Purification and Properties of Xanthine Oxidase from *Enterobacter cloacae*

Yozo MACHIDA and Toru NAKANISHI

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.,
Machida-shi, Tokyo 194, Japan

Received July 31, 1980

Enterobacter cloacae KY 3074 grown in a medium containing xanthine, hypoxanthine, guanine, or their nucleosides and nucleotides produced xanthine oxidase. The purified enzyme preparation showed a major protein band and a few minor bands in acrylamide gel electrophoresis. Molecular oxygen was the most effective electron acceptor. Ferricyanide and 2,6-dichlorophenolindophenol also served as electron acceptors, but NAD and NADP did not. Xanthine and hypoxanthine were good substrates, and guanine was also an effective substrate. The activity was inhibited by Ag^{2+} , Cu^{2+} , PCMB, and ascorbate. The spectrum of the *Enterobacter* enzyme resembled that of some known xanthine oxidizing enzymes, and this suggests a similarity in the prosthetic groups of these enzymes. The molecular weight of the native enzyme and subunit was 128,000 and 69,000, respectively.

Xanthine-oxidizing enzymes can utilize various electron acceptors for the oxidation of hypoxanthine and xanthine to uric acid. Most of the xanthine-oxidizing enzymes from microorganisms are xanthine dehydrogenases (EC 1.2.1.37)^{1~4)} and show the highest activity in the presence of electron acceptor as ferricyanide, 2,6-dichlorophenolindophenol, dyes, or ferredoxin. However, some microbial xanthine dehydrogenases use NAD as the most effective electron acceptor.^{5~8)}

Xanthine oxidase (EC 1.2.3.2) distributes among mammalian milks, but in microorganisms the enzyme activity is weak as compared with xanthine dehydrogenase activity.

Woolfolk and Downard reported on the bacterial xanthine oxidase from *Arthrobacter* S-2.⁹⁾ With molecular oxygen, their oxidase oxidized xanthine in a rate of only 64% of that observed with ferricyanide as the electron acceptor. Therefore, molecular oxygen does not seem to be the most effective electron acceptor for the enzyme.

We investigated xanthine oxidase (XOD) activities of the microorganisms grown in a medium containing xanthine, and found *Enterobacter cloacae* KY3074 to be prominent in the enzyme activity. The *Enterobacter* xanthine oxidase gave the highest activity with molecular oxygen.

This paper reports on the purification and some properties of xanthine oxidase of *E. cloacae* KY3074.

MATERIALS AND METHODS

Cultivation of cells and preparation of crude enzyme extracts. The microorganisms examined were cultured aerobically in the medium containing 0.2% xanthine, 0.1% peptone, 0.5% corn steep liquor, 0.05% KH_2PO_4 , 0.1% K_2HPO_4 , and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at pH 7.2 for 72 hr at 30°C. The grown cells were collected by centrifugation, suspended in 50 mM Tris·HCl buffer (pH 8.0), and disrupted with the Kubota Ultrasonic Generator (KMS-250) for 10 min. The disrupted mixture was centrifuged and the supernatant was used as a crude enzyme extract.

Enzyme assay. Xanthine oxidase was assayed by the following two methods: Assay method I was performed by measuring absorbance at 283 nm due to uric acid produced by oxidation of xanthine. The standard assay mixture was 3 ml as a total volume and contained 100 μmol Tris·HCl buffer (pH 7.3), 2 μmol xanthine and enzyme. The reaction was performed at 37°C for 10 min, and was stopped by the addition of 12 ml of 0.2 N HCl, and the absorbance was measured at 283 nm. The reference value was provided by adding HCl prior to the addition of enzyme and measured as above. The molecular extinction coefficient of uric acid under the condition was 1.238×10^4 .¹⁰⁾ One unit of enzyme activity was defined as the enzyme amount that produced 1 μmol uric acid per min at pH 7.3 and 37°C. Assay method II was done by the method of Allain *et al.*¹¹⁾ The standard assay mixture

contained 100 μ mol Tris·HCl buffer (pH 7.3), 2 μ mol xanthine, 5 μ mol 4-aminoantipyrine, 5 μ mol phenol, 2.5 units peroxidase, and enzyme in total volume of 3 ml. The reaction was performed at 37°C for 10 min, and the production of H_2O_2 by oxidation of xanthine was measured in absorbance at 500 nm. The molecular extinction coefficient of 6.3×10^3 at pH 7.3 was used. One unit of enzyme activity was defined as 1 μ mol H_2O_2 production from xanthine per min at pH 7.3 and 37°C.

Protein assay. The amount of protein was determined by the method of Lowry *et al.*¹²⁾ using bovine serum albumin as a standard. In chromatographic procedures, the absorbance at 280 nm was measured.

Electrophoresis. Analytical disc electrophoresis was carried out according to the method by Davis¹³⁾ using columns of 7.5% polyacrylamide gel with the pH 8.3 buffer system. Protein samples were loaded on the columns and a constant current of 4 mA per column was applied at room temperature. The gel was stained for protein with Amido Black 10B and for the enzymatically active zone by incubating it in the standard assay mixture of the enzyme assay method II.

SDS-Electrophoresis. SDS-electrophoresis was performed by a modification of Weber and Osborn¹⁴⁾ with columns of 10% polyacrylamide gel containing 0.1% SDS. Electrophoresis was performed at a constant current of 8 mA per column at room temperature. Cytochrome *c* (molecular weight, 12,400), myoglobin (17,800), chymotrypsinogen (25,000), ovalbumin (45,000), bovine serum albumin (68,000), and phosphorylase *b* (92,500) were used as standards.

Determination of molecular weight. Gel filtration was performed according to the method of Andrews¹⁵⁾ with a column of Sephadex G-200 (2.6 $\times$ 75 cm) equilibrated with the 50 mM Tris·HCl buffer (pH 8.0) containing 0.1 M KCl. Elution was performed with the same buffer, and eluates were collected in 3 g fractions. Ovalbumin (45,000), bovine serum albumin (68,000) and alcohol dehydrogenase (148,000) were used as standards.

Isoelectric focusing. Isoelectric focusing was carried out according to Vesterberg and Svensson.¹⁶⁾ The enzyme protein was applied to a column (110 ml) of carrier ampholite with a pH range of 3.5 to 5.0. A constant potential of 900 V was applied to the column at 5°C for 22 hr and the column was fractionated into 2 g fractions.

Chemicals. Most of the nucleotide-related compounds were obtained from Kyowa Hakko Kogyo Co. Peroxidase, purine, uric acid, xanthosine and 4-aminoantipyrine were obtained from Sigma, Nakarai Chemicals, Koso Chemicals Co., P-L Biochemicals, and

Wako Pure Chemicals Industries, respectively. Other compounds were of reagent grade and from commercial sources.

RESULTS

Xanthine oxidase activity in various microorganisms

Brevibacterium imperiale, *Brevibacterium fusum*, *Corynebacterium rathayi*, *Pseudomonas gelidicola*, and *Sarcina aurantiaca* were capable of growing with a medium containing xanthine among many bacteria, yeasts, actinomycetes, and molds. Of these bacteria, xanthine oxidase activity was observed in only *Enterobacter cloacae* KY3074 by using assay method II.

The following experiments were made with *Enterobacter cloacae* KY3074.

Induction of xanthine oxidase

The enzyme activity of the cells grown in media containing various purines, nucleosides, or nucleotides was compared (Table I). This enzyme activity was found in the cells grown in media containing guanine, hypoxanthine, xanthine, or their nucleosides and nucleotides.

TABLE I. XANTHINE OXIDASE ACTIVITY OF THE CELLS GROWN ON VARIOUS PURINES

Each substrate was added instead of xanthine to the medium described in MATERIALS AND METHODS at concentration of 20 mM. *Enterobacter cloacae* KY3074 was cultured for 2 days, and cells were collected by centrifugation. Xanthine oxidase activity was indicated with the activity in one ml of cultured fluid.

Purine base	XOD activity (munits/ml)
None	12
Purine	23
Adenine	11
Guanine	81
Xanthine	75
Hypoxanthine	177
Adenosine	27
Guanosine	191
Xanthosine	113
Inosine	113
GMP*	172
IMP*	84
Uric acid	38

GMP*, guanosine-5'-monophosphate;
IMP*, inosine-5'-monophosphate.

Purification of the enzyme

Unless otherwise stated, all purification procedures were carried out at about 5°C in 50 mM Tris·HCl buffer (pH 8.0) and centrifugation was carried out at $10,000 \times g$ for 15 min.

The grown cells (wet weight 55 g) were harvested by centrifugation, suspended in the buffer and disrupted with Dyno Mill. Extracts were centrifuged and the supernatant solution was used as crude extract.

Solid ammonium sulfate was added to the crude extract and precipitates from between 0.3 and 0.65 saturations were collected by centrifugation. The precipitates were dissolved in the buffer and dialyzed. A precipitate formed during dialysis was removed by centrifugation.

The supernatant solution was loaded on a column of DEAE-cellulose (1 liter column) previously equilibrated with the buffer, and the column was washed with the buffer and then the enzyme was eluted with an increasing linear gradient concentration from 0 to 0.6 M NaCl in the buffer. The active fractions were combined and precipitated at 0.8 saturation of ammonium sulfate.

The precipitates were dissolved in the buffer and subjected to a column of Sepharose 4B (1 liter column) equilibrated with the buffer, and the column was eluted with the buffer.

The active fractions were collected and subjected to gel filtration on a column of Sephadex G-150 (1 liter column). The column was eluted with the buffer, active fractions were collected and then loaded on a column of DEAE-Sephadex A-50 (500 ml column) previously equilibrated with the buffer containing 0.1 M NaCl. The column was washed with the same buffer and then the enzyme was eluted with an increasing linear gradient concentration from 0.1 to 0.6 M NaCl in the buffer. The active fractions were collected and dialyzed against 10 times volume of 5 mM sodium phosphate buffer (pH 6.8) overnight.

The dialyzed solution was loaded on a column of hydroxyapatite (100 ml column) equilibrated with 5 mM phosphate buffer (pH 6.8) and the enzyme was eluted with a

linear gradient of buffer concentration from 5 to 80 mM phosphate buffer (pH 6.8). The active fractions were combined and dialyzed against 50 mM Tris·HCl buffer (pH 8.0) and the dialyzed solution was used as the purified enzyme. The results of the purification of the enzyme are summarized in Table II. The enzyme was purified about 400 fold from the crude extract in a yield of 14%.

TABLE II. PURIFICATION OF XANTHINE OXIDASE FROM *Enterobacter cloacae*

Step	Total unit (U)	Total protein (mg)	Sp. activity (U/mg)	Recovery (%)
Crude extract	4800	128880	0.037	100
Ammonium sulfate (30~65% sat)	2381	32500	0.073	50
DEAE-cellulose	1589	1268	1.25	33
Sepharose 4B	1258	678	1.85	26
Sephadex G-150	1176	469	2.51	24
DEAE-Sephadex A-50	874	72.6	12.0	18
Hydroxyapatite	662	43.6	15.2	14

Homogeneity of the enzyme preparation

As shown in Fig. 1, the purified enzyme gave main protein band and a few minor protein bands and an activity band with a mobility identical to that of the main protein band in analytical disc electrophoresis.

FIG. 1. Polyacrylamide Gel Electrophoresis of the Purified Xanthine Oxidase.

A, stained with Amino Black 10B; B, stained for enzyme activity as described in MATERIALS AND METHODS; C, SDS-electrophoresis.

Molecular weight and subunit structure

Gel filtration method with Sephadex G-200 was used to estimate the molecular weight of the purified enzyme. The molecular weight was calculated to be about 128,000 (Fig. 2).

FIG. 2. Determination of Molecular Weight of Xanthine Oxidase.

A, ovalbumin; B, bovine serum albumin; C, alcohol dehydrogenase; D, xanthine oxidase.

FIG. 3. Determination of Molecular Weight of Xanthine Oxidase Subunit.

A, cytochrome *c*; B, myoglobin; C, chymotrypsinogen; D, ovalbumin; E, bovine serum albumin; F, phosphorylase *b*; G, xanthine oxidase.

In SDS-electrophoresis, a single protein band (Fig. 1) corresponding to a molecular weight of 69,000 was obtained, as shown in Fig. 3. It is suggested that the enzyme consists of two identical subunits.

Isoelectric point

The isoelectric point of the enzyme was found to be at pH 3.8 by isoelectric focusing.

Spectral characteristics

Absorption spectra of the oxidized and hydrosulfite-reduced forms of the enzyme are shown in Fig. 4. The oxidized enzyme ex-

FIG. 4. Absorption Spectra of the Purified Xanthine Oxidase.

—, oxidized form; ----, hydrosulfite-reduced form.

hibited absorption maxima at 280 and 450 nm, shoulder at 425 and 473 nm. Reduction of the enzyme with sodium hydrosulfite resulted in elimination of the absorption maximum at 450 nm. The spectral characteristics suggest that the enzyme is an iron-containing flavo-protein.

Effect of pH, temperature, and various compounds of the activity

The effect of pH and temperature on the activity was studied with xanthine as substrate. The enzyme was found to be most active at pH 7.1 and 35 to 45°C (Fig. 5).

The effect of various metal ions and other compounds on the activity was presented in Tables III and IV. The activity was inhibited

TABLE III. EFFECT OF METAL IONS ON THE ENZYME ACTIVITY

Reactions were performed with assay method I involving 50 munits of XOD and metal ions of indicated concentration.

Metal ion	Concentration (mM)	Inhibition (%)
MgSO ₄	1	0
MnCl ₂	1	0
CoSO ₄	1	21.3
CuCl ₂	0.01	79.6
FeCl ₃	1	44.7
AgNO ₃	0.001	54.8

FIG. 5. Effect of pH and Temperature on the Activity of Xanthine Oxidase.

(A) Activity was measured with assay method I except that reaction pH was varied. The reaction mixture contained 50 m units of XOD, 2 μ mol xanthine and various buffers. Following buffers were used: $\circ$, acetate; $\bullet$, potassium phosphate-sodium borate.

(B) Activity was measured with assay method I except that reaction temperature was varied. The reaction mixture contained 50 m units of XOD, 2 μ mol xanthine and 100 μ mol borate buffer (pH 7.0).

TABLE IV. EFFECT OF VARIOUS COMPOUNDS ON THE ENZYME ACTIVITY

Reactions were performed with assay method I involving 50 munits of XOD and additives of concentrations indicated.

Compound	Concentration (mM)	Inhibition (%)
EDTA	1	0
NaN ₃	1	0
α,α' -Dipyridyl	1	5.2
<i>o</i> -Phenanthroline	1	7.2
8-Hydroxyquinoline	1	2.1
H ₂ O ₂	1	43.3
KCN	1	2.1
Iodoacetic acid	1	3.1
PCMB*	0.01	34.7
L-Ascorbic acid	0.01	61.7

PCMB*, *p*-chloromercuribenzoate.

by Ag⁺, Cu²⁺, Fe³⁺, Co²⁺, H₂O₂, PCMB, and ascorbate.

Effect of pH and temperature on the stability

The enzyme was found to be stable at pH range between 6.2 and 6.8, when kept at 60°C

for 30 min. The activity remained 100% at temperatures below 50°C for 30 min and was lost completely at temperatures above 70°C.

Substrate specificity

The compounds listed in Table V were examined by xanthine oxidase assay method II. Xanthine, hypoxanthine, 2-hydroxypurine, guanine, and purine was oxidized at considerable rates, and the activity was highest with xanthine. Benzaldehyde and 6-mercaptopurine were oxidized slowly, whereas adenine, uric acid, acetaldehyde, and NADH were not oxidized.

TABLE V. SUBSTRATE SPECIFICITY

Enzyme reactions were performed with assay method II involving 50 munits of XOD and 5 μ mol of the substrate listed. The enzyme did not oxidize uric acid, purine nucleosides, theophylline, theobromine, and acetaldehyde.

Substrate	Relative activity (%)
Purine	23.3
Hypoxanthine	92.3
2-Hydroxypurine	88.5
6-Mercaptopurine	4.4
Xanthine	100
Adenine	0
Guanine	53.3
Benzaldehyde	9.8
NADH	0

TABLE VI. ACTIVITIES WITH VARIOUS ELECTRON ACCEPTORS

Enzyme reactions were performed anaerobically in mixture (2.0 ml) containing 73 μ mol of Tris·HCl buffer (pH 7.3), 1 μ mol xanthine, 58 munits of XOD, and one of the compounds listed at the indicated concentration at 37°C, and activities were determined by measuring absorbance changes at the wavelengths indicated between 15 and 75 seconds.

Electron acceptor	Concentration (mM)	Wavelength observed (nm)	Relative activity (%)
O ₂	dissolved	295	100
NAD	0.1	340	0
NADP	0.1	340	0
Ferricyanide	0.5	420	21.2
2,6-Dichlorophenol-indophenol	0.04	600	20.4
Nitro blue tetrazolium	0.1	540	6.0
Cytochrome <i>c</i>	0.1	550	0

When the compounds listed in Table VI were examined as electron acceptor for the oxidation of xanthine, the activity was highest with O_2 , and also ferricyanide or 2,6-dichlorophenolindophenol served as electron acceptor, but slight activity was noted with NAD, NADP, and cytochrome *c*.

K_m values obtained with Lineweaver-Burk plots of the activities were 0.11, 0.15, and 0.3 mM for xanthine, hypoxanthine, and guanine, respectively.

Stoichiometry of the reaction

The stoichiometries for the reaction were shown in Table VII. During the oxidation of xanthine, hypoxanthine, or guanine, almost stoichiometric amounts of O_2 consumption and H_2O_2 generation were observed.

TABLE VII. STOICHIOMETRY OF THE REACTION

(A) O_2 consumption was measured manometrically at 37°C for 30 min with a Warburg apparatus. The experiments were performed in mixture (2.0 ml) containing 50 μ mol Tris·HCl buffer (pH 7.3), 5 μ mol 4-aminoantipyrine, 5 μ mol phenol, 2.5 units of peroxidase, 2.6 units of XOD (side arm), and substrates listed at the concentration.

(B) H_2O_2 generation was measured by using substrates listed instead of xanthine in the assay method II involved 0.92 unit of XOD.

A

Substrate	Substrate added (μ mol)	O_2 consumed (μ mol)
Xanthine	1.25	1.22
	2.50	2.25
Hypoxanthine	1.25	2.17
	2.50	4.65

B

Substrate	Substrate added (μ mol)	H_2O_2 generated (μ mol)
Xanthine	0.15	0.15
Hypoxanthine	0.15	0.27
Guanine	0.15	0.14

DISCUSSION

We found the xanthine oxidase activity in *Enterobacter cloacae* KY3074 among the bacteria grown in a medium containing xanthine.

The crude extract of the other bacteria

grown on the xanthine media indicated a weak xanthine oxidase activity, when uric acid production from xanthine was measured with O_2 as electron acceptor (assay method I). Woolfolk and Downard has also reported that some bacteria indicate a weak xanthine oxidase activity as our experiments.⁴⁾

Although the purine catabolism of *E. cloacae* KY3074 is easily inferred from the reports that some of Enterobacteriaceae can utilize purine bases as a source of carbon and nitrogen,¹⁷⁾ the apparent enzyme activity was only observed in this strain among Enterobacteriaceae. Furthermore, this strain grew in a medium containing uric acid and produced uricase, as previously reported.¹⁸⁾

Xanthine oxidase was induced in the bacterial cells by guanine, xanthine, and especially hypoxanthine, all of which were substrate of the enzyme. The effective enzyme induction by hypoxanthine may be related to the two step oxidation of the compound uptaken from the surrounding medium by the enzyme. The enzyme induction by guanine is not clear as to whether this is done by guanine itself or xanthine produced from guanine by guanine deaminase or by both of them. Guanosine and GMP having a moderately higher solubility than guanine induced the enzyme as effectively as hypoxanthine. This result may suggest that guanine can induce the enzyme essentially as well as hypoxanthine can, but the very low solubility prevents the uptake of guanine into the cells and effective enzyme induction by the compound. Because adenine or adenosine slightly induced the enzyme, adenine deaminase activity or adenosine deaminase activity may be very weak or does not exist in this strain.

Enterobacter xanthine oxidase was usually stable in a state of the crude extract and during purification processes. The purified enzyme preparation had a few minor protein bands in polyacrylamide gel electrophoresis, but in SDS-electrophoresis almost single protein band was obtained. The minor protein bands may be due to decomposition or coagulation of the native enzyme during the purification processes as reported for *Arthrobacter*

enzyme.⁹⁾

The enzyme may be a dimer consisted of identical subunit with a molecular weight of 69,000. The molecular weight of the enzyme was almost identical with *Streptomyces* xanthine dehydrogenase,⁸⁾ somewhat smaller than *Arthrobacter* xanthine oxidase,⁹⁾ and was about a half or one-third of those of xanthine oxidizing enzymes from other sources.^{2,5~7,19~22)}

The isoelectric point was at pH 3.8, and was lower than those of other xanthine oxidizing enzymes.⁸⁾

The spectrum of this enzyme was similar to those of some xanthine oxidizing enzymes.^{6,8,9,22)} The spectrum of *Enterobacter* enzyme included the peak at 280 and 450 nm, and the shoulder at 425 and 473 nm. The peak at 450 nm and shoulders were reduced with sodium hydrosulfite. The spectral characteristics suggest that *Enterobacter* enzyme is flavoprotein containing non-hem iron as other xanthine oxidizing enzymes.

The enzyme had a relatively wide substrate specificity for various purine bases. The enzyme oxidized xanthine, hypoxanthine, and also guanine at considerable rates. During the oxidation of guanine the release of ammonia due to guanine deaminase was not detected. The determination of ammonia was performed by measuring the reductive amination of α -ketoglutarate catalized by glutamate dehydrogenase. Therefore, the XOD oxidizes guanine and the oxidation product appears to 8-hydroxyguanine same as that of xanthine dehydrogenase from *Streptomyces*.⁸⁾

The xanthine dehydrogenase from *Streptomyces* can oxidize guanine, but the enzyme has a higher affinity for guanine than for hypoxanthine and xanthine in contrast to *Enterobacter* enzyme.

The K_m value for xanthine was higher than those of milk enzyme and other related enzymes, and was similar to those of *Arthrobacter* enzyme.⁹⁾

The enzyme oxidized benzaldehyde same as milk enzyme,²³⁾ but did not oxidize NADH.

In the *Enterobacter* enzyme, O_2 , ferricyanide, and 2,6-dichlorophenolindophenol served as

an electron acceptor. Of these electron acceptors, O_2 was the most effective electron acceptor, and this was very different from other xanthine oxidizing enzymes.^{7,8,23)} Although the xanthine oxidase has been reported from *Arthrobacter* by Woolfolk and Downard⁹⁾ O_2 is not the most effective electron acceptor, but ferricyanide is most effective for the enzyme. Furthermore, also in milk xanthine oxidase ferricyanide is most effective.²²⁾ Milk enzyme and *Arthrobacter* enzyme is similar to aldehyde oxidase in the electron acceptor specificity. In these enzymes, O_2 shows only about 80% activity of ferricyanide as electron acceptor.²³⁾

From these results, we concluded that the *Enterobacter* enzyme is a new type of xanthine oxidase given the highest activity toward molecular oxygen.

REFERENCES

- 1) W. H. Bradshaw and H. A. Barker, *J. Biol. Chem.*, **235**, 3620 (1960).
- 2) S. T. Smith, K. V. Rajagopalan and P. Handler, *J. Biol. Chem.*, **242**, 4108 (1967).
- 3) C. A. Woolfolk, B. S. Woolfolk and H. R. Whiteley, *J. Biol. Chem.*, **245**, 3167 (1970).
- 4) C. A. Woolfolk and J. S. Downard, *J. Bacteriol.*, **130**, 1175 (1977).
- 5) I. L. Sin, *Biochim. Biophys. Acta*, **410**, 12 (1975).
- 6) E. S. Lyon and R. H. Garrett, *J. Biol. Chem.*, **253**, 2604 (1978).
- 7) T. Sakai and H. K. Jun, *Agric. Biol. Chem.*, **43**, 753 (1979).
- 8) T. Ohe and Y. Watanabe, *J. Biochem.*, **86**, 45 (1979).
- 9) C. A. Woolfolk and J. S. Downard, *J. Bacteriol.*, **135**, 422 (1978).
- 10) J. L. Mahler, *Anal. Biochem.*, **38**, 65 (1970).
- 11) C. C. Allain, L. S. Poon, C. S. G. Chan, W. Richmond and C. Fu, *Clin. Chem.*, **20**, 470 (1974).
- 12) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 13) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
- 14) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
- 15) P. Andrews, *Biochem. J.*, **91**, 222 (1964).
- 16) O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, **20**, 820 (1966).
- 17) G. D. Vogels and C. V. D. Drift, *Bacteriol. Rev.*, **40**, 403 (1976).
- 18) Y. Machida and T. Nakanishi, *Agric. Biol. Chem.*, **44**, 2811 (1980).

- 19) R. Y. Andres, *Eur. J. Biochem.*, **62**, 591 (1976).
 - 20) N. J. Lewis, P. Hurt, H. M. Sealy-Lewis and C. Schazzocchio, *Eur. J. Biochem.*, **91**, 311 (1978).
 - 21) K. V. Rajagopalan and P. Handler, *J. Biol. Chem.*, **242**, 4079 (1967).
 - 22) V. Massey, P. E. Brumby, H. Komai and G. Palmer, *J. Biol. Chem.*, **244**, 1682 (1969).
 - 23) R. C. Bray, "The Enzymes," Vol. XII, ed. by P. D. Boyer, Third Edition, Academic Press Inc., New York, N. Y., 1975, p. 299.
-