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THE ROLE OF SULFHYDRYL GROUPS IN THE ACTION AND STRUCTURE OF MAMMALIAN α-AMYLASE

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SUMMARY

Hog pancreatic α-amylase contains 2 –SH groups and 1 tightly bound Ca^{2+} per enzyme molecule. We have prepared derivatives of this enzyme by using 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), iodoacetamidonaphthol, and Hg^{2+}. These derivatives retain enzymatic activity thereby demonstrating that the –SH groups of this enzyme are not required for enzymatic activity. The pH profile of these derivatives is the same as that of the native enzyme although the specific activity at each pH is depressed to a varying extent, depending on the derivative. The reaction with DTNB and iodoacetamidonaphthol proceeds only after prior removal of the tightly bound Ca^{2+}. The Hg^{2+} derivatives, however, may be formed without prior removal of the Ca^{2+}. Upon reacting the amylase with one mole of Hg^{2+} an –S–Hg–S– bridge is formed. This leads us to conclude that the 2 –SH groups lie very close to each other. These derivatives may be useful for an X-ray crystallographic study of this enzyme.

INTRODUCTION

Porcine pancreatic α-amylase (1,4-α-D-glucan gluconohydrolase, EC 3.2.1.1) contains 2 –SH groups and 1 tightly bound Ca^{2+} per enzyme molecule [1]. Previous reports [2] have claimed that the 2 –SH groups are essential for enzymatic activity. The 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) derivative has been investigated in some detail and recently, Telegdi and Straub [3] have suggested that reaction of α-amylase with DTNB yields a product devoid of 5-thio-2-nitrobenzoic acid (TNB^-) but containing an internal disulfide bridge. In the course of our investigations we were able to verify the proximity of the two –SH groups by inserting a Hg^{2+} bridge between them. Because X-ray crystallographic studies of this enzyme are now underway [4], a clear understanding of the role of the –SH groups and their proximity to each other is imperative.

Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid).

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MATERIALS AND METHODS

α-Amylase was prepared from undiluted hog pancreatin (Miles, Marschall Division, Elkhart, Ind.) by the method of Loyter and Schramm [5]. Amylase activity was assayed by the method of Bernfeld [6] but was performed at 30 °C. 1 unit of amylase activity is defined as the amount of enzyme which produces 1 mg equivalent maltose hydrate from starch in 3 min at 30 °C. DTNB and 1,4-dithiothreitol were obtained from Calbiochem. Iodoacetamidonaphthol was purchased from Nutritional Biochemicals Corp. Bio-Gel P-2 and Chelex-100 were purchased from BioRad. $^{203}Hg^{2+}$ (acetate salt) was purchased from the Radiochemical Centre, Amersham, England. Sartorius membranes were purchased from Sartorius Membranfilter, Göttingen, West Germany. All other chemicals were of the highest purity available.

Hg^{2+} concentrations were determined by titration with Cl^- using diphenylcarbazone as the end-point indicator [7] except when using mercuric acetate. In this case, the Cl^- titration was found to be inaccurate and the titration was performed with bis-(2-hydroxyethyl)dithiocarbamate using Cu^{2+} as the end-point indicator [8].

Polyacrylamide gel electrophoresis

10% polyacrylamide/bis gels were prepared with 1% sodium dodecylsulfate according to the method of Weber and Osborn [9] with the addition of 2.5 mM 2-mercaptoethanol. α-Amylase at pH 7.0 and 8.5 was incubated at room temperature in 0.1 M or 0.5 M Tris–HCl with 10 mM dithiothreitol. 10 and 100 μg samples of enzyme were then applied to the gels and electrophoresis performed in phosphate buffer with 5 mM 2-mercaptoethanol and 1% sodium dodecylsulfate. 10 mA/tube were applied until the marking dye was 1 cm from the end of the gels. The gels were stained with Coomassie blue. Bovine serum albumin, chymotrypsinogen and cytochrome *c* were used as standard molecular weight markers.

Reaction with DTNB and isolation of $(TNB)_2$–amylase

Reaction of amylase with DTNB was performed at pH 8.0 in 0.02 M *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) with 0.01 M EDTA to which a 20-fold molar excess of DTNB over enzyme was added. The reaction was followed spectrophotometrically at room temperature using the Gilford 2400 at 412 nm. The molar extinction coefficient for TNB^- used was 13 600 [10]. After reaction with DTNB, a 1-ml sample (2 mg/ml) was gel filtered on a Bio-Gel P-2 column (1.0 cm × 10 cm) equilibrated and eluted with 0.02 M HEPES buffer (pH 8.0) to remove excess DTNB and free TNB^-. 20 μl of pure 2-mercaptoethanol (14 M) was added to a 1-ml sample (0.6 mg/ml protein) from the protein peak which had no absorption at 412 nm. After addition of the 2-mercaptoethanol, absorption at 412 nm was monitored spectrophotometrically.

Reaction with 4-iodoacetamidonaphthol

A 10-fold molar excess of 4-iodoacetamido-1-naphthol was reacted with amylase in 0.02 M HEPES buffer (pH 8.7) with 0.01 M EDTA. After the reaction was completed, the samples were gel filtered on a Bio-Gel P-2 column (1.8 cm × 37 cm) equilibrated and eluted with 0.02 M piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid)

(PIPES) buffer (pH 7.2). The protein peak was pooled by monitoring protein concentration spectrophotometrically using $A^{1\%}_{280\ nm} = 24.1$ [11]. One aliquot of the protein peak was assayed for unreacted –SH groups by using DTNB at pH 8.0 in 0.01 M EDTA. The pH of another portion of the protein peak was titrated to pH 11 using 10 M NaOH and $A_{340\ nm}$ measured spectrophotometrically. The extinction coefficient for 4-*S*-(β-mercaptoethyl)acetamidonaphthol at 340 nm was determined and found to be 9600 at pH 11. This value was used to calculate the number of acetamidonaphthol residues incorporated into the enzyme.

Reaction with Hg^{2+}

Amylase, $2.8 \cdot 10^{-5}$ M in 0.1 M HEPES pH 8.0 was incubated for up to 1 h at room temperature with varying ratios of $Hg(NO_3)_2$ to enzyme by addition of 10^{-3} M $Hg(NO_3)_2$. Aliquots were then reacted with DTNB at pH 8.0 in 0.01 M EDTA to determine the number of –SH groups per enzyme available for reaction. The results obtained were independent of the incubation time of the enzyme with mercury. The reaction with mercury is instanteneous.

Reaction with $^{203}Hg^{2+}$

Amylase in 0.02 M HEPES pH 6.9 was incubated with $^{203}Hg(NO_3)_2$ in various ratios of Hg^{2+} to enzyme. The sample (1.0 ml) was then gel filtered on a Bio-Gel P-2 column (1.0 cm × 28 cm) with 0.5 cm Dowex A-1 (Chelex 100) at the bottom on the column to remove non-specifically bound or free mercury. Protein concentration was determined as described above. ^{203}Hg was counted in a Packard Tricarb scintillation counter.

Demonstration of –S–Hg–S– bridge

α-Amylase was extensively dialyzed against 0.02 M PIPES buffer to remove other anions. The enzyme was then diluted in the same buffer to a final concentration of 2 mg/ml ($4 \cdot 10^{-5}$ M) to which was added $^{203}Hg^{2+}$ (acetate salt) in a 1.2 to 1.0 molar ratio of Hg^{2+} to enzyme. The amylase–mercury(II) reaction was instanteneous and incubation times of up to 2 h did not alter the results. At the end of the incubation time, addition of DTNB to an aliquot of the reaction mixture in the presence of 0.01 M EDTA failed to demonstrate any reactable –SH groups. The ^{203}Hg(II)–amylase derivative was then gel filtered at 4 °C on a Bio-Gel P-2 column (1.2 cm × 17 cm) with 0.5 cm of Chelex-100 at the base. The column had been equilibrated and eluted with 0.02 M PIPES buffer (pH 7.0). Fractions from the leading edge of the protein peak were pooled and concentrated with a Sartorius membrane using vacuum dialysis. The final solution (1 ml) contained 5.8 mg/ml enzyme. The ^{203}Hg/enzyme ratio was determined by counting aliquots in a Packard Tricarb scintillation counter. Additional aliquots were used for the determination of reactable –SH groups using DTNB. –SH group reactivity was measured within 15 min after exposure to 0.01 M EDTA or 8 M urea at pH 8.5. Control experiments in which the native enzyme was incubated up to 4 h in 0.01 M EDTA (pH 7.0), both in the presence and the absence of 8 M urea revealed between 1.8 to 2.0 exposed –SH groups per enzyme molecule. The ability of 0.01 M EDTA to remove the tightly bound Ca^{2+} and thus expose the two –SH groups has been demonstrated [12].

Specific activity and pH profile of the amylase derivatives

$(TNB)_2$–enzyme, (acetomido-naphthol)$_2$-enzyme and the mercury(II) derivatives were gel filtered at 4 °C on a Bio-Gel P-2 column equilibrated and eluted with 0.02 M PIPES buffer (pH 7.0) immediately after their preparation. Activity of the various enzyme derivatives was assayed immediately after gel filtration. The assays were performed in the presence of $1 \cdot 10^{-4}$ M $CaCl_2$ and $1 \cdot 10^{-2}$ M NaCl at 30 °C using the assay of Bernfeld [6]. The pH profiles were determined using the following 0.02 M buffers: Acetate, pH 5, 5.65; PIPES, pH 6.0, 6.6, 7.0; HEPES, pH 7.4, 8.0; Glycine–NaOH, pH 8.5, 8.7, 8.9, 9.4, 9.6.

RESULTS

The molecular weight of α-amylase

A single amylase band was found in all sodium dodecylsulfate gels in the presence of dithiothreitol and β-mercaptoethanol at a migration distance corresponding to a molecular weight of 50 000. An identical band was found when the electrophoresis was performed in the absence of 2-mercaptoethanol and without pre-treatment with dithiothreitol.

Reaction of enzyme with DTNB

As previously reported [11, 12], the 2 –SH groups of porcine pancreatic α-amylase are masked in the native state of the enzyme. If Ca^{2+} is removed by EDTA or if the enzyme is denatured in urea, the –SH groups become exposed and react with DTNB. After removal of Ca^{2+} by EDTA, the change in absorption at 412 nm corresponded to the release of 2.0 TNB^- per enzyme molecule. After gel filtration of the reacted enzyme and addition of 2-mercaptoethanol, 1.8 moles of TNB^- per mole of enzyme were liberated from the protein. This demonstrates that the product of the reaction of amylase with excess DTNB is the enzyme–$(TNB)_2$ derivative.

Reaction of enzyme with iodoacetamidonaphthol

After reaction of amylase with iodoacetamidonaphthol and gel filtration of the product, DTNB addition failed to cause a change in the absorption at 412 nm implying that no more –SH groups were available for reaction. When the gel filtered product of the iodoacetamidonaphthol–enzyme reaction was titrated to pH 11, the absorption at 340 nm corresponded to 1.7 moles of acetamidonaphthol bound per mole enzyme. Therefore, as with DTNB, the derivative formed with iodoacetamidonaphthol is the enzyme–(acetamidonaphthol)$_2$ species.

Reaction with $Hg(NO_3)_2$

Titration of –SH groups with $Hg(NO_3)_2$ (Fig. 1) gave a linear plot. One Hg^{2+} per enzyme was required to prevent both –SH groups from reacting with DTNB.

Reaction with $^{203}Hg(NO_3)_2$

The results of titration of amylase with varying ratios of $^{203}Hg^{2+}$ to enzyme are shown in Table I.

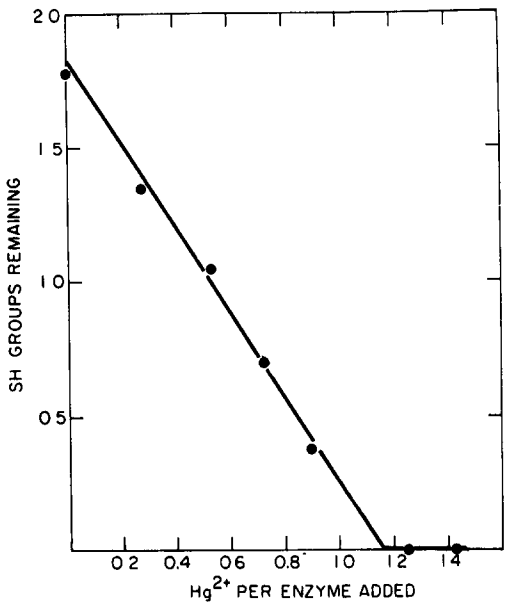


Fig. 1. The titration of amylase -SH-groups by Hg^{2+}. Mercuric nitrate was added to the enzyme in the ratios Hg^{2+} per enzyme as indicated in the figure. The disappearance of -SH groups was monitored by DTNB as described in the text. The reaction mixture did not contain EDTA.

Demonstration of an -S–Hg–S- bridge

The native enzyme used in this experiment was found to react 1.7 -SH groups per enzyme in the presence of DTNB and 0.01 M EDTA and 1.5 -SH groups per enzyme in the presence of DTNB and 8 M urea. After reaction with $^{203}Hg^{2+}$ (acetate salt) and gel filtration, the derivatized enzyme, which had full enzymatic activity, was found to contain 0.8 molecules Hg^{2+} per enzyme molecule. This derivative failed to react with DTNB in the presence of 0.01 M EDTA or 8 M urea or both (Table II). Prolonged incubation of the mercury derivative with 0.01 M EDTA very slowly exposes -SH groups for reaction with DTNB (0.4 -SH groups re-exposed after 1 h). Therefore, our measurements were performed immediately after exposure to EDTA. These results strongly suggest that a -S–Hg–S- bridge had, indeed, been formed.

TABLE I

BINDING OF $^{203}Hg^{2+}$ BY α-AMYLASE

Experimental details are given in the text.

Moles $^{203}Hg(NO_3)_2$ per enzyme added	$^{203}Hg^{2+}$ incorporated per enzyme*
1.0	0.81
2.5	0.97
3.0	1.71
5.0	1.91

* The molecular weight used for the calculation was 50 000.

TABLE II

THE FORMATION OF AN -S-Hg-S- BRIDGE

All reactions were allowed to proceed at pH 8.5. The number of -SH groups per enzyme reacting with DTNB was determined spectrophotometrically using the molar extinction coefficient for TNB^- = 13 600 [10]. Other experimental details are given in the text.

Hg^{2+} bound per enzyme	Number of -SH groups reacting with DTNB	Reaction conditions
0.0	1.7	0.01 M EDTA
0.0	1.5	8 M urea
0.8	0.2	0.01 M EDTA
0 8	0 2	8 M urea
0.8	0.2	0.01 M EDTA + 8 M urea

Control experiments in which the enzyme was incubated up to 4 h in 0.01 M EDTA in the presence or in the absence of 8 M urea revealed 1.7–2.0 -SH groups per enzyme molecule. This indicates that no oxidation of the -SH groups occurs.

The specific activity and pH profile of the amylase derivatives

The activity at pH 7.0 of the $(TNB)_2$–enzyme, $(acetamidonaphthol)_2$–enzyme, Hg(II)–enzyme and $Hg(II)_2$–enzyme derivatives are shown in Table III. The pH profiles of Hg(II)–amylase, $Hg(II)_2$–amylase, $(TNB)_2$–amylase and (acetamidonaph-

TABLE III

THE SPECIFIC ACTIVITIES OF -SH GROUP DERIVATIVES OF α-AMYLASE AT pH 7.0

The activity of the enzyme was assayed in the presence of $1 \cdot 10^{-4}$ M $CaCl_2$ and $1 \cdot 10^{-2}$ M NaCl at 30 °C using the assay of Bernfeld [6]. Extra Ca^{2+} was added since, in the preparation of the $(acetamidonaphthol)_2$–enzyme, EDTA must be added (see experimental details). A control in which the enzyme was treated with EDTA alone yielded 10% loss of activity of the enzyme. As is mentioned in the text, the reaction of amylase with Hg^{2+} does not require the removal of Ca^{2+} and therefore does not require EDTA treatment of the enzyme.

Derivative	Spec. act. (units/mg protein)
None	1500 ± 100
Hg(II)–enzyme	1400 ± 100
$Hg(II)_2$–enzyme	1300 ± 100
$(Acetamidonaphthol)_2$–enzyme	760
$(TNB)_2$–enzyme	450

$thol)_2$–amylase are identical to that of the native enzyme although depressed (Fig. 2). The specific activity of the Hg(II)–amylase derivative is only slightly less than that of the native enzyme at each pH tested. Both of the mercury derivatives remained unchanged, both in terms of molar ratio of bound mercury and specific activity, after 1 week of dialysis against 0.02 M HEPES buffer (pH 6.9) at 4 °C.

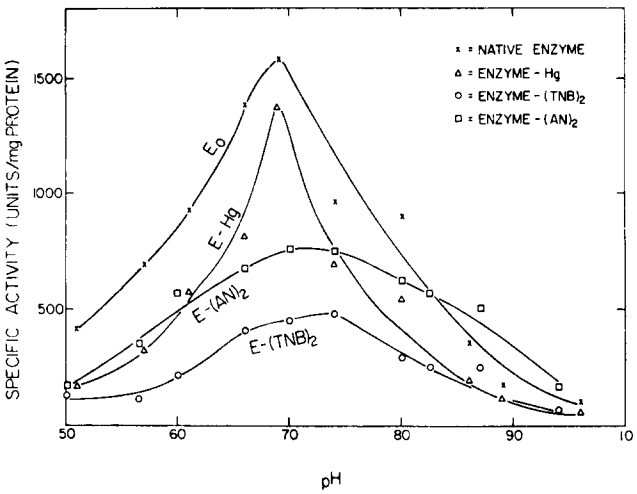


Fig. 2. pH profile of α-amylase –SH derivatives. The pH profile of native α-amylase is unchanged by the formation of several types of –SH derivatives. The absolute catalytic activity at each pH is, however, depressed to a varying extent in the derivatized enzyme species. Experimental details are given in the text.

DISCUSSION

The molecular weight of α-amylase

The molecular weight of α-amylase is 50 000 as determined by gel electrophoresis. Even in the presence of reducing agents (dithiothreitol and β-mercaptoethanol) and sodium dodecylsulfate, no subunit bands were noted. This finding confirms earlier reports [1] which claimed that mammalian α-amylase consists of one polypeptide chain of 50 000 molecular weight and is at variance with the recent report of Robyt et al. [13]. Robyt et al. used a commercially available preparation of α-amylase which was probably partially degraded by accompanying proteolytic activity. Robyt's preparation had a specific activity of 500–600 units/mg which also reflects the degraded status of the enzyme. The procedure used to prepare the enzyme in our studies [5] more efficiently separates the proteolytic enzymes from α-amylase. It should be noted that the specific activity of commercial α-amylase preparations is always substantially lower than the enzyme used in our studies (1500 units/mg). In addition, DFP was added to all of our solutions containing α-amylase.

The role of –SH groups in amylase activity

Binding of Hg^{2+} to the –SH groups of α-amylase causes only a very small decrease in the catalytic activity of the enzyme. Furthermore, the attachment of two bulky residues, such as the TNB^- or acetamidonaphthol groups, to the –SH groups, results in derivatives with high specific activity (Table III). These findings demonstrate that the –SH groups are not essential for amylase activity.

When the $(TNB)_2$–enzyme is reduced with 2-mercaptoethanol, 1.8 TNB^- groups per enzyme molecule are liberated. This shows that the reaction of amylase with excess DTNB produces the $(TNB)_2$–enzyme species. The same type of reaction

also occurs when α-amylase is incubated with iodoacetamidonaphthol in the presence of EDTA.

Both DTNB and iodoacetamidonaphthol interact with the enzyme only after the tightly bound Ca^{2+} is removed from the enzyme by EDTA or by denaturation with urea [11]. Mercury (Hg^{2+}), probably because of its smaller size, can react with the –SH groups of α-amylase even without the prior removal of Ca^{2+} (Fig. 3). Interestingly, as previously reported [11], I_2 can also react with the –SH groups of amylase without prior removal of the tightly bound Ca^{2+} but, in this case, the derivative is inactive.

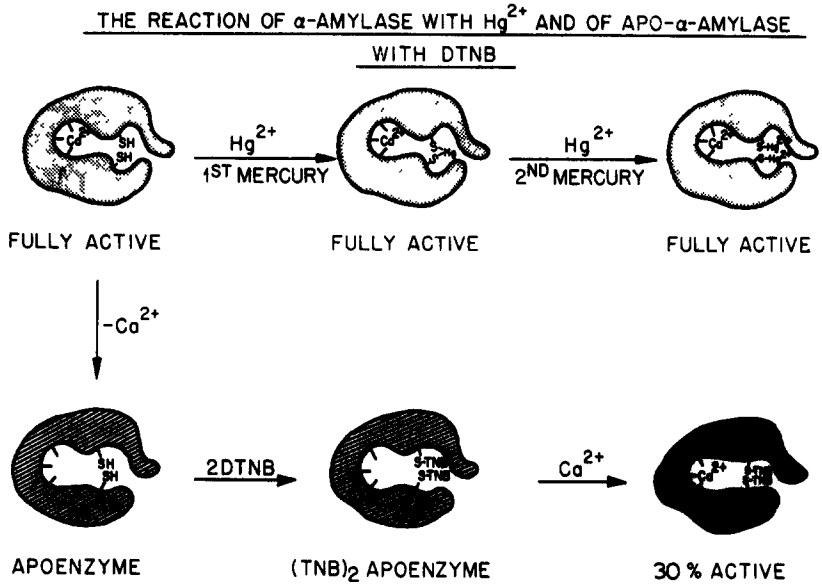
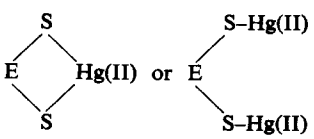


Fig. 3. Proposed scheme for the reaction of –SH groups with DTNB and mercury. The reaction of DTNB proceeds only after prior removal of the tightly bound Ca^{2+} atom by EDTA. Evidence in favor of the formation of an –S–Hg–S– bridge is given in the text.

The proximity of the two –SH groups

It is readily apparent from Fig. 1 and Table II that the binding of one Hg^{2+} per amylase molecule masks both –SH groups. This suggests that the two –SH groups are close enough to each other to form an –S–Hg–S– bridge (Fig. 3). Even in the presence of 8 M urea or 0.01 M EDTA, the one Hg^{2+} bond prevents 2 –SH groups per enzyme molecule from reacting with DTNB. This result eliminates the possibility that the second –SH group is simply masked by a solitary derivatized –SH group. The stability of 2 $-S-Hg^{2+}$ is comparable to the stability of –S–Hg–S– as is well known from model compounds [14]. Indeed, one can form either of the species:



,depending on the Hg^{2+} to –SH ratio in the reaction mixture (Table I). Therefore, it is not surprising that we were able to form the $Hg(II)_2$-derivative by increasing the Hg^{2+}/enzyme ratio in the reaction mixture. From the evidence presented here, we must conclude that the 2 –SH groups of porcine pancreatic α-amylase lie very close to each other. The proximity of the two –SH groups was recently demonstrated by Telegdi and Straub [3] who succeeded to form an intramolecular S–S bond upon reacting the enzyme stoichiometrically with DTNB.

Since both Hg(II)-derivatives maintain almost full activity [15] (Table III), it seems that no significant alteration in the protein structure occurs upon reaction of the –SH groups with Hg^{2+}. They may therefore be useful in X-ray crystallographic studies [4] of this enzyme. Recently, we have succeeded in removing Ca^{2+} from α-amylase and replacing it with other divalent cations such as Ba^{2+} [15]. These divalent metal derivatives may also be useful for X-ray crystallographic studies since, in some cases, full enzymatic activity is retained.

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REFERENCES

1 Fischer, E. M. and Stein, E. A. (1960) The Enzymes, Vol. 4, pp. 313–343, Academic Press, New York
2 Cozzone, P. and Pommier, G. (1972) Proc. 8th FEBS Meet., Amsterdam, Abstr. 250, North Holland, Amsterdam
3 Telegdi, M. and Straub, F. B. (1972) Proc. 8th FEBS Meet., Amsterdam. Abstr. 252, North Holland, Amsterdam
4 McPherson, A. and Rich, A. (1972) Biochim. Biophys. Acta 285, 493–497
5 Loyter, A. and Schramm, M. (1962) Biochim. Biophys. Acta 65, 200–206
6 Bernfeld, P. (1955) Methods in Enzymology, Vol. 1, pp. 149–153, Academic Press, New York
7 Celon, E. (1969) Mikrochim. Acta, 592–598
8 Fritz, J. S. and Sutton, S. A. (1956) Anal. Chem. 28, 1300–1303
9 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
10 Ellman, G. (1959) Arch. Biochem. Biophys. 82, 70–74
11 Schramm, M. (1964) Biochemistry 3, 1231–1234
12 Levitzki, A. and Steer, M. L. (1974) Eur. J. Biochem., in the press
13 Robyt, J. F., Chittenden, C. G. and Lee, C. T. (1971) Arch. Biochem. Biophys. 144, 160–167
14 Sperling, R., Burstein, Y. and Steinberg, I. Z. (1969) Biochemistry 8, 3810–3820
15 Steer, M. L. and Levitzki, A. (1973) FEBS Lett. 31, 89–92