were not so tested, previous samples prepared by identical procedures in this laboratory have been examined by atomic absorption spectroscopy and found to contain negligible concentrations of non-heme iron, copper, manganese, and cobalt. Further, as noted under Results, our control NMR experiments, especially with enzyme inhibitor, indicate that paramagnetic impurities as free metal ions are not significant. Ferric horse cytochrome *c*, metmyoglobin, and bovine serum albumin were purchased from Sigma Chemical Co. and used as received. Sodium [^{15}N]nitrite, 97.2% enriched, was purchased from Prochem/Isotopes. The standard buffer for all experiments was 40 mM potassium/potassium phosphate in 70% H_2O–30% ^2H_2O, pH 7.0 (glass electrode determined), sterilized by passage through a 0.45-μm filter.

Spectra were obtained on a Nicolet spectrometer operating at 30.4 MHz for ^{15}N in the Fourier transform mode. Samples of 1.5 mL were contained in 10-mm sample tubes at 20 °C. Nitrite chemical shifts were initially referenced against natural abundance formamide, but since shifts were never significant, later experiments at a fixed transmitter frequency contained no standard other than nitrite. Spin–spin relaxation times (T_2) were estimated from full line widths at half-height. Spin–lattice relaxation times (T_1) were determined by progressive saturation employing a $(90–\tau–90–\tau)_n$ pulse sequence. This technique choice was critical because nitrite has a relatively long relaxation time (T_1) yet requires extensive signal accumulations. Progressive saturation requires less total spectrometer time than inversion–recovery techniques and gives equivalent results when the effective T_2 is shorter than T_1. This condition was fully satisfied in these experiments. Figure 1 shows typical data obtained. Statistical standard deviations of T_1 obtained from least-squares fits of data in a given experiment averaged 2%, but this may overestimate the true accuracy.

Table I: Spin–Lattice (T_1) Relaxation Times for [^{15}N]Nitrite

sample	T_1 (s)	line width (Hz)
$^{15}NO_2^-$	13.2	5
100 mM $^{15}NO_2^-$ + 2 mM BSA	13.1	6
100 mM $^{15}NO_2^-$ + 2 mM cyt *c*	13.5	5
250 mM $^{15}NO_2^-$ + 2 mM metMb	7.9	4
80 mM $^{15}NO_2^-$ + 0.17 mM cyt cd_1	9.1	13
80 mM $^{15}NO_2^-$ + 0.5 mM cyt cd_1	2.8	10
1 M $^{15}NO_2^-$ + 0.5 mM cyt cd_1	4.7	14
1 M $^{15}NO_2^-$ + 0.5 mM cyt cd_1 + 50 mM KCN	10.6	8

Results

It has been established that the major mechanism for spin–lattice relaxation in $^{15}NO_2^-$ is spin rotation with a minor contribution from intramolecular dipole–dipole interactions (Saluvere & Lippmaa, 1970). In the present experiments decoupler irradiation of the solvent ^1H_2O with optimized conditions increased the ^{15}N signal by only 30%, presumably through a heteronuclear Overhauser effect. Since decoupler irradiation led to sample heating, and this was deemed unfavorable for the enzyme solutions, it was omitted in the T_1 experiments. The present experimentally determined T_1 of 13.2 s for free solution $^{15}NO_2^-$ (see Table I) was independent of concentration. It differs from the previous report of ca. 20 s (Saluvere & Lippmaa, 1970). The difference is ascribed to the use of air-saturated buffer in the present case vs. argon-degassed solution in the previous case. The use of oxygen-containing buffer in the present case was by design. The longer T_1 in degassed solution would make signal accumulation even more difficult. Furthermore, the solvent choice of 70% ^1H_2O–30% ^2H_2O represented a compromise between the necessity of a deuterium lock and a desire to take advantage of the intramolecular dipole–dipole contribution to T_1.

Control experiments summarized in Table I were performed to assess the contribution of nonspecific protein interactions on the spectrum of $^{15}NO_2^-$. Viscosity differences represented in the bovine serum albumin control had negligible effect. The bulk solution effect due to the presence of a paramagnetic macromolecule was assessed by examining mixtures of ferric cytochrome *c* and $^{15}NO_2^-$ and metmyoglobin and $^{15}NO_2^-$. Ideally, the perfect control would be readily available and would have electronic spin properties (magnitude, relaxation times, and symmetry) equal to those of the nitrite reductase but with no specific nitrite interaction. However, these characteristics of the nitrite reductase are sufficiently unique that such an ideal control is not evident. Roughly speaking, ferric cytochrome *c* and metmyoglobin bracket the electronic properties of nitrite reductase. As shown in Table I, ferric cytochrome *c* had no effect on the $^{15}NO_2^-$ spectrum. Metmyoglobin at a relatively high concentration did not affect the chemical shift or line width but did cause a partial reduction of T_1. Metmyoglobin constitutes an ambiguous control because it is well-known that the ferrous state reacts strongly with nitrites (Fox & Ackerman, 1968) and myoglobin has some affinity for small anions (Antonini & Brunori, 1971). So, the T_1 effect could be due to some type of specific interaction. However, the effect is small in comparison to that with nitrite reductase.

In the presence of oxidized cytochrome cd_1, line widths approximately doubled, presumably reflecting a halving of T_2. No significant chemical shift from free nitrite was observed.

No new lines were observed in a range of ±400 ppm. However, because of the low sensitivity of ^{15}N and the low cytochrome concentration, a resonance corresponding to a complex could be well below the present detection limits. The addition of enzyme did exert a strong perturbation on the observed T_1 with the effect dependent on protein concentration as shown in Table I. Cyanide, an inhibitor of the enzyme (Yamanaka et al., 1961) that binds at both the heme *c* and heme d_1 (Walsh et al., 1979), caused a return of T_1 to a value approaching that of the free anion case. At a fixed concentration of enzyme, the dependence of the observed T_1 on $^{15}NO_2^-$ concentration was measured from 10 mM to 1 M with these limits imposed by sensitivity and enzyme solubility considerations. Values varied gradually from 2.6 to 4.7 s. The interpretation of this dependence will be discussed in the subsequent section.

Discussion

The data obtained on mixtures of nitrite and oxidized cytochrome cd_1 can be interpreted in terms of several limiting cases involving the rate of exchange between bulk solution and a hypothetical complex and the association strength of the complex. If the rate of exchange is very slow, such that the lifetime in the complex is much greater than T_1 for free anion, then the bound site would be invisible to the present type of NMR experiment, i.e., no change on the free anion spectrum or relaxation times. For moderate or fast exchange, with lifetimes on the order of T_1 or much shorter than T_1, a bound site, in principle, could affect chemical shifts and both T_1 and T_2 [see Dwek (1973) for a comprehensive review of theory and practice]. The present experiments provide no data on the chemical shift of $^{15}NO_2^-$ in the bound site, and therefore the constant chemical shift of bulk nitrite and its broadened line may not be analyzed in detail. It may be noted qualitatively that moderate or intermediate exchange rates could produce line broadening but no shift. The mechanism of T_1 perturbation due to binding is not clear; it may involve change in the rotational correlation time of $^{15}NO_2^-$, relaxation due to proximity to iron electron spin, increased intramolecular dipole–dipole interactions with protein nuclei, or some combination of these.

A perturbation on T_1 has been observed in excess of control experiments, so there is an exchangeable site visible on the NMR time scale. However, it is not possible to rigorously rule out the existence of an additional strong binding site to the oxidized form of the enzyme, especially if the additional site has a slow exchange rate. No information would be obtained on this hypothetical, additional site; it is undetectable by ^{15}N NMR. On the other hand, there is an observable site, and its sensitivity to the enzyme inhibitor cyanide indicates proximity to the enzyme's prosthetic groups. At the present time, there is no reason to suppose the existence of more than one type of nitrite binding site.

The magnitude of the association constant for the nitrite–enzyme complex determines the concentration behavior for the observed T_1. The usual substrate binding equilibrium is assumed:

$$E + S \rightleftharpoons ES$$

where E represents free enzyme, S represents nitrite, and ES represents the complex with association constant $K_s = [ES]/([E][S])$. In the present circumstances the bulk substrate concentration, S_0, is in excess over starting enzyme, E_0, i.e., $S_0 \gg E_0$, so the mole fraction of bound substrate, *ES*, is given by

$$ES/S_0 = K_sE_0/(1 + K_sS_0)$$

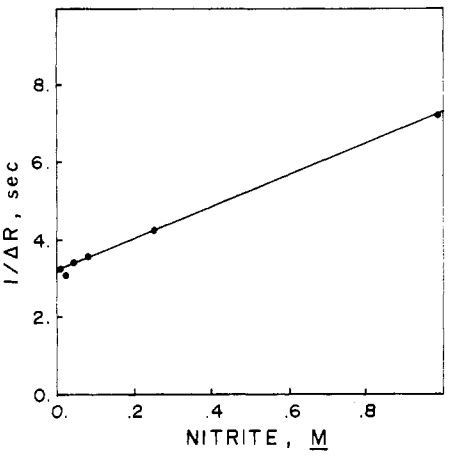

FIGURE 2: Plot of reciprocal relaxation rate change vs. bulk nitrite concentration, S_0. The value $1/\Delta R$ is $[1/T_1(\text{obsd}) - 1/T_1(\text{free nitrite})]^{-1}$. As described in the text, the slope gives the value of $1/(\alpha E_0)$ and the intercept gives the value of $1/(\alpha K_s E_0)$ from which a value of 1.3 M^{-1} was obtained for K_s.

Regardless of the mechanism of T_1 perturbation, in the presence of fixed E_0, the change in relaxation rate is proportional to the mole fraction bound (Dwek, 1973):

$$1/T_1(\text{obsd}) - 1/T_1(\text{free anion}) = \alpha ES/S_0 = \alpha K_sE_0/(1 + K_sE_0)$$

where α is a constant whose identity and value depend upon the mechanism of relaxation in the bound site. For the present purpose it suffices to note that α is constant as S_0 varies. For strong association ($K_sS_0 \gg 1$) the relaxation rate change at fixed E_0 would be proportional to $1/S_0$. For weak association ($K_sS_0 \ll 1$), the change could be constant with respect to S_0. As S_0 varied from 10 mM to 1 M, the observed T_1 showed an increase that was intermediate in extent. A plot of the reciprocal of the rate change vs. S_0 (Figure 2) gave a reasonable straight line from which an estimate of K_s may be made. The determined value was 1.3 M^{-1}. There may be considerable inaccuracy in this estimate of K_s because of the relatively small T_1 differences for large changes in nitrite concentration, but K_s is clearly on the order of 1 M^{-1}.

During catalytic turnover of the nitrite reductase, steady-state kinetics have yielded a value of 5 μM for the Michaelis–Menten K_M or 2×10^5 M^{-1} expressed as an apparent association constant (Saraste & Kuronen, 1978; Timkovich et al., 1982). The ^{15}N data for oxidized enzyme and nitrite indicate an association 5 orders of magnitude different from the kinetically significant interaction. One can conclude that reduced cytochrome cd_1 is the form that interacts strongly with the substrate nitrite.

References

Antonini, E., & Brunori, M. (1971) in *Hemoglobin and Myoglobin in their Reactions with Ligands*, pp 40–52, North-Holland, Amsterdam.

Dwek, R. A. (1973) in *Nuclear Magnetic Resonance in Biochemistry: Applications to Enzyme Systems*, Chapters 2, 6, 9, Clarendon Press, Oxford.

Fox, J. B., Jr., & Ackerman, S. A. (1968) *J. Food Sci. 33*, 364–370.

Kuronen, T., Saraste, M., & Ellfolk, N. (1975) *Biochim. Biophys. Acta 393*, 48–54.

Muhoberac, B. B., & Wharton, D. C. (1980) *J. Biol. Chem. 255*, 8437–8442.

Parr, S. R., Barber, D., & Greenwood, C. (1976) *Biochem. J. 157*, 423–430.

Saluvere, T., & Lippmaa, E. (1970) *Chem. Phys. Lett. 7*, 545–548.
Saraste, M., & Kuronen, T. (1978) *Biochim. Biophys. Acta 513*, 117–131.
Silvestrini, M. C., Colosimo, A., Brunori, M., Walsh, T. A., Barber, D., & Greenwood, C. (1979) *Biochem. J. 183*, 701–709.
Timkovich, R., Dhesi, R., Martinkus, K. J., Robinson, M., & Rea, T. M. (1982) *Arch. Biochem. Biophys. 215*, 47–58.
Walsh, T. A., Johnson, M. K., Greenwood, C., Barber, D., Springall, J. P., & Thomson, A. J. (1979) *Biochem. J. 177*, 29–39.
Wharton, D. C., & Wintraub, S. T. (1980) *Biochem. Biophys. Res. Commun. 97*, 236–242.
Yamanaka, T., & Okunuki, K. (1963) *Biochim. Biophys. Acta 67*, 394–406.
Yamanaka, T., Ota, A., & Okunuki, K. (1961) *Biochim. Biophys. Acta 53*, 294–308.

Photoaffinity Labeling of *Escherichia coli* Ribosomes by an Aryl Azide Analogue of Puromycin. On the Identification of the Major Covalently Labeled Ribosomal Proteins and on the Mechanism of Photoincorporation†

Allen W. Nicholson,‡ Clifford C. Hall, William A. Strycharz, and Barry S. Cooperman*

ABSTRACT: *p*-Azido[^{3}H]puromycin [6-(dimethylamino)-9-[3′-deoxy-3′-[(*p*-azido-L-phenylalanyl)amino]-β-D-ribofuranosyl]purine] has been used to photoaffinity label the *Escherichia coli* ribosome. Initial studies with this compound, reported earlier [Nicholson, A. W., & Cooperman, B. S. (1978) *FEBS Lett. 90*, 203–208], indicated a very diffuse labeling pattern with many proteins labeled to significant extents. In the present work, much of this previous apparent labeling is shown to arise from both light-independent noncovalent binding and light-independent incorporation of photolyzed *p*-azidopuromycin with ribosomal protein, and procedures are described for measuring true covalent photoincorporation. When these new procedures are used, *p*-azidopuromycin is shown to photoincorporate into ribosomal protein and RNA. The protein labeling pattern, as determined by both polyacrylamide gel electrophoresis and immunoprecipitation, is quite specific and is essentially unchanged whether 2537 Å or 3500 Å lamps are used. The extent of photoincorporation into proteins falls in the order S18 > L23 > L18/22 > L15 > S7, S14. When β-mercaptoethanol is present during photolysis as a photoaffinity label scavenger, S18 and most other S protein labeling is suppressed, and the order of labeling becomes L23 > L18/22 > L15, S7 > S1. This result suggests that high S18 labeling is not site specific but is due rather to its high chemical reactivity. The specific suppression of S18 labeling by pretreatment of the ribosomes with *N*-ethylmaleimide supports this view. L23 labeling by *p*-azidopuromycin is azide dependent and proceeds by a mechanism which is most probably different from that responsible for photoincorporation of puromycin [Cooperman, B. S., Jaynes, E. N., Brunswick, D. J., & Luddy, M. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2974–2978; Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, R., & Cooperman, B. S. (1978) *Biochemistry 17*, 561–569] yet under the appropriate conditions both of these compounds label L23 to the highest extent of any ribosomal protein. This constitutes strong evidence for the site specificity of L23 labeling.

Photoaffinity labeling has become an important method for identifying functional sites on the *Escherichia coli* ribosome (Cooperman, 1978, 1980; Kuechler & Ofengand, 1980). We have been pursuing such studies with the antibiotic puromycin (Cooperman et al., 1975; Jaynes et al., 1978; Grant et al., 1979a,b). Puromycin is a substrate for the peptidyltransferase activity of the ribosome, and accordingly, localization of its site of binding on the ribosome should provide direct information on the peptidyltransferase center. In these studies we found that puromycin photoincorporated into ribosomes, that protein L23 was the major labeled protein, and that such labeling was site specific. Because of the uncertainties to which photoaffinity labeling studies are subject (Cooperman, 1976; Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979), it is important to verify and extend results obtained in any one study by altering the labeling process in a deliberate fashion. One such approach is to compare the labeling patterns obtained when different photolabile derivatives of the same parent ligand are photoincorporated into the receptor. Accordingly, we have synthesized *p*-azido[^{3}H]puromycin [6-(dimethylamino)-9-[3′-deoxy-3′-[(*p*-azido-L-phenylalanyl)amino]-β-D-ribofuranosyl]purine], a functionally competent analogue of puromycin (Symons et al., 1978; Krassnigg et al., 1978; Nicholson & Cooperman, 1978; Nicholson et al., 1982), and used it to photoaffinity label the ribosome. A preliminary report of our findings has already appeared (Nicholson & Cooperman, 1978) in which labeling was found to be distributed over a large number of proteins (as judged by one- and two-dimensional polyacrylamide gel electrophoresis), with the most

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104 (A.W.N., C.C.H., and B.S.C.), and the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706 (W.A.S.). *Received September 30, 1981.* This work was supported by National Institutes of Health Grant AI16806 and National Science Foundation Grant PCM 84278 awarded to B.S.C. A.W.N. was a National Institutes of Health predoctoral trainee (5T32 GM 07229). W.A.S. was supported by National Science Foundation Grant PCM 7818490 administered by M. Nomura. This is paper 2 of the series.

‡ Present address: Department of Genetics, Rockefeller University, New York, NY 10021.

0006-2960/82/0421-3797$01.25/0