한국농화학회지 Vol. 21, No. 3, September 1978
J. Korean Agricultural Chemical Society

Studies on Xanthine Oxidase from Bovine Thyroid Glands

[Part 2] Composition and Some Properties

Hyo-sa Lee
College of Agriculture, Kyungpook National University
(Received May 13, 1978)

소의 갑상선에 있는 크산친 옥시다아제에 관한연구

〔제 2 보〕 효소의 조성과 특성

이 효 사
경북대학교 농과대학
(1978년 5월 13일 수리)

SUMMARY

Xanthine oxidase from bovine thyroid glands was found to contain FAD, molybdenum and iron in a ratio 1:0.36:1.6. The molecular weight of the thyroid enzyme was similar to that of the milk enzyme when estimated by gel filtration and polyacrylamide gel electrophoresis. The optimum pH for the enzyme activity was 7.8. The pH of the isoelectric point was determined to be 6.2 by electrofocusing. Sodium dodecyl sulfatepolyacrylamide gel electrophoresis experiment indicated that the enzyme was dissociated into subunits and that the molecular weight for the smallest subunit was 65,000 daltons.

Absorption spectra were dissimilar between milk and thyroid xanthine oxidase.

INTRODUCTION

Xanthine oxidase(EC 1.2.3.2) is regarded as one of the most complex of metalloflavoproteins. It has been generally agreed that highly purified milk enzyme contains flavin adenine dinucleotide(FAD), molybdenum, and iron in the ratio of 1:4:4[1-3]. A number of evidence has been accumulated that the variations of the enzyme activity in xanthine oxidase preparations is correlated with the content of cofactors, molybdenum[1-5] and FAD[6-8]. Recently, several groups of investigators have studied with xanthine oxidase isolated from enzyme-poor sources such as rat brain[9] and mouse skeletal muscle [10] and have found that the enzyme has considerably low specific activity as compared with milk or liver enzyme. However, the study concerning the composition of the enzyme isolated from enzyme-poor sources has not been attempted to search for a possible correlation between the enzyme activity and cofactors.

The previous paper described the isolation of thyroid xanthine oxidase and the purification to electrophoretic homogeneity. The study reported here was undertaken to characterize thyroid xanthine oxidase further in terms of composition of cofactors, physico-chemical and spectral properties, and subunit structure. The results indicate that xanthine oxidase from thyroid glands

contains FAD, molybdenum, and iron but quantitatively less as compared with the enzyme isolated from enzyme-rich sources. The absorption spectrum of the enzyme is dissimilar to that of milk enzyme. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis suggests that the enzyme may consist of four subunits with a molecular weight of 65,000 daltons.

MATERIALS AND METHODS

All chemicals and assays were as described in the preceding paper.

Isoelectric point determination. The isoelectric point of the enzyme was determined by the procedure of LKB-Produkter AB[11] in an LKB 8101 Ampholine electrofocusing apparatus. Ampholine(pH 5-8) used as a carrier ampholite. Ten mg of the purified enzyme, which was dialyzed against 0.005M Tris-HCl, pH 8.1, was applied to the column. The electrofocusing experiment was carried out at 0°C for 48 hr. The pH of the effluent was measured with an Orion Model 701 digital pH meter.

Molecular weight determination by gel filtration. The molecular weight of the enzyme was estimated on a Sephadex G-200 column(1× 78cm) by the procedure of Andrews[12]. The molecular weight was calculated by plotting the data according to the method developed by Whitaker[13]. The following standard proteins were employed as molecular weight markers: apoferritin (480,000), γ-globulin (human)(160,000), serum albumin(67,000) and egg albumin (45,000). Blue dextran 2,000(2×10^6) was used to determine the void volume of the column. Each protein(0.5mg) in 2ml of 0.1M phosphate buffer, pH7.4, was separately applied to the column. The proteins were eluted at a constant flow rate of 1.5ml/20 min/tube in cold room. The protein was measured by the absorbancy of the effluents at 280nm.

Molecular weight determination by disc gel electrophoresis. The molecular weight of the enzyme was determined electrophoretically by the slightly modified procedure of Hedrick and Smith[14]. The ratio of bisacrylamide to acrylamide monomer concentration was kept constant for the variation of the resolving gel concentrations.The following standard proteins were employed as molecular weight marker: Thyroglobulin (680,000), milk xanthine oxidase(290,000) γ-globulin(156,000), bovine serum albumin (68,000) and egg albumin(45,000). The samples containing 50μg of protein in 0.1ml were electrophorized at room temperature at 2.5 ma/tube.

SDS-Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by the method of Shapiro *et al.*[15]. Standard protein samples and milk and thyroid xanthine oxidase were dissolved in 0.01M phosphate buffer, pH 7.1, containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and incubated at 37°C for 3 hr.The samples were then dialyzed overnight against 0.01M phosphate, pH 7.1, with 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol at room temperature. The dialyzed samples containing 50μg of protein were electrophorized in 5% acrylamide gel. The gels were stained with Coomassie brilliant blue for 15 hr and destained in acetic acid/methanol/water solution(14:40:160, v/v/v).

FAD determination. The FAD content of electrophoretically homogeneous thyroid xanthine oxidase was fluorometrically determined by the method of Burch[16]. The enzyme solution(0.3ml) containing 3-6mg of protein was mixed with 3ml of 11% trichloroacetic acid to release FAD. After 15min the sample was centrifuged in cold room and an aliquot of 0.2ml was neutralized with 1ml of 0.2M K_2HPO_4 and another sample allowed to sit for 2 days in the dark to hydrolyze the FAD to flavin mononucleotide(FMN). Riboflavin(0.5μg/ml) was carried through the entire procedure. The fluorescent measurement of the samples was measured in the direct recording mode using a Perkin-Elmer Model MPF-3 Fluorescence Spectrophotometer. The excitation wave length was set at 450nm and the floures cent(emission) wave length at 520nm.

Molybdenum determination. The molybde-

num content of thyroidal xanthine oxidase was determined by the dithiol method of Bingley[17]. The electrophroetically homogeneous enzyme containing an equivalent of 0.6mg of protein was digested with concentrated H_2SO_4 and HNO_3.

Iron determination. The iron was estimated as the ferrous Tris-0-phenanthroline chelate after trichloroacetic acid denaturation[18]. The same amount of the enzyme was used as emplo yed for molybdenum determination.

Copper determination. The content of copper was determined by a micro adaptation[18] of the method of Martens and Githens[19]. The enzyme (0.6mg protein) was digested with concentrated H_2SO_4 and HNO_3 and then the copper was extr-acted from the acidic aqueous medium by reacting with 0.01% zinc dibenzyl-dithiocarbamate in carbon tetrachloride.

RESULTS AND DISCUSSION

pH Effect on the enzyme activity. Most investigators have found that the pH optimum of xanthine oxidase is between pH 7 and 8.5. It has been shown that the maximum activity of the milk enzyme is dependent on pH, buffer, and ionic strength of the reaction mixture[20]. Figure 1 shows the effect of pH on thyroid xan

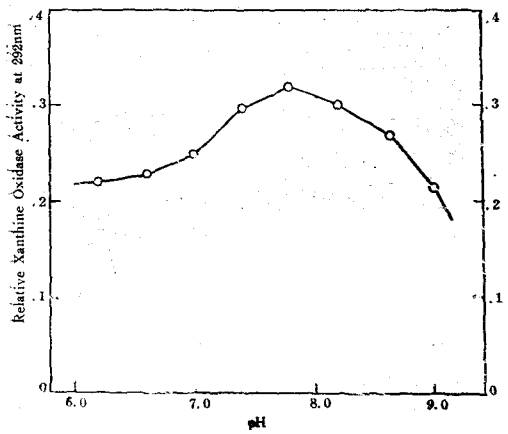

Fig. 1. pH Effect on thyroid xanthine oxidase activity. The enzyme activity was determined under the condition described in the text except the buffer varied: 0.1M phosphate buffer(K^+), pH 6.2-7.8, and 0.1M Tris buffer(Cl^-), pH8.2-9.0.

thine oxidase activity in the pH range of 6.2-9.0, using 0.1M phosphate, pH 6.2-7.8, and 0.1 M Tris-HCl buffer, pH 8.2-9.0. The optim um pH for the enzyme activity was found to be pH 7.8 in 0.1M phosphate buffer.

Isoelectric point. The isoelectric point of thyroid xanthine was pH 6.2 as determined by electrofocusing using ampholine with a pH range of 5-8 (Fig. 2). This result agrees with the isoelectric point of 6.2 for milk enzyme as dete rmined by a cataphoresis experiment[21]. How-

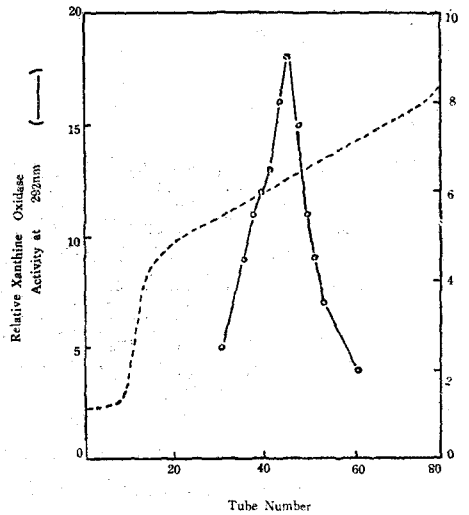

Fig. 2. Electrofocusing elution pattern of thyroid xanthine oxidase. The experimental conditions were described in the text. Each tube contained 1.5ml of effluent.

ever, Avis *et al.*[1] reported that milk enzyme has somewhat lower isoelectric point, pH 5.3-5.4, by estimating electrophoretic mobilities at different pH values in an acetate buffer with an ionic strength of 0.2.

Molecular weight. The molecular weight of thyroidal xanthine oxidase estimated from data presented in Fig. 3 and Fig. 4 was 240,000 by gel filtration with Sephadex G-200 and 290,000 by analytical gel electrophoresis. These values are similar to those for the milk enzyme which was simultaneously determined as a standard protein. However, a wide range of molecular weights for xanthine oxidase obtained from several sources has been reported. Also, the differences appears

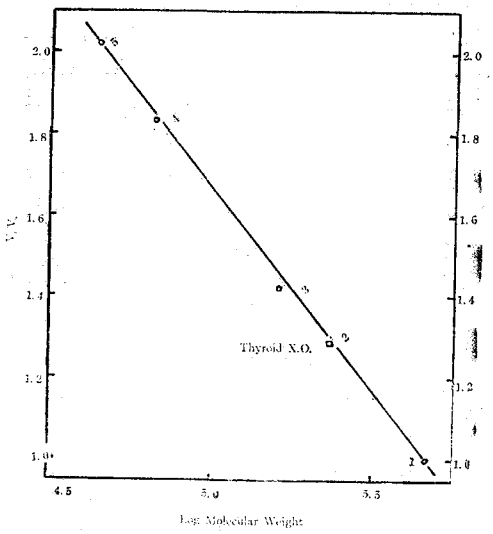

Fig. 3. Determination of the molecular weight of thyroid xanthine oxidase on Sephadex G-200. The void volume(Vo) of the column was estimated with Blue Dextran 2000. V: elution volume for each protein. Standard proteins: 1) apoferritin, 2) milk xanthine oxidase, 3) γ-globulin, 4) bovine serum albumin, 5) egg albumin.

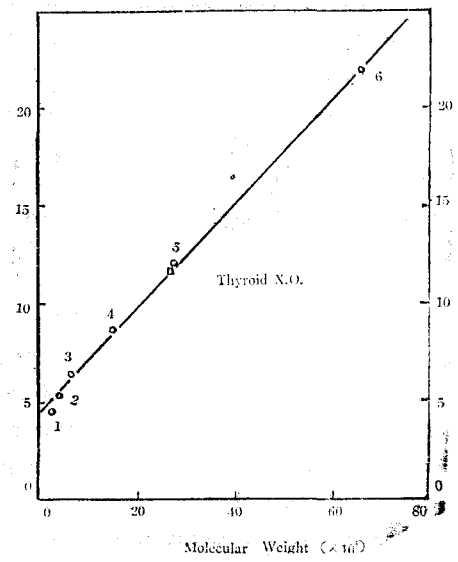

Fig. 4. Determination of the molecular weight of thyroid xanthine oxidase by disc gel electrophoresis. The electrophoresis experimental conditions were described in the text. Four different resolving gel concentrations; 5%, 7%, 9%, and 11% were employed to estimate the slope from a log R_f(relative migration distance of protein to dye) versus gel concentration plot. Standard proteins as molecular weight markers: 1) bovine carbonic anhydrase (30,000), 2) egg albumin (45,000), 3) bovine serum albumin (68,000), 4) γ-globulin(156,000), 5) milk xanthine oxidase(290,000), and 6) thyroglobulin(680,000).

to be dependent upon the methods used for enzyme preparation and the methods employed for molecular weight determination. The largest difference in molecular weight has been found with the pig liver enzyme. Brumby and Massey[22] have determined 385,000 by the Archibald method and 190,000 by gel filtration. The range of the molecular weight for the milk enzyme lies between 240,000 and 362,000 depending upon the enzyme preparations and the measuring methods[1-3,23,24].

Subunits of thyroid xanthine oxidase. The electrophoretically pure thyroid enzyme was subjected to gel electrophoresis in sodium dodecyl sulfate. Three distinct protein bands appeared in the gel(Fig. 5). The molecular weights of the three proteins dissociated from the thyroid enzyme were estimated to be 182,000, 128,000 and 65,500(Fig. 6). These results indicate that the thyroidal xanthine oxidase may consist of four subunits with a molecular weight of 65,500. Recently, it has been reported that milk xanthine oxidase is dissociated into a subunit with molecular weight of 150,000 by sodium dodecyl sulfate[25]. Earlier, the similar result was obtained by treating the enzyme with guanidine and acid[26]. However, both sodium dedecyl sulfate and guanidine treatment did not bring the molecular weight below 100,000.

Composition of thyroidal xanthine oxidase. The enzyme prepared from preparative polyacryamide gel electrophoresis was subjected to determination of the common cofactors of xanthine oxidase. The results are given in Table 1. A salient feature is the fact that thyroid xanthine oxidase also contains FAD, molybdenum, and

Fig. 5. Diagram of SDS gel electrophoresis of thyroid xanthine oxidase. The electrophoresis experimental conditions were described in the text. The proteins were migrated from cathode to anode. I, II, III protein bands with R_f of 0.52, 0.60, and 0.76.

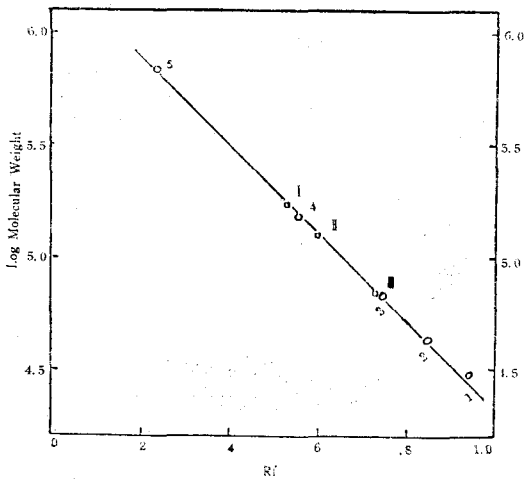

Fig. 6. Estimation of the molecular weight of thyroid xanthine oxidase subunits appeared SDS gel electrophoresis. The standard proteins used are designated by arabic numbers: 1) bovine carbonic anhydrase, 2) egg albumin,3) bovine serum albumin, 4) γ-globulin, and 5) thyroglobulin. The deaggregated fractions of thyroid xanthine oxidase are designated by Roman numbers, I, II, and IIII.

iron. Surprisingly, the contents of all three cofactors of thyroid enzyme are considerably lower than those of xanthine oxidase from milk [2,3] and liver[27]. The molecular weight of thryoid xanthine oxidase was estimated to be 290,000 on the basis of one mole of FAD per mole of thyroid enzyme. This value is reasonalbly close to the molecular weight determined by gel filtration and gel electrophoresis method.

The composition of xanthine oxidase has been extensively investigated in order to correlate the variations of the enzyme activity with the cont ents of the cofactors in the enzyme preparations. It was observed by Avis *et al.*[1] that the variability of the enzyme activity is somehow assoc iated with the content of molybdenum in the milk enzyme preparations. They assumed that the milk enzyme preparations include two inactive forms; demolybdo-xanthine oxidase and inactivated xanthine oxidase. Recently, Hart, *et al.*[3] reported that the demolybdoxanthine oxid ase has a FAD : Mo : Fe ratio of 1 : 0 : 4 while the inactivated enzyme has the ratio 1 : 1 : 4 as found for the active form.It was concluded that demolybdo form is a natural product secreted along with the active form and that the inact ivated form is originated during storage of enzyme samples or enzyme preparation. Recent studies have shown that cofactors are directly responsible for the enzyme activity since the removal of FAD from the enzyme[6,8] and the substitution of molybdenum with tungsten[5] decrease the enzyme activity and there constituted enzymes with cofactors restore the enzyme activity. In view of the ratio of the cofactors, thyroid xanthine oxidase is different from not only an active form but both inactive forms of milk xanthine oxidase. The low content of cofactors in thyroid xanthine oxidase may be accounted for by either the nature of the enzyme or the loss of the cofactors during the enzyme purification. Hewever, it seems unlikely that the great amount of iron is dissociated from the enzyme during the purification if thyroid xanthine oxidase is assumed to have a similar struc-

tural characteristics to the most studied milk enzyme. The low content of cofactors in thyroid xanthine oxidase conforms to the results that the highly purified enzyme has a low specific activity as observed in the preceding paper. Since Roussos and Morrow[28] reported that the most active intestinal enzyme contains copper rather than molybdenum, the determination of copper content in thyroid enzyme was attempted. Copper was not detected in the analytical method which is capable of determining the low limit of 0.1 atom of copper per mole of FAD.

Absorption spectrum. The spectrum of thyroid xanthine oxidase shown in Fig. 7 has somewhat different features when compared with the enzyme isolated from milk[2,21,29,30] and liver[27]. This spectrum shows an indication of the presence of less flavin in the 450 nm region

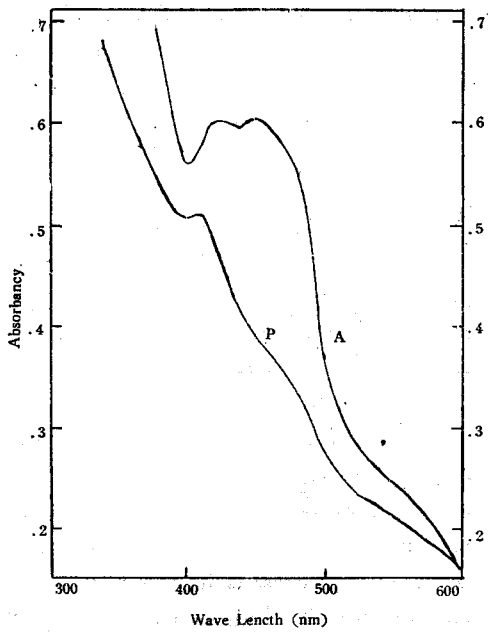

Fig. 7. Absorption spectrum of xanthine oxidase. Curve A: milk xanthine oxidase, Curve B: thyroid xanthine oxidase. Milk enzyme purified from calcium phosphate gel-cellulose column was dialyzed against 5mM Tris-HCl buffer, pH8.1, before scanning the enzyme spectrum. Thyroid enzyme eluted from polyacrylamide gel with 0.1 M Tris-HCl buffer, pH 8.1, was used for the absorption spectrum.

in contrast to the milk enzyme and displays a shoulder at 400-410nm. Thyroid enzyme spectrum is not similar to either that of deflavo enzyme[2] or that of molybdenum free milk xanthine oxidase[5]. Neither spectrum of desulfo milk enzyme[31] shows the similarity to that of thyroid enzyme. At the present time, it is difficult to explain the dissimilarity between the absorption spectrum of the milk enzyme and that of the thyroid enzyme. The dissimilarity may be due to the different contents of cofactors between two enzymes.

적 요

소의 갑상선에 있는 xanthine oxidase 효소도 flavin adenine dinucleotide(FAD), 모리브덴 및 철을 cofactor로서 가지고 있었으며 그 비율은 1 : 0.36 : 1.6이었다 갑상선 xanthine oxidase의 분자량은 gel filtration과 gel electrophoresis방법으로 측정하였을 때 우유에서 추출한 xanthine oxidase의 분자량과 큰 차이가 없었다. 그 효소의 활성도는 pH7.8에서 가장 높았고 isoelectric point는 electrofocusing으로 측정하였을 때 pH 6.2 였다.

SDS-polyacrylamide gel electrophoresis 실험 결과는 소의 갑상선에서 추출된 xanthine oxidase가 세개의 subunit로 분해되었음을 지적했고 최소 단위분자량 65,000정도의 polypeptide 4개로서 완전한 xanthine oxidase 한 분자를 구성하고 있을 가능성을 보였다. 갑상선 효소의 absorption spectrum을 우유에서 추출된 효소와 비교하였을 때 상당한 상이점을 나타내었다.

REFERENCES

1. Avis, P. G., Bergel, F., and Bray, R. C. :J. Chem. Soc., p. 1219(1956).
2. Massey, V. V., Brumby, P. E., Komai, H., and Palmer, G.: J. Biol. Chem., **244**, 1682 (1964).
3. Hart, L. I., McGartoll, M. A., Chapman,H. R., and Bray, R. C.: Biochem. J., **116**, 851 (1970).
4. Johnson, J. L., Rajagopalan, K. V., and Cohen, H. J.: J. Biol. Chem., **249**, 859 (1974).
5. Johnson, J. L., Waud, W. R., Cohen, H.

J., and Rajagopalan, K. V.: J. Biol. Chem., **249**, 5056(1974).
6. Komai, H., Massey, V., and Palmer, G.: J. Biol. Chem., **244**, 1962(1969)
7. McGartoll, M. A., Pick, F. M., Swann, J. C., and Bray, R.C.: Biochim.Biophys. Acta, **212**, 523(1970).
8. Kanda, M., Brady, F. O., Rajagopalan, K. V., and Handler, P.: J. Biol. Chem., **247**, 765(1972).
9. Markley, H. G., Faillace, L. A.,and Mezey, E.: Biochim. Biophys. Acta, **309**, 23(1973).
10. Lalanne, M., and Willemot,J.: Int. J. Bioch em., **6**, 479(1975).
11. LKB-Produkter AB, "LKB 8100 Ampholine Electrofocusing Equipment Instruction Mannual," S-16125 Brommal, Sweden (1972).
12. Andrews, P.: Biochem. J.,**91**, 222(1964).
13. Whitaker, J. R.: Anal. Chem., **35**, 1950 (1963).
14. Hedrick, J. L., and Smith, A. J.: Arch. Biochem. Biophys.,**126**, 155(1968).
15. Shapiro, A. L., Vinuela, E., and Maizel, J. V.: Biochem. Biophys. Res. Commun., **28**, 815(1967).
16. Burch, H. B. in Methods in Enzymology (Colowick, S. P. and Kaplan, N.O., Eds.) Vol. III, p. 960, Academic Press, New York (1957).
17. Bingley, J. B.: J. Agr. Food Chem., **7**, 269 (1959).
18. Brumby, P. E., and Massey, V. in Methods in Enzymology(Colowick, S. P. and Kaplan, N. O., Eds.) Vol. X, p. 463, Academic Press, New York (1967).
19. Martens, R. I., and Githens,R. E. Sr.:Anal. Chem., **24**, 991(1952).
20. Fridovich, I., and Handler, P.: J. Biol. Chem., **233**, 1578 (1958).
21. Ball, E. G.: J. Biol. Chem., **128**, 51(1939).
22. Brumby, P. E., and Massey, V.: Biochem. J., **89**, 46p. (1963).
23. Avis, P. G.,Bergel, F., Bray, R. C., James, D. W. F., and Schooter, K. V.: J. Biol. Chem. Soc., 1212(1956).
24. Andrews, P., Bray, R. C., Edwards, P.,and Shooter, K. V.: Biochem. J., **93**, 627(1964)
25. Nagler, L. G., and Vartanyan, L. S.: Biokhimiya, **38**, 561(1973); Chem. Abstr., **79**, 143851(1973).
26. Nelson, C. A., and Handler, P.:J. Biol. Chem., **243**, 5368 (1968).
27. Kielley, R. K.: J. Biol. Chem., **216**, 405 (1955).
28. Roussos, G. G., and Morrow, B. H.: Arch. Biochem. Biophys., **114**, 599(1966).
29. Mackler, B., Mahler, H. R., and Green, D. E.: J. Biol. Chem., **210**, 149(1954).
30. Morell, D. E.: Biochem.J., **51**, 657(1952).
31. Massey, V., and Edmondson, D.: J. Biol. Chem., **245**, 6595 (1970).