INVOLVEMENT OF A B-TYPE CYTOCHROME IN THE ASSIMILATORY NITRATE REDUCTASE OF NEUROSPORA CRASSA*

By Reginald H. Garrett† and Alvin Nason

McCollum Pratt Institute, The Johns Hopkins University, Baltimore, Maryland

Communicated by W. D. McElroy, August 9, 1967

The enzyme systems which catalyze the reduction of nitrate to nitrite have generally been classified into two broad types: (*a*) *assimilatory nitrate reductase*, which is responsible for the first step in the reduction of nitrate for the ultimate biosynthesis of nitrogen-containing cell constituents (e.g., amino acids and purines and pyrimidines), and (*b*) *respiratory* or *dissimilatory nitrate reductase*, which utilizes nitrate as the terminal acceptor (in place of oxygen) in the electron transport or respiratory pathway, usually under anaerobic or partially anaerobic conditions.[1–5] Comparisons of the properties of the assimilatory nitrate reductase of *Neurospora* with those of the respiratory nitrate reductase of *E. coli* have clearly indicated that the above two enzyme types share several similarities (including the possession of molybdenum) and are considerably more alike than hitherto believed.[3–5] Thus far the major distinguishing feature of respiratory nitrate reductase, as originally suggested by Sato,[1] has been the involvement of one or more cytochromes as electron carriers in the enzymatic reduction of nitrate to nitrite, a characteristic notably absent in the assimilatory system. The reasons for the inhibitory effect of molecular oxygen on the respiratory nitrate reductase only are not entirely clear. The effect is probably due both to an inhibition of adaptive formation of the enzyme[6] and to a competition for electrons by the oxygen itself, perhaps by way of an accompanying cytochrome oxidase.

In the course of large-scale purification of the soluble assimilatory NADPH-nitrate reductase (NADPH:nitrate oxidoreductase, E.C. 1.6.6.2), from *Neurospora crassa* grown under highly aerobic conditions, a *b*-type cytochrome was found to be associated with the fractions having nitrate reductase activity. The present paper reports that the properties of this cytochrome associated with the *Neurospora* nitrate reductase (including its specific reduction and oxidation by NADPH and nitrate, respectively) imply it to be an integral part of the electron transfer scheme involved in assimilatory nitrate reduction. The cytochrome spectrum of the partially purified enzyme is clearly different from that of cytochrome b_1 of the *E. coli* respiratory nitrate reductase, and to a lesser extent from those of several other *b*-type cytochromes reported in the literature, but is very similar to those of bakers' yeast lactic dehydrogenase cytochrome b_2 and mammalian cytochrome b_5. Despite its similarity in this and several other respects to yeast lactate dehydrogenase, the *Neurospora* nitrate reductase has no lactate dehydrogenase activity. Nor does the yeast lactate dehydrogenase system possess nitrate reductase activity. The previous failure to observe this hemoprotein in the assimilatory enzyme can be attributed to the relatively low yield and degree of purity heretofore obtained with *Neurospora crassa* nitrate reductase.

Materials and Methods.—Cytochrome *c* (type III) and FAD were provided by the Sigma Chemical Company. NADPH was obtained from the Sigma Chemical

Company and from Calbiochem. The contents of one vial of bakers' yeast lactate dehydrogenase (YLDH, cytochrome b_2) (L(+)-lactate:cytochrome *c* oxidoreductase, E.C. 1.1.2.3), 5–7 units per vial, supplied by Worthington Biochemical Corporation, were dissolved in 1 ml of 0.1 *M* phosphate buffer, pH 7.3, and used without further treatment.

Nitrate reductase was prepared from wild-type *Neurospora crassa* (5297a) mycelia grown aerobically on Fries medium modified to contain sodium nitrate as the sole nitrogen source. The purification procedure, to be published elsewhere, entailed adjustment of the pH of the crude extract to 5.0, removal of the resultant precipitate, $(NH_4)_2SO_4$ fractionation, phase separation to remove nucleic acids, *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) chromatography, hydroxylapatite chromatography, and, finally, Sephadex G-200 gel filtration. The purest fraction obtained showed two bands of equal density after amidoschwartz staining on polyacrylamide gel electrophoresis at pH 8.3, according to Clark.[7] Only one of the bands possessed nitrate reductase activity.

Absorption spectra were determined with a Cary model 14 recording spectrophotometer equipped with the 0–0.1, 0.1–0.2 OD slidewire.

Nitrate reductase activity was determined by the method of Nason and Evans,[8] except that 0.05 ml of 10^{-4} *M* flavin-adenine dinucleotide (FAD) and either 0.05 ml of 2×10^{-3} *M* NADPH or 0.10 ml 0.1 *M* potassium DL-lactate, pH 7.0, as indicated, were used; 0.1 *M* phosphate buffer, pH 7.2, replaced the pyrophosphate buffer, and an incubation period of ten minutes was used instead of five minutes. One unit of nitrate reductase is that amount of enzyme which results in the formation of 1 mμM of nitrite under the above assay conditions.

Cytochrome *c* reductase activity was determined by observing the increase in absorbancy at 550 mμ with a Beckman DU spectrophotometer in a reaction mixture consisting of 0.05 ml 10^{-4} *M* FAD, 0.05 ml 2 per cent aqueous cytochrome *c*, 0.05 ml

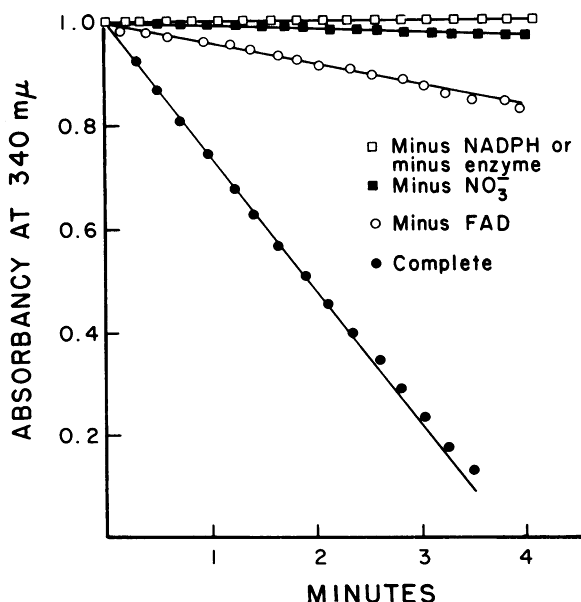

FIG. 1.—Requirements for TPNH oxidation by *N. crassa* nitrate reductase. Complete reaction mixture consisted of 0.10 ml 0.1 *M* $NaNO_3$, 0.05 ml 10^{-4} *M* FAD, 0.01 ml enzyme (6.82 γ protein, specific activity = 19,500), 0.79 ml 0.1 *M* phosphate buffer, pH 7.2, and 0.05 ml 2×10^{-3} *M* NADPH. Measurements were made with a Gilford recording spectrophotometer under aerobic conditions.

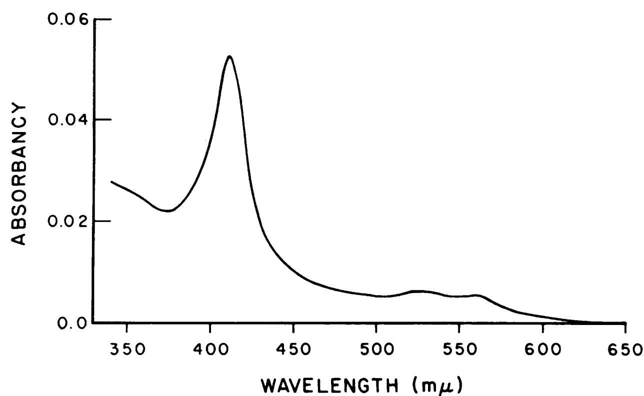

Fig. 2.—Absorption spectrum of purified nitrate reductase. Sephadex G-200 fraction, 272 γ protein per ml, specific activity = 119,000, vs. water.

2×10^{-3} *M* NADPH or 0.10 ml 0.1 *M* potassium DL-lactate, pH 7.0, as indicated, and enzyme plus 0.10 *M* Tris buffer, pH 8.0, to give 1 ml final volume. Ferricyanide reductase activity was assayed in an identical reaction mixture except that 0.05 ml 10^{-2} *M* potassium ferricyanide was used in place of cytochrome *c*, the decrease in absorbancy at 420 mμ being recorded. Trichlorophenol indophenol (TCPIP) reductase activity was also determined as above except that 0.05 ml 10^{-3} *M* TCPIP was used in place of cytochrome *c*, the decrease in absorbancy at 600 mμ being recorded.

Protein was measured by a modified Lowry procedure and by determination of the 280 mμ/260 mμ ratio as described by Layne.[9]

Results.—*N. crassa* nitrate reductase activity requires nitrate for NADPH oxidation, is markedly enhanced in the presence of added FAD (Fig. 1), and proceeds

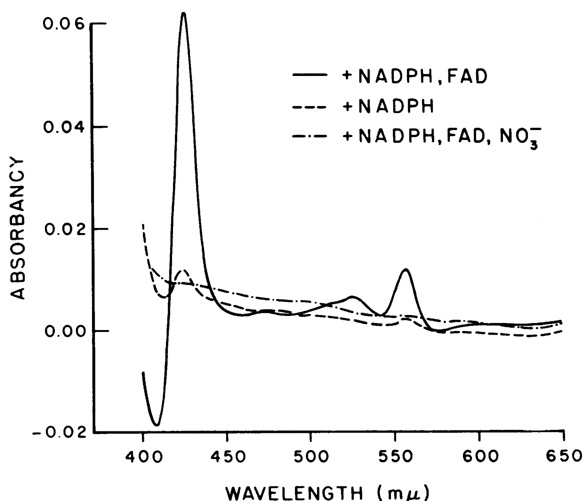

FIG. 3.—Oxidized vs. reduced difference spectra of nitrate reductase.
- - - -, *Curve 1:* Sample cuvette contained 0.75 ml Sephadex G 200 nitrate reductase fraction, 635 γ protein per ml, specific activity = 119,000, +0.05 ml 2×10^{-3} *M* NADPH. Reference cuvette contained 0.75 ml of the same fraction + 0.05 ml H_2O.
———, *Curve 2:* 0.02 ml of 10^{-5} *M* FAD were added to both cuvettes detailed under curve 1.
· - · - ·, *Curve 3:* 0.05 ml 0.1 *M* $NaNO_3$ and 0.05 ml 2×10^{-3} *M* NADPH were added to the above sample cuvette; 0.05 ml 0.1 *M* $NaNO_3$, and 0.05 ml H_2O were added to the above reference cuvette. Addition of a few crystals of sodium dithionite to the sample cuvette after obtaining curve 3 gave a spectrum essentially the same as curve 2. All recordings were made with samples under aerobic conditions.

TABLE 1

COMPARISON OF SPECTRAL PROPERTIES OF *N. crassa* NITRATE REDUCTASE WITH THOSE OF SEVERAL *b*-TYPE CYTOCHROMES FROM VARIOUS SOURCES AS REPORTED IN THE LITERATURE

			Reduced Peaks					
			α		β		γ or Soret	
Substance	Ref.	Oxidized peak λ_{max}	λ_{max}	Relative peak height*	λ_{max}	Relative peak height	λ_{max}	Relative peak height
N. crassa nitrate reductase	—	412–413	557	1.0†	528	0.5†	423–424	5.1†
Bakers' YLDH (cytochrome b_2)	10	413	556.5	1.0	528	0.48	423	6.0
Rabbit liver microsomal cytochrome b_5	11	413	556	1.0	526	0.48‡	423	6.6‡
E. coli respiratory nitrate reductase (cytochrome b_1)	12	430	560	—	532	—	432	—
b-Type cytochrome from *Sclerotinia libertiana*	13	413	554 and 561	—	528	—	424	—
From mung bean seedlings (*Phaseolus aureus*)								
Cytochrome *b*-555	16	413	555–559	—	529		423	—
Cytochrome b_3(559)	17	415	559	—	529		425	—
Cytochrome *b*-561	14	418	561	—	531		427	—

* Defined as 1.0 for the α-peak.
† Obtained from oxidized vs. reduced difference spectra (Fig. 3) and uncorrected for absorption of oxidized form at the given wavelength. Relative absorption of oxidized vs. reduced form is appreciable in the Soret region.
‡ Estimated from the spectrum reported in reference 11.

rapidly in air, as established previously.[8] An appreciable production of nitrite occurred concomittantly (not shown) in the complete reaction mixture only.

Absorption spectra: The visible absorption spectrum of purified nitrate reductase has a major peak at 413 mμ (Fig. 2), whereas the oxidized-reduced difference spectrum (Fig. 3), using NADPH or dithionite as the reductant shows an *alpha* peak at 557 mμ, a *beta* peak at 528 mμ, and a *gamma* or Soret peak at 423 mμ. A comparison of these spectral properties with those reported for several *b*-type cytochromes from various sources is given in Table 1. The maxima exhibited by nitrate reductase are typical of a *b*-type cytochrome and are very close, if not identical, to those reported for YLDH cytochrome b_2[10] and rabbit liver microsomal cytochrome b_5.[11] The spectrum is significantly different from that observed for the cytochrome b_1 of the *E. coli* particulate respiratory nitrate reductase system,[12] from that of the purified *b*-type cytochrome derived from the fungus *Sclerotinia libertiana*,[13] and from those of the soluble *b*-types obtained from mung bean.[14–17]

As shown in Figure 3, a slight reduction of the cytochrome occurs upon addition of NADPH alone. However, the subsequent addition of FAD to the cuvette results in essentially complete reduction of the cytochrome. If nitrate is now added, the reduced cytochrome is rapidly oxidized (in the absence of nitrate, only a slow autooxidation of reduced cytochrome occurs). 10^{-3} *M* KCN does not inhibit the appearance of reduced peaks.

TABLE 2

RELATIONSHIP OF CYTOCHROME *b* CONTENT, NITRATE REDUCTASE ACTIVITY, AND PROTEIN CONCENTRATION IN THE PURIFICATION OF *Neurospora* NITRATE REDUCTASE

Fraction	Specific activity	Mg protein/ ml in cuvette	Nitrate reductase activity (units per ml in cuvette)	Absorbance at 557 mμ (reduced minus oxidized)*	Ratio of activity units × 10^{-6} per ml to A_{557}	Ratio of $A_{557} \times 10^3$ to mg protein/ml
Crude extract	636	4.69	2,980	0.0105	0.3	2.24
50% $(NH_4)_2SO_4$ ppt.	2,050	9.13	18,700	0.006	3.1	0.66
Pooled DEAE eluates	8,320	1.79	14,900	0.005	3.0	2.79
2nd 50% $(NH_4)_2SO_4$ ppt.	15,500	5.30	82,000	0.019	4.3	3.59
35–40% $(NH_4)_2SO_4$ ppt.	18,200	3.00	54,600	0.0095	5.7	3.17
40–45% $(NH_4)_2SO_4$ ppt.	18,750	2.62	49,100	0.009	5.5	3.44
Concentrated hydroxylapatite eluate	75,000	0.288	21,600	0.005	4.3	17.4
Sephadex G-200 fraction	119,000	0.272	32,300	0.0055	5.9	20.2

* Enzyme fractions were reduced by addition of dithionite and the resultant peak height measured within 30 sec.

Cytochrome b content and nitrate reductase activity: Table 2 shows that the ratio between the concentration of cytochrome *b* (as measured by the difference in absorbancy at 557 mμ between oxidized and dithionite-reduced enzyme fractions) and nitrate reductase activity does not vary significantly over a 60-fold purification range starting with the first ammonium sulfate fractionation step, whereas the ratio between cytochrome *b* and protein concentrations increases more or less directly with the purity of the preparation.

Nitrate reductase-YLDH relationship: In view of the striking spectral similarities between the nitrate reductase preparation and YLDH-cytochrome b_2, an examination of both enzymes was undertaken to determine if they possessed any common catalytic activities. Using a partially purified *Neurospora* nitrate reductase fraction, NADPH proved to be an effective electron donor when nitrate, ferricyanide, trichlorophenol indophenol, or cytochrome *c* was the electron acceptor, whereas lactate failed to reduce any of these substances under the same conditions. Neither substitution of riboflavin 5'-phosphate (FMN) for FAD nor omission of flavin in the assay mixtures yielded any activity with lactate as substrate. Moreover, the addition of lactate to *Neurospora* nitrate reductase, with and without added flavin, did not cause the appearance of reduced cytochrome *b* peaks in contrast to the positive results obtained with NADPH (see Fig. 3). Apparently lactate does not act as an electron donor for nitrate reductase.

Similarly, nitrate failed to serve as an electron acceptor for YLDH (as measured by nitrite production) regardless of whether NADPH or lactate was used as the reductant. As further evidence, the spectrum of reduced cytochrome b_2 obtained by addition of NADPH or lactate to YLDH remained unchanged (i.e., the cytochrome was not oxidized) upon addition of nitrate (not shown). Using YLDH, lactate

caused the reduction of cytochrome *c*, ferricyanide, and trichlorophenol indophenol, as expected. NADPH also reduced these acceptors in the presence of YLDH, although less effectively.

Discussion.—The experimental evidence reported in the present paper strongly implicating a *b*-type cytochrome as an electron carrier in the *Neurospora* assimilatory nitrate reductase system is as follows: (*a*) occurrence in the enzyme fractions of a heme component possessing spectral properties identical with those of yeast lactate dehydrogenase (cytochrome b_2) and liver microsomal cytochrome b_5, (*b*) a constant ratio of cytochrome concentration to nitrate reductase activity during purification, (*c*) a similar flavin requirement for NADPH reduction of the *b*-type cytochrome component of the enzyme as for over-all nitrate reductase activity, and (*d*) oxidation of NADPH-reduced cytochrome *b* by the addition of nitrate.

However, kinetic studies, namely a comparison of the rates of reduction and oxidation of the nitrate reductase cytochrome *b*-type component by NADPH and nitrate, respectively, with the over-all rate of nitrate reductase activity will be necessary to establish conclusively that the hemeprotein is directly involved. Experiments of this type are somewhat complicated in the present situation, since high concentrations of the enzyme are needed for reliable spectral rate measurements in contrast to the exceedingly low enzyme concentration necessary for determining over-all nitrate reductase activity.

The present finding that *Neurospora* assimilatory nitrate reductase possesses a hemoprotein component, designated here tentatively as cytochrome *b*-557 (*N. crassa*), in line with the nomenclature practice of Hackett and his colleagues,[14–17] raises an interesting point. It clearly eliminates the one remaining major criterion, namely the supposed lack of cytochrome involvement, which until now was believed to distinguish the assimilatory type of nitrate reductase from the respiratory type.[1–5] Perhaps a sounder basis for the two classes of nitrate reductase may prove to reside in their physical orientation and localization within the cell—with the respiratory type presumably being suitably complexed to the electron transport chain, and the assimilatory type possibly localized in another part of the cell.

The striking spectral resemblance of *Neurospora* nitrate reductase to the YLDH-cytochrome b_2 is paralleled by several other properties. Both enzyme systems are soluble, possess a dissociable flavin, and are of relatively high molecular weight. The behavior of *Neurospora* nitrate reductase on Sephadex indicates a molecular weight of greater than 100,000. By contrast, all these characteristics, other than spectral similarity, are different for cytochrome b_5 and its reductase. Nevertheless, YLDH cannot catalyze the reduction of nitrate. Nor can nitrate reductase utilize lactate as a reductant for its cytochrome component or for the over-all reduction of nitrate.

Finally, a tentative modification of the originally proposed electron transfer scheme for *Neurospora* assimilatory nitrate reductase[18] which fits the above data is as follows:

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{Cytochrome } b\text{-557} \rightarrow \text{Mo} \rightarrow NO_3^-$$

Cytochrome *b*-557 is placed in the above sequence after FAD because of the striking flavin requirement for maximal reduction, and before Mo since the appearance of reduced cytochrome *b* peaks is unaffected by cyanide concentrations

that are inhibitory to nitrite production. The site of the CN^- inhibition has been indicated to be at the molybdenum moiety.[5] Exogenous cytochrome *c* accepts electrons in the above scheme after FAD since the flavin requirement for cytochrome *c* reduction is similar to that for nitrate reduction. However, reduced cytochrome *c* will not reduce nitrate. The possibility that cytochrome *b*-557 is also an electron carrier in the reduction of cytochrome *c* has not yet been determined. The above NADPH-cytochrome *c* reductase activity is induced in parallel with the induction of nitrate reductase activity in the presence of nitrate.[19] By contrast, the constitutive NADPH-cytochrome *c* reductase(s) of *N. crassa* grown on ammonium salts instead of nitrate show no requirement for FAD (unpublished observations of S. R. Targan, R. H. Garrett, and A. Nason). The evidence that NADPH-nitrate reductase activity is due to a single protein is inconclusive, especially since electrophoretic patterns show that the most purified enzyme preparations are still not homogenous. Nevertheless, the fact that this over-all activity is still retained after a 200-fold purification makes it seem reasonable that, as a physical entity, nitrate reductase constitutes a physiologically discrete enzyme system which has been inductively formed for the purpose of nitrate assimilation.

Summary.—A *b*-type cytochrome, spectrally similar to YLDH-cytochrome b_2 and liver microsomal cytochrome b_5, has been found in partially purified preparations of *Neurospora crassa* assimilatory nitrate reductase. The concentration of this cytochrome, tentatively designated as cytochrome *b*-557 (*N. crassa*), exhibits a constant ratio with nitrate reductase activity and an increasing ratio with protein concentration during purification. Reduction of the cytochrome *b*-557 component of nitrate reductase by NADPH is insensitive to cyanide and shows the same flavin requirement as over-all nitrate reductase activity, with the reduced cytochrome *b*-557 being subsequently oxidized by nitrate. Although they both contain spectrally identical cytochrome *b*, YLDH cannot catalyze the reduction of nitrate, and nitrate reductase cannot utilize lactate as a substrate for reduction of its cytochrome *b* or for the over-all reduction of nitrate. Cytochrome *b*-557 is tentatively postulated to act as an electron carrier between FAD and molybdenum in the *Neurospora* assimilatory nitrate reductase in the following sequence:

$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{Cytochrome } b\text{-557} \rightarrow \text{Mo} \rightarrow NO_3^-$$

* Contribution no. 511 of the McCollum-Pratt Institute. This investigation was supported in part by research grant GM-02332 from the National Institutes of Health, U.S. Public Health Service.

† Predoctoral fellow of the U.S. Public Health Service.

[1] Sato, R., in *Inorganic Nitrogen Metabolism*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins University, 1956), p. 163.

[2] Nason, A., and H. Takahashi, *Ann. Rev. Microbiol.*, **12**, 203 (1958).

[3] Nason, A., *Bacteriol. Rev.*, **26**, 16 (1962).

[4] Takahashi, H., S. Taniguchi, and F. Egami, in *Comparative Biochemistry*, ed. M. Florkin and H. S. Mason (New York: Academic Press, 1963), vol. 5, p. 92.

[5] Nason, A., in *The Enzymes*, ed. P. D. Boyer, H. Lardy, and K. Myrbäck (New York: Academic Press, 1963), vol. 7, p. 587.

[6] Pichinoty, F., and L. D'Orano, *Nature*, **191**, 879 (1961).

[7] Clark, J. T., *Ann. N.Y. Acad. Sci.*, **121**, 428 (1964).

[8] Nason, A., and H. J. Evans, *J. Biol. Chem.*, **202**, 655 (1953).

[9] Layne, E., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. 3, p. 447.

[10] Morton, R. K., J. McD. Armstrong, and C. A. Appleby, in *Haematin Enzymes*, ed. J. E. Falk, R. Lemberg, and R. K. Morton (Oxford: Pergamon Press, 1961), vol. 2.
[11] Strittmatter, P., in *The Enzymes*, ed. P. D. Boyer, H. Lardy and K. Myrbäck (New York: Academic Press, 1963), vol. 8, p. 113.
[12] Taniguchi, S., and E. Itagaki, *Biochim. Biophys. Acta*, **44,** 263 (1960).
[13] Yamanaka, T., T. Horio, and K. Okunuki, *Biochim. Biophys. Acta*, **40,** 349 (1960).
[14] Shichi, H., and D. P. Hackett, *J. Biol. Chem.*, **237,** 2955 (1962).
[15] *Ibid.*, p. 2959.
[16] Shichi, H., D. P. Hackett, and G. Funatsu, *J. Biol. Chem.*, **238,** 1156 (1963).
[17] Shichi, H., H. E. Kasinsky, and D. P. Hackett, *J. Biol. Chem.*, **238,** 1162 (1963).
[18] Nicholas, D. J. D., and A. Nason, *J. Biol. Chem.*, **211,** 183 (1954).
[19] Kinsky, S. C., and W. D. McElroy, *Arch. Biochem. Biophys.*, **73,** 466 (1958).