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INTERACTION OF GLUCOSE OXIDASE WITH BLUE DEXTRAN*

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SUMMARY

The preparation and properties of a glucose oxidase–Blue Dextran complex are described. At pH 7.4, in 0.1 *M* phosphate buffer, glucose oxidase and Blue Dextran interact strongly with each other. Under these conditions a soluble complex is formed, as shown by ultrafiltration experiments using a Diafio XM-300 membrane (nominal molecular weight cut-off 300,000). In this complex, the enzyme is about 40% more active than when free in solution, and is also more stable towards acid denaturation. Comparative studies were carried out using a high-molecular-weight Dextran fraction devoid of dye residues (Dextran T-2000). The results obtained suggest that the dye moiety of Blue Dextran is directly involved in the binding of the protein, and possibly also in the enhancement of its enzymatic activity.

INTRODUCTION

Blue Dextran and its derivatives have been found to bind to a variety of proteins[1–13]. It has been pointed out[14,15] that some of these proteins require the presence of a dinucleotide [*e.g.*, flavin–adenine dinucleotide (FAD), nicotinamide–adenine dinucleotide (NAD) or nicotinamid–adenine dinucleotide phosphate (NADP)] or a mononucleotide [*e.g.*, riboflavin 5′-phosphate (FMN) or adenosine 5′-triphosphate (ATP)] as cofactor for their activity and that these compounds are located in a characteristic cleft provided by the tertiary structure of the proteins; this cleft has accordingly been given the generic name of "dinucleotide fold"[13,14]. Binding of Blue Dextran to some enzymes was also shown to inhibit their catalytic activity. The inhibition of enzymes such as lactate dehydrogenase (LDH) and phosphoglycerate kinase is competitive with respect to the cofactor, and it was therefore suggested that Blue Dextran could be used as a specific reagent for detecting the presence of the dinucleotide fold[13].

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It should be noted that Blue Dextran was also found to bind to other proteins, such as chymotrypsinogen A, ovalbumin, cytochrome *c* and hemoglobulin[12]. These proteins, however, are devoid of cofactors, and thus the binding of Blue Dextran occurs at different types of sites. A different behaviour was encountered with subtilisin. This enzyme contains in its three-dimensional conformation a structural feature resembling the dinucleotide fold mentioned above[16,17], nevertheless it does not bind to Blue Dextran[13].

Glucose oxidase (GO) (EC 1.1.3.4.) is a FAD-containing enzyme[18], and it was therefore of interest to investigate its interaction with Blue Dextran. The studies reported below showed that GO indeed binds tightly to the dyed polysaccharide. However, unlike the situation encountered with the nucleotide-containing enzymes mentioned above, Blue Dextran does not inhibit the enzymatic activity of GO; indeed, under appropriate conditions, binding is even associated with an enhancement of the activity of this enzyme.

EXPERIMENTAL

Materials

Glucose oxidase (mol.wt. 150,000) from *Aspergillus niger* was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.). The enzyme showed a single band upon electrophoresis on sodium dodecyl sulphate–acrylamide gel at pH 7.0, and on a cellulose acetate sheet at pH 6.8.

Dextran T-2000 (mol.wt. 2×10^6) and Blue Dextran (containing the polycyclic chromophore at a degree of substitution of 0.017) were obtained from Pharmacia (Uppsala, Sweden). Flavin–adenine dinucleotide (FAD) was purchased from Nutritional Biochemical Corp. (Cleveland, OH, U.S.A.). All other chemicals used were of reagent grade.

Methods

(a) Analytical procedures. Electrophoresis on cellulose acetate sheets[19] and on SDS-acrylamide gel[20] was performed as described.

Protein concentrations in stock solutions of GO were usually determined from absorbance measurements at 280 nm, using the values $2.7 \cdot 10^5$ l cm^{-1} mole^{-1} and 150,000 for the molar extinction coefficient and molecular weight respectively. The former value is based on independent determinations of protein concentration using the Lowry method[21], with bovine serum albumin as standard.

(b) Determination of enzymatic activity. The enzymatic activity of GO was determined essentially according to Swoboda[22]. The assay was carried out at room temperature and pH 6.2 at an enzyme concentration of 2–5 μg/ml. Oxidation of the glucose was followed by monitoring the initial rate of oxygen uptake, using a Clark O_2 electrode, Model YSI 4004 (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). The results are expressed in terms of arbitrary units (AU) of instrument reading. Under the assay conditions used, the activity of 1 mg of the GO preparation is 660 AU.

The enzymatic activity of the GO–Blue Dextran complex was determined according to the procedure given above for the free native enzyme. Control experiments were performed by omitting either the enzyme or the glucose from the assay system.

(c) Binding of glucose oxidase to Blue Dextran and Dextran T-2000. Complex formation between GO and Blue Dextran at room temperature, in 0.1 *M* sodium phosphate buffer, pH 7.4, was studied by the ultrafiltration technique, using an Ultrafiltration Cell Model 12 (Amicon, Lexington, MA, U.S.A.) equipped with an Diaflo XM-300 ultrafiltration membrane. This membrane was chosen as it is permeable to the free enzyme (mol.wt. 150,000) but not to the Blue Dextran (mol.wt. $2 \cdot 10^6$) or to the enzyme–Blue Dextran complex. Thus, to a solution of Blue Dextran (20 mg) in 1 ml buffer, various amounts (0.2–2.0 mg) of GO (as a 10 mg/ml solution, activity 6600 AU/ml) were added, and the solution was brought to a final volume of 2 ml, using the same buffer. After incubation of the mixture for 16 h at room temperature, samples were withdrawn and assayed for enzymatic activity. Then 1 ml buffer was added, the mixture was passed through the Diaflo membrane and 1 ml filtrate was collected; this procedure was repeated four times. No enzymatic activity could be detected in the last filtrate. The filtrate portions (4 × 1 ml) were pooled and the enzymatic activity of the mixture was determined. The residual solution containing the GO–Blue Dextran complex was assayed for enzymatic activity, brought to a final volume of 3 ml and used in all subsequent studies.

A few experiments were also carried out with Dextran T-2000, *i.e.*, a Dextran preparation having the same molecular weight as Blue Dextran but lacking the dye moiety. Complex formation between GO and Dextran T-2000 (ratios 1:15–1:100 w/w) was brought about by a procedure similar to the one described above for Blue Dextran. However, more washings were needed to reach the point where no more enzymatic activity could be detected in the last filtrate.

(d) Determination of the acid stability of the Blue Dextran-bound glucose oxidase. The stability of the GO–Blue Dextran complex in acidic media was tested at room temperature and pH 1.9, using a complex in which the enzyme to polysaccharide ratio was 1:20 w/w. After incubation of the complex-containing acidic solution for different time intervals, samples were withdrawn and enzymatic activity was determined at pH 6.2 in the presence and absence of excess of FAD, as follows. (1) The recovered enzymatic activity was measured as described above after raising the pH of the acidic aliquot to pH 6.2 (using 0.1 *M* sodium phosphate buffer), adding FAD (2 moles per mole of apoprotein, as a 10 μg/ml solution in the same buffer) and incubating for 1 h at room temperature. (2) The residual enzymatic activity was measured in a similar manner, except that no supplementary FAD was added to the assay mixture. For comparison, parallel experiments were carried out with the native enzyme.

(e) Determination of the temperature stability of the Blue Dextran-bound glucose oxidase. The temperature stability of the bound GO was evaluated from measurements of the residual enzymatic activity of the complex, after incubation at 45°C and 55°C for various time periods in 0.1 *M* phosphate buffer, pH 7.4. The enzymatic activity was determined at room temperature and pH 6.2, under the standard assay conditions described above.

(f) Circular dichroism (CD) measurements. CD spectra were measured at room temperature with a Cary Model 60 spectropolarimeter, equipped with a Model 6002 CD attachment. Cells of 0.1–10 mm light path length (Optical Cell Co., Beltsville, MD, U.S.A.; Hellma, Mulheim/Baden, G.F.R.) were used.

The spectrum of Blue Dextran was measured using solutions containing 6–12

mg polysaccharide per ml of 0.1 *M* sodium phosphate buffer, pH 7.4. The data are reported as mean residue ellipticity, $[\theta]$, in degrees cm^2 $dmole^{-1}$, and were calculated using a mean residue weight of 180, corresponding to that of glucose.

The spectrum of GO was measured using solutions containing 0.2–8.0 mg enzyme per ml of the same buffer.

The spectra of the GO–Blue Dextran complexes were measured using the preparations obtained as described above. The solutions contained a constant amount (20 mg) of Blue Dextran and various amounts (0.2–2.0 mg) of enzyme, all in 3 ml of 0.1 *M* sodium phosphate buffer, pH 7.4. A 1-mm light path length cell was used, and the data obtained are reported as direct instrument readings (ellipticity in degrees).

RESULTS

Interaction of glucose oxidase with Blue Dextran

Upon incubation of GO with Blue Dextran (ratios 1:10–1:100 w/w, corresponding to molar ratios of about 1:1–1:10), a soluble complex was formed. In all the cases studied and under the experimental conditions employed (room temperature, 0.1 *M* phosphate buffer, pH 7.4), quantitative attachment of the enzyme to Blue Dextran took place. This was indicated by the finding that all the enzymatic activity was present in the residual solution after filtration of the mixture through the Diaflo membrane, while essentially no activity was detected in the filtrate. These results are presented in Fig. 1.

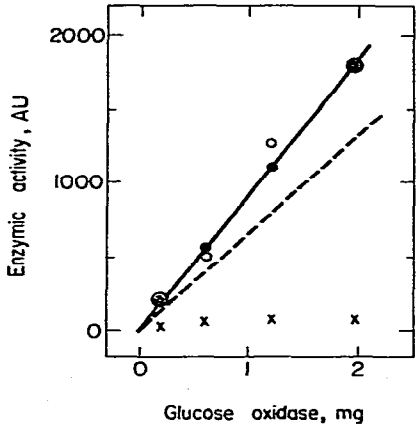

Fig. 1. Enzymatic activity of the GO–Blue Dextran complexes, as compared to the activity of equivalent amounts of native GO. ●, Activity of the complex before ultrafiltration; ○, activity of the residual solution after ultrafiltration of the complex. The enzymatic activity in the ultrafiltrate of the corresponding complex preparation is also included (×). The dashed line indicates the activity of GO prior to complex formation. The enzymatic activity is expressed in arbitrary units (AU), defined in *Methods*. For further details see Experimental.

We also found that, as a result of its binding to Blue Dextran, GO exhibits somewhat enhanced activity. These results are also presented in Fig. 1. It can be seen that the activity of GO in the complex with Blue Dextran is about 40% higher than that measured in the absence of the polysaccharide. In control experiments, oxygen uptake in the assay system was tested with Blue Dextran and glucose in the absence of

enzyme, as well as with the GO–Blue Dextran complex in the absence of glucose. In neither case could oxygen uptake be detected.

For comparison, similar experiments were carried out in which Blue Dextran was replaced by Dextran T-2000. In these experiments some of the enzyme could be withdrawn from the original solution by repeated filtrations through the Diaflo membrane. Only part of the enzyme (15–70%) was found in the complex, the more so the higher the polysaccharide content within the mixture. The complex of the enzyme with Dextran T-2000, unlike the GO–Blue Dextran complex, showed the same enzymatic activity as an equivalent amount of native enzyme.

Acid stability of native and Blue Dextran-bound glucose oxidase

The activities of the native enzyme and of the GO–Blue Dextran complex (1:20 w/w), after being kept for different periods of time at pH 1.9, were measured under the standard conditions given in *Methods*. The results are presented in Fig. 2A. The residual enzymatic activity (*i.e.*, the activity in the absence of added FAD, see *Methods*) decreased upon increasing the time of incubation in the acidic medium, and disappeared altogether after 2 h of incubation. Similar results were obtained with the native enzyme.

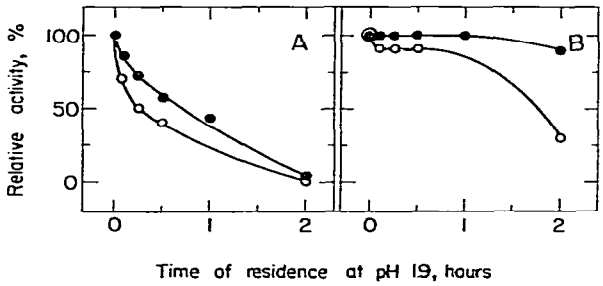

Fig. 2. Enzymatic activity of native enzyme (○) and of the Blue Dextran-bound enzyme (●), after being kept for different periods of time at pH 1.9 at room temperature. The data are expressed in terms of relative activity, measured at pH 6.2 under the standard assay conditions (see *Methods*). A, Residual activity; B, recovered activity after addition of FAD (2 equivalents per mole of protein) and incubation for 1 h at room temperature in 0.1 *M* sodium phosphate buffer, pH 6.2.

The ability of the acid-treated Blue Dextran-bound GO to recover enzymatic activity upon addition of excess of FAD was also investigated, and compared to that of the native enzyme. The results are presented in Fig. 2B. It can be seen that both enzyme preparations recovered almost full activity when incubation at pH 1.9 did not exceed 1 h. Prolonged incubation periods (up to 2 h) at pH 1.9 did not affect the reconstitution capacity of the Blue Dextran-complexed enzyme; under similar conditions the native enzyme, however, recovered only 30% of its original activity.

Temperature stability of Blue Dextran-bound glucose oxidase

The temperature stability at 45°C and 55°C of different preparations of Blue Dextran-bound GO in 0.1 *M* sodium phosphate buffer at pH 7.4 was measured and compared to the corresponding behaviour of the native enzyme. All the preparations investigated showed a steady decrease in activity with time; after incubation for 3 h, the residual activity dropped to about 60% at 45°C and to 20% at 55°C.

Circular dichroism measurements

The CD spectrum of Blue Dextran in the range of 250–460 nm is presented in Fig. 3. Positive bands are seen at 275 nm and 430 nm, and a negative, more complex spectrum is observed in the 290–400 nm region. The corresponding absorption spectrum is also included in Fig. 3; absorption maxima are observed at 280 nm and 380 nm, and a shoulder appears at about 330 nm. In the absorption spectrum of Blue Dextran an additional band is present between 500 and 700 nm, with a maximum at about 620 nm (not shown). In this region CD measurements could be performed only below 600 nm, due to the high absorbance of the sample. No CD bands could be detected in the 500–600 nm region.

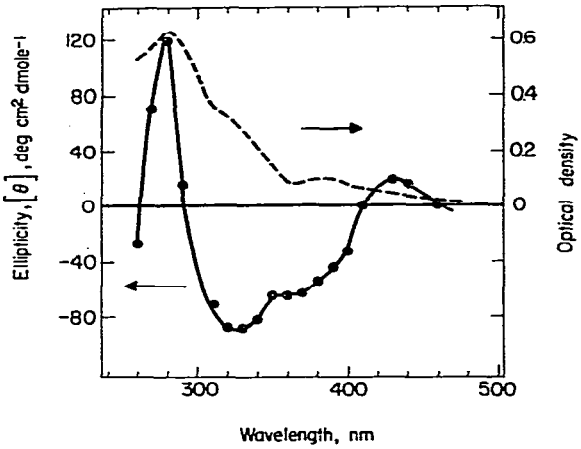

Fig. 3. Spectral properties of Blue Dextran. – – –, Absorption spectrum of a 0.2 mg/ml solution in 50 m*M* sodium phosphate buffer, pH 7.0, measured in a cell of 1-cm light path length; ———, CD spectrum in 0.1 *M* sodium phosphate buffer, pH 7.4.

In the region 260–300 nm, the CD spectrum of GO exhibits a maximum at 275 nm. At higher wavelengths the ellipticity values are very low[23,24], and at the concentration used in the GO–Blue Dextran complex no contribution of the enzyme could be detected in the corresponding spectrum.

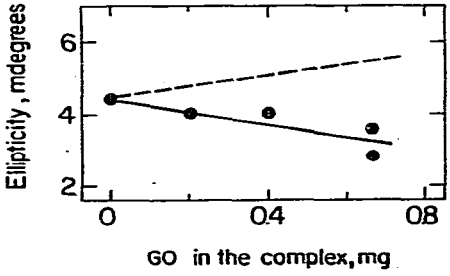

Fig. 4. Ellipticity at 280 nm (in mdegrees instrumental reading) of GO–Blue Dextran complex preparations. Each sample contained 20 mg Blue Dextran and the indicated amount of enzyme, all in 3 ml of 0.1 *M* sodium phosphate buffer, pH 7.4. Measurements were carried out using a cell of 1-mm light path length. The dashed line represents the ellipticity values expected for a non-interacting mixture, and obtained by summation of the contributions of the two components.

The CD spectra of the GO–Blue Dextran complexes studied were similar to the spectrum of the polysaccharide. Some differences were observed in the 260–300 nm region, and the results are presented in Fig. 4. It can be seen that the complex formation is accompanied by a decrease in the rotatory strength of the band(s) at 280 nm, the ellipticity measured at 280 nm being lower than the sum of the contributions of the individual components. No such changes could be detected in the 200–260 nm and 300–600 nm regions.

DISCUSSION

Blue Dextran has been proposed as a specific reagent for the identification and purification of dinucleotide fold-containing proteins, and has been shown to operate as a competitive inhibitor of LDH and phosphoglycerate kinase with respect to their corresponding cofactors[13]. Glucose oxidase is a flavoprotein, containing flavin–adenine dinucleotide as coenzyme[18]. Although we have no information concerning the existence of a dinucleotide fold in this enzyme, it was nevertheless of interest to determine whether Blue Dextran also binds to GO, and inhibits its enzymatic activity.

Using ultrafiltration techniques it was demonstrated (see Results) that GO indeed binds to Blue Dextran, with the formation of a soluble and stable complex. The dye moiety of the blue polysaccharide seems to be directly involved in the binding of GO. Thus, while the enzyme binds quantitatively to Blue Dextran under the conditions employed, only a weak complex is formed with Dextran T-2000, the corresponding polysaccharide devoid of the dye moiety. In the last case, the amount of enzyme bound depends on the ratio of the two components in the mixture.

The involvement of the dye moiety in GO binding by Blue Dextran is further inferred by the results of circular dichroism measurements. Thus, while the CD spectra of GO–Dextran T-2000 complexes are similar to that of the native enzyme, in the spectra of GO–Blue Dextran complexes the band at 280 nm has a rotatory strength that differs from what one would expect for a mixture of non-interacting components (see Fig. 4).

We also investigated the enzymatic activity of GO when complexed to Blue Dextran. Measurements were performed by determining the oxygen uptake during the initial stage of the enzyme-catalyzed reaction. This method has been shown[18,25] to correlate well with other procedures for the assay of glucose oxidase. A variety of additives, including low-molecular-weight sugars as well as dextran, have been found not to interfere with the O_2 uptake assay[25]. Furthermore, control experiments have shown that no oxygen is consumed by mixtures containing Blue Dextran and glucose but no enzyme, or enzyme and Blue Dextran but no glucose. It can thus be concluded that the assay used for measuring GO activity in the complex is reliable for determining GO activity under the experimental conditions employed.

Binding of GO to Blue Dextran is associated with a somewhat unexpected effect on the activity of the enzyme. Unlike LDH and phosphoglycerate kinase, which are inhibited by Blue Dextran[13], GO showed an enhanced enzymatic activity when complexed to the dyed polysaccharide (see Fig. 1). Our results indicate that this is due to the presence of the dye moiety, as no enhanced activity was exhibited by the GO–Dextran T-2000 complex.

The experimental data given concerning the acid stability of native and Blue

Dextran-bound GO indicate that the polysaccharide derivative prevents irreversible conformational changes in the bound apoenzyme after the dissociation of FAD at pH 1.9. Thus, whereas the native enzyme recovered only about 30% of its original activity after incubation for 2 h at pH 1.9 even after addition of excess of FAD (see Fig. 2B), the Blue Dextran-bound enzyme recovered almost full activity under similar conditions.

Under the experimental conditions employed, complex formation between GO and Blue Dextran did not significantly alter the thermal stability of the enzyme. Similar behaviour has been reported[26] for GO in admixtures with poly(vinylpyrrolidone) or poly(vinyl alcohol). Improved thermal stability of GO has been observed[26], however, in the presence of a copolymer containing hydrophobic residues, and that only at very high excess of additive (*i.e.*, polymer:enzyme $\geqslant$ 1000:1 w/w).

The involvement of dye molecules in the GO-catalyzed oxidation of glucose has been considered previously[18,27–29]. Thus, 2,6-dichlorophenolindophenol[27,28], toluylene blue, thionine, methylene blue, pyocyanine and safranine T[29] have been shown to operate as terminal hydrogen acceptors (in addition to O_2), while the donor was the reduced FAD, formed as an intermediate in the reaction. If the dye moiety in the GO–Blue Dextran complex was to act in a similar manner, *i.e.*, as a hydrogen sink, one would then expect the oxygen consumption (as indicated by the O_2 electrode) to be lower than that measured in the absence of Blue Dextran (in the case of the dye being competitive with O_2), or equal to it (in the case of the dye being active in parallel to O_2). In our studies, however, a different effect was observed, namely, in the presence of Blue Dextran the oxygen uptake was significantly higher than in its absence. One can thus conclude that Blue Dextran does not act in a similar manner to the above mentioned hydrogen acceptors.

Activation of GO by Blue Dextran may be due to modifications of the microenvironment of the enzyme. The complexes studied here, for example, contained equimolar amounts of GO and Blue Dextran or excess of the latter, and one can envisage the dye moiety binding to the enzyme in a non-specific manner, *i.e.*, at a site not directly related to the enzymatic activity. As a result of this the enzyme is "microencapsulated" into the polysaccharide. Another possible explanation is that the anthraquinone nucleus of the blue dye moiety is involved directly in the hydrogen transfer process; the dye would then operate as an artificial coenzyme instead of or in addition to FAD. A similar rôle has already been proposed for the structurally related compound 2-methylnaphthoquinone, in a steroid oxidation process[30].

We also addressed ourselves in the present work to the question of whether GO being an FAD-enzyme contains a dinucleotide fold in its structure. In view of the fact that Blue Dextran binds to GO but does not inhibit it, and accepting the criteria suggested for the detection of this structural feature[13], it can be concluded that GO does not possess a dinucleotide fold of the type present in enzymes such as alcohol dehydrogenase, LDH, adenylate kinase or phosphoglyceromutase.

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REFERENCES

1 G. Kopperschläger, R. Freyer, W. Diezel and E. Hofmann, *FEBS Lett.*, 1 (1968) 137.
2 R. Haeckel, B. Hess, W. Lauterborn and K. H. Wüster, *Hoppe-Seyler's Z. Physiol. Chem.*, 349 (1968) 699.
3 G. E. J. Staal, J. F. Koster, H. Kamp, L. Van Milligen-Boersma and C. Veeger, *Biochim. Biophys. Acta*, 227 (1971) 86.
4 G. E. J. Staal, J. Visser and C. Veeger, *Biochim. Biophys. Acta*, 185 (1969) 39.
5 A. C. W. Swart and H. C. Hemker, *Biochim. Biophys. Acta*, 222 (1970) 692.
6 H. D. White and W. P. Jencks, *American Chemical Society Meeting, 1970*, Abstr. No. 43.
7 J. J. Marshall, *J. Chromatogr.*, 53 (1970) 379.
8 K. G. Blume, R. W. Hoffbauer, D. Busch, H. Arnold and G. W. Löhr, *Biochim. Biophys. Acta*, 227 (1971) 364.
9 G. Kopperschläger, W. Diezel, R. Freyer, S. Liebe and E. Hofmann, *Eur. J. Biochem.*, 22 (1971) 40.
10 H.-J. Böhme, G. Kopperschläger, J. Schulz and E. Hofmann, *J. Chromatogr.*, 69 (1972) 209.
11 L. D. Ryan and C. S. Vestling, *Arch. Biochem. Biophys.*, 160 (1974) 279.
12 R. L. Easterday and I. M. Easterday, in R. B. Dunlap (Editor), *Immobilized Biochemicals and Affinity Chromatography*, Plenum, New York, 1974, pp. 123–133.
13 S. T. Thompson, K. H. Cass and E. Stellwagen, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 669.
14 M. G. Rossmann, D. Moras and K. W. Olsen, *Nature (London)*, 250 (1974) 194.
15 G. E. Schulz and R. H. Schirmer, *Nature (London)*, 250 (1974) 142.
16 C. S. Wright, R. A. Alden and J. Kraut, *Nature (London)*, 221 (1969) 235.
17 J. Drenth, W. G. J. Hol, J. N. Jansonius and R. Koekoek, *Cold Spring Harbor Symp. Quant. Biol.*, 36 (1971) 107.
18 R. Bentley, in P. D. Boyer, H. Lardy and K. Myrbach (Editors), *The Enzymes*, Vol. 7, Academic Press, New York, 1963, pp. 567–586.
19 A. J. Kalb, *Biochim. Biophys. Acta*, 168 (1968) 532.
20 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
21 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
22 B. E. P. Swoboda, *Biochim. Biophys. Acta*, 175 (1969) 365.
23 D. E. Edmondson and G. Tollin, *Biochemistry*, 10 (1971) 113.
24 B. Solomon, unpublished results.
25 M. McLean and D. Hearn, *26th Nat. Meet. American Association Clinical Chemists, 1974.*
26 J. J. O'Malley and R. W. Ulmer, *Biotechnol. Bioeng.*, 15 (1973) 917.
27 D. Keilin and E. F. Hartree, *Biochem. J.*, 42 (1948) 221.
28 K. Kusai, *Annu. Rep. Sci. Works, Fac. Sci. Osaka Univ.*, 8 (1960) 43.
29 W. Franke and M. Deffner, *Justus Liebigs Ann. Chem.*, 541 (1939) 117.
30 H. J. Ringold, M. Gut, M. Hayano and A. Turner, *Tetrahedron Lett.*, (1962) 835.