# The Reaction of Bovine Thrombin with N-Butyrylimidazole. Two Different Reactions Resulting in the Inhibition of Catalytic Activity†

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ABSTRACT: N-Butyrylimidazole has been found to be a potent inhibitor of purified bovine thrombin. The rate and extent of inhibition of thrombin by N-butyrylimidazole could be reduced by the presence of benzamidine, a competitive inhibitor, or by the ester substrate, p-tosyl-L-arginine methyl ester. Spectral studies of the reaction of N-butyrylimidazole with thrombin demonstrated the modification of approximately 1 mol of tyrosine/mol of enzyme at maximum inhibition. In addition to the reaction with tyrosine, N-butyrylimidazole also appears to react with a residue at the "active site" as judged by a decrease in the number of active sites available in the modified enzyme for titration with p-nitrophenyl-p'-guanidinobenzoate. The time course of ester hydrolysis by butyrylated thrombin showed a distinct lag phase suggesting partial reactivation of the enzyme under assay conditions. Partial reactivation of the modified enzyme also occurred spontaneously upon standing in 0.5 M NaCl but was much faster in presence of imidazole (0.03 M, pH 7.6). It is suggested that, in addition to reaction with tyrosine, there is a reaction of N-butyrylimidazole with either the histidine and/or serine residue at the active site of thrombin resulting in a derivative unstable under esterase assay conditions such as that described for the reaction of N-acetylimidazole with trypsin (L. L. Houston and K. A. Walsh (1970), *Biochemistry 9*, 156).

Previous studies from this and other laboratories on the reaction of bovine thrombin with tetranitromethane (Sokolovsky and Riordan, 1969; Lundblad and Harrison, 1971), N-acetylimidazole (Kotoku *et al.*, 1970; Lundblad *et al.*, 1973), and acetic anhydride (Landaburu and Seegers, 1959) have implicated tyrosyl residues in the function of this enzyme. The reaction of thrombin with either tetranitromethane or N-acetylimidazole resulted in the modification of 4–5 tyrosyl residues per molecule with concomitant partial inhibition of fibrinogen-clotting activity. It has been suggested that the reaction of tetranitromethane or N-acetylimidazole with tyrosyl residues in proteins may not be as much a reflection of accessibility to solvent as an indication of a hydrophobic environment (Myers and Glazer, 1971; Herskovits and Fuchs, 1972). In view of this consideration, the reaction of N-butyrylimidazole, a reagent more hydrophobic than N-acetylimidazole, with purified bovine thrombin was studied in the hopes of further elucidating the role of tyrosyl residues in this highly specific proteolytic enzyme. A further reason for the selection of the butyryl derivative was based on our failure in earlier studies (Lundblad *et al.*, 1973) to observe a reaction of N-acetylimidazole with active site residues in thrombin similar to that reported by Houston and Walsh (1970) for trypsin. This was somewhat surprising considering the homology between these two enzymes. The recent observation (Lundblad, 1973a) that p-nitrophenyl butyrate was a better substrate for thrombin than p-nitrophenyl acetate suggested that the reaction of N-butyrylimidazole with thrombin may show the same characteristics as the reaction of N-acetylimidazole with trypsin.

## Experimental Procedure

*Materials.* Crude bovine thrombin (Topical Thrombin) was purchased from Parke-Davis, Detroit, Mich., and purified by chromatography on Sulfopropyl Sephadex C-50 as previously described (Lundblad, 1971; Lundblad *et al.*, 1973). Only enzyme preparations having a specific activity of 1800 NIH units/mg or greater were utilized in this investigation. This level of specific activity is consistent with a 90% or greater level of α-thrombin. These preparations, when assayed with an active site reagent, p-nitrophenyl-p'-guanidinobenzoate, consistently showed a level of 80% or greater active enzyme. Sulfopropyl Sephadex C-50 and Sephadex G-25 (coarse) were obtained from Pharmacia Fine Chemicals. Benzamidine hydrochloride was a product of Eastman Chemicals, p-tosyl-L-arginine methyl ester (Tos-ArgOMe)[1] was a product of Schwarz/Mann, and N-acetylimidazole was either a product of Pierce Chemical Corporation or synthesized by the method of Boyer (Boyer, 1952). The copolymer of glutamic acid and tyrosine (Glu:Tyr; 1.16:1.00) was a product of Miles-Yeda. p-Nitrophenyl-p'-guanidinobenzoate was obtained from the Cyclo Chemical Corporation. N-Butyrylimidazole was synthesized from imidazole (recrystallized from benzene) and butyryl chloride as described by Fife (Fife, 1965). All other chemicals were of reagent grade and used without further purification.

*Methods.* The reaction of bovine thrombin with N-butyrylimidazole or N-acetylimidazole was performed as previously described for the acetyl derivative (Lundblad *et al.*, 1973). Enzymatic activity toward either fibrinogen or Tos-

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[1] Abbreviations used are: Tos-ArgOMe, p-tosyl-L-arginine methyl ester; NphOGdnBz, p-nitrophenyl-p'-guanidinobenzoate.

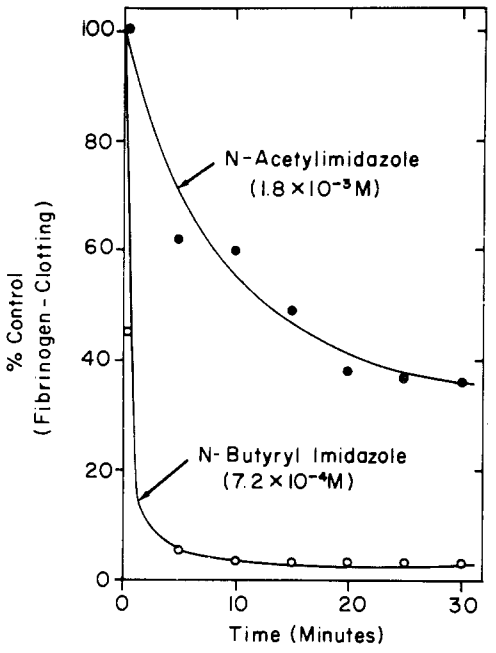


FIGURE 1: The inactivation of thrombin by *N*-acetylimidazole or *N*-butyrylimidazole. A solution (2.4 ml) of thrombin (0.8 mg/ml, $2.2 \times 10^{-5}$ M) in 0.02 M sodium phosphate (pH 7.6) was added to a tube containing the respective *N*-acylimidazole in a quantity to give the indicated final concentration. The reaction was allowed to proceed at room temperature (23°) with 0.1-ml portions removed at the indicated times and diluted tenfold in 0.09 M Tris–0.06 M NaCl (pH 7.6). The diluted samples were immediately assayed for their ability to clot fibrinogen as described in the text.

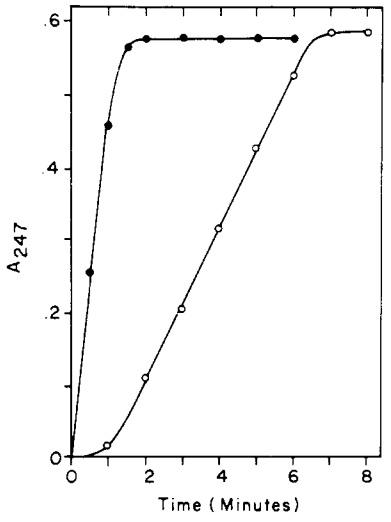


FIGURE 2: The effect of butyrylation on thrombin esterase activity. Control thrombin and butyrylated thrombin were prepared by gel filtration as described under Table I. A portion (0.1 ml) of control thrombin (●) or butyrylated thrombin (○) was added to 2.9 ml of 0.001 M Tos-ArgOMe and the increase in absorbance at 247 nm determined as a function of time at room temperature.

ArgOMe was determined as previously described (Lundblad, 1971).

The extent of tyrosine modification in both thrombin and the Glu-Tyr copolymer was estimated by the decrease in absorbance at 278 nm (Myers and Glazer, 1971). It is assumed that O-butyrylation of tyrosine results in the same spectral changes as O-acetylation and that the $\Delta\epsilon_M$ of 1210 $M^{-1}$ $cm^{-1}$ can be used for the determination of the extent of tyrosine modification (Myers and Glazer, 1971). Active site titrations were accomplished using NphOGdnBz (Chase and Shaw, 1967; 1969). Protein concentration was determined by the ninhydrin reaction (Moore, 1968) after alkaline hydrolysis (Fruchter and Crestfield, 1965) using crystalline bovine serum albumin as a standard.

Table I: Differential Inactivation of Thrombin Proteinase and Esterase Activity by *N*-Butyrylimidazole.

| Sample[a] | % Control[b] | |
|---|---|---|
| | Fibrinogen Clotting | Esterase |
| Butyrylthrombin, I[c] | 15 | 61 |
| Butyrylthrombin, II[d] | 6 | 50 |
| Butyrylthrombin, II[c] (20 min) | 14 | 54 |

[a] Butyrylthrombin was prepared by incubation of thrombin with *N*-butyrylimidazole as described under Figure 1 and then passed over a G-25 Sephadex column to remove excess reagent. [b] The results are expressed as per cent of a control preparation manipulated as the experimental in the absence of *N*-butyrylimidazole. [c] Sample allowed to stand at ambient temperature for 20 min following gel filtration. [d] Sample assayed immediately after gel filtration.

## Results

A comparison of the respective abilities of *N*-butyrylimidazole and *N*-acetylimidazole to inhibit the fibrinogen-clotting activity of bovine thrombin is shown in Figure 1. It can be readily observed that *N*-butyrylimidazole at a 33-fold molar excess leads to a far more rapid and extensive inhibition of thrombin than *N*-acetylimidazole at a 82-fold molar excess. That the difference in the rate of inhibition of thrombin by the acetyl and butyryl derivatives was not a reflection of a higher avidity of the butyryl compound for reaction with tyrosine was obtained by studies of a model compound. *N*-Acetylimidazole and *N*-butyrylimidazole were allowed to react with a copolymer of tyrosine and glutamic acid; *N*-acetylimidazole was more effective in the modification of tyrosyl residues than *N*-butyrylimidazole.

Butyrylated thrombin and native thrombin were then compared with respect to their ability to hydrolyze Tos-ArgOMe. The time course of the hydrolysis of the ester by the two enzyme preparations is presented in Figure 2. The marked lag phase shown in the first minute by butyrylated thrombin suggests that the modified enzyme is completely inactive or nearly so, and that it is reactivated under the conditions of assay. It is observed that the final rate of hydrolysis attained by the butyrylated enzyme is still less than that of the native enzyme. In this and subsequent experiments, the esterase activity is calculated from the linear portion of the curve shown in Figure 2. Taken together, these two observations suggest that butyrylation involves the modification of two different sites, and the modification is readily reversed at only one of these sites under the conditions of esterase assay. The butyrylation of thrombin inhibits the fibrinogen-clotting activity far more than esterase activity as shown in Table I.

The residue which is deacylated under the conditions described above would appear to be part of the catalytically active site of the enzyme. To test this hypothesis, the butyrylated enzyme was titrated with the active site reagent, *p*-

Table II: Active Site Titration of Acylated Thrombin.

| Sample[a] | % Control | Active Sites/ Mole of Thrombin[b] |
|---|---|---|
| Control thrombin | 100 | 0.83 |
| Butyrylthrombin | 20 | 0.34 |
| Acetylthrombin | 68 | 0.81 |

[a] The samples were obtained as described under Table I. [b] The number of active sites per mole of thrombin was determined by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate.

Table III: Reactivation of Butyrylthrombin in Dilute Imidazole.

| Sample[a] | Clotting Activity % Control | Esterase Activity % Control |
|---|---|---|
| Control thrombin | 100 | 100 |
| Butyrylthrombin | 1.4 | 47 |
| Butyrylthrombin imidazole (pH 7.6)[b] | 26 | 60 |

[a] Prepared by gel filtration as described under Table I. [b] Prepared as described under Figure 3.

Table IV: Reaction of Butyrylthrombin with Hydroxylamine.

| Sample | Clotting Time[a] (sec) | NIH U/mg | % Control[b] | Tos-Arg-OMe (units/mg) | % Control[c] |
|---|---|---|---|---|---|
| Butyryl-thrombin[d] | 23.2; 23.5 | 544 | 28 | 22.0 | 63 |
| Butyryl-thrombin + 1.0 M $NH_2OH$[e] | 13.7/14.2 | 1940 | 100 | 35.0 | 100 |

[a] Clotting time with fibrinogen as described in the text. [b] The % control with respect to fibrinogen-clotting activity. [c] The % control with respect to Tos-ArgOMe hydrolysis. [d] Butyrylthrombin prepared as described in Table I; clotting times on a 1:20 dilution. [e] Butyrylthrombin (0.4 ml) was mixed with 0.4 ml of 2.0 M $NH_2OH$ (pH 7.5) and allowed to stand for 6 min; clotting time determined on a 1:10 dilution.

nitrophenyl-*p'*-guanidinobenzoate. As shown in Table II, the butyrylated enzyme showed a decreased reactivity with NphOGdnBz compared to either a control or an acetylated preparation of thrombin. Reactivity of the butyrylated enzyme toward NphOGdnBz was observed to increase upon standing in 0.5 M NaCl after gel filtration of the reaction mixture containing *N*-butyrylimidazole. Under these conditions the number of active sites per molecule increased from 0.28 to 0.58 with only a slight increase in fibrinogen-clotting activity. This spontaneous reactivation of the modified enzyme is similar to the reactivation of "heavily acetylated"

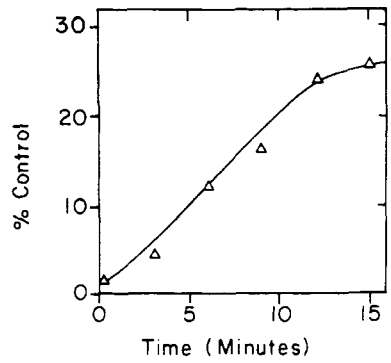


FIGURE 3: Time course study of the reactivation of butyrylated thrombin by dilute imidazole. Thrombin was reacted with *N*-butyrylimidazole as described under Figure 1 and freed of reagents by gel filtration on G-25 Sephadex in 0.5 M NaCl. The desalted modified enzyme (0.45 ml) was mixed with 0.05 ml of 0.30 M imidazole (pH 7.6). Fibrinogen-clotting activity as described under Figure 1 was determined as a function of reaction time at room temperature.

trypsin which occurred during concentration by ultrafiltration (Houston and Walsh, 1970).

Trypsin has been shown to react with *N*-acetylimidazole at a number of sites including the catalytically active site (Houston and Walsh, 1966, 1970). This modification at the catalytically active site was reversed upon treatment with dilute imidazole at pH 7.6. Figure 3 shows the recovery of fibrinogen-clotting activity in butyrylated thrombin upon standing in 0.03 M imidazole (pH 7.6). It should be noted that the reactivation observed under these reaction conditions is only partial with respect to fibrinogen-clotting activity. Table III shows that there is only a slight increase in esterase activity as compared to fibrinogen-clotting activity but the lag phase in ester hydrolysis previously observed is eliminated upon treatment with the dilute imidazole.

Thrombin which had been modified with *N*-butyrylimidazole could be completely reactivated with neutral hydroxylamine as shown in Table IV. This observation suggested that the inhibition of thrombin by *N*-butyrylimidazole involved the modification of tyrosyl residues in addition to the residue or residues described above. There is a characteristic decrease in the absorbance of tyrosine at 278 nm upon acylation (Riordan and Vallee, 1963; Simpson *et al.*, 1963) and it is possible to quantitate the extent of modification in proteins by measuring the decrease in absorbance at 278 nm as a function of time (Myers and Glazer, 1971). The extent of modification of tyrosyl residues in thrombin upon treatment with *N*-butyrylimidazole was then examined utilizing this spectrophotometric method. Figure 4 shows the change in the absorbance of thrombin at 278 nm during reaction with either *N*-butyrylimidazole or *N*-acetylimidazole. The change in absorbance at 278 nm observed upon reaction with *N*-butyrylimidazole is far less than that seen with *N*-acetylimidazole. The extent of modification with *N*-acetylimidazole as shown in Table V observed in the present experiments is consistent with the earlier observations from this laboratory (Lundblad *et al.*, 1973) while the butyrylated enzyme had less than 2 mol of tyrosine modified/mol of enzyme at maximum inhibition. The reaction of *N*-acetylimidazole or *N*-butyrylimidazole with the amino groups of thrombin was also examined. While the former reagent modified approximately 20% of the free amino groups as judged by the ninhydrin reaction, the latter reagent led to the modification of approximately one-half this number. It is not believed that the reaction of these com-

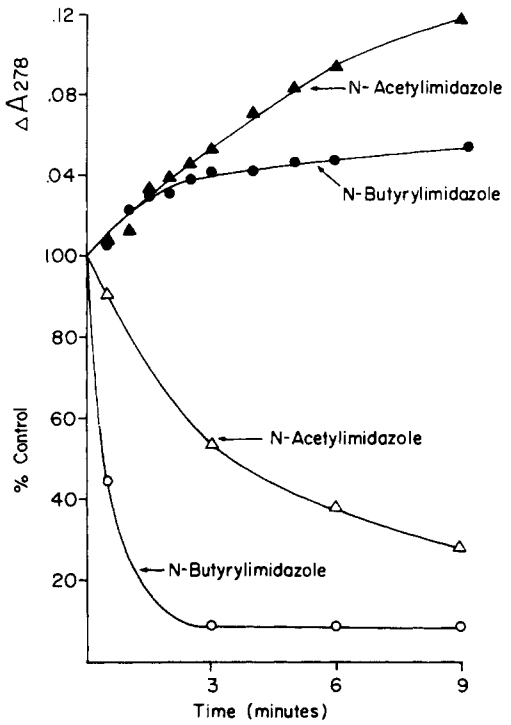


FIGURE 4: The relationship between tyrosine modification and changes in fibrinogen-clotting activity in bovine thrombin upon treatment with *N*-acetylimidazole (triangles) and *N*-butyrylimidazole (circles). The reactions were performed as described under Figure 1 and either fibrinogen-clotting activity (open symbols) or absorbance at 278 nm (solid symbols) was determined as a function of time.

pounds with the free amino groups is in any way responsible for the observed changes in catalytic activity. If this were the case, complete reactivation with neutral hydroxylamine would not have been observed, as *N*-acyl compounds are stable under these conditions (Simpson *et al.*, 1963; Connellan and Shaw, 1970).

The extent of inhibition of thrombin by *N*-butyrylimidazole could be decreased by either benzamidine or Tos-ArgOMe (Table VI). As with the reaction of *N*-acetylimidazole with thrombin (Lundblad *et al.*, 1973), the presence of benzamidine did protect the enzyme from inactivation but increased the extent of tyrosine modification.

Finally, in order to further explore the hypothesis that the more stable acylated site (presumably involving tyrosine) is affecting substrate binding, the affinity of the butyrylated enzyme for the ester substrate, Tos-ArgOMe, was examined and compared with the native and acetylated enzyme. The reaction of thrombin with either *N*-acetylimidazole or *N*-butyrylimidazole does not result in a change in the observed $V_{max}$, but instead in an increase in the observed $K_m$ values. The $K_m$ with respect to Tos-ArgOMe at pH 8.0 for the butyrylated enzyme was found to be $8.1 \times 10^{-4}$ M as compared to $2.1 \times 10^{-4}$ M for a control preparation; acetylated thrombin showed an intermediate value of $4.1 \times 10^{-4}$ M.

## Discussion

The data above suggest that there are two operationally distinguishable sites of reaction of *N*-butyrylimidazole with thrombin which lead to changes in catalytic activity. The evidence obtained from both spectrophotometric measurements as well as neutral hydroxylamine reversal certainly suggests the involvement of tyrosine in the reaction of *N*-butyrylimidazole with thrombin. There does, however, appear to be a reaction of *N*-butyrylimidazole with thrombin at a site other than tyrosine which results in changes in catalytic activity. The unstable nature of this derivative as well as the decreased active site availability suggests that the reaction of *N*-butyrylimidazole with bovine thrombin is similar to that reported by Houston and Walsh (1966, 1970) for the interaction of *N*-acetylimidazole with trypsin. These investigators showed that reaction of trypsin with *N*-acetylimidazole under appropriate conditions resulted in the formation of a derivative which had partially inhibited esterase activity when assayed a pH 7.8 but was virtually inactive upon "active site titration" at pH 2.66. It is not possible to assay thrombin at low pH as irreversible inactivation occurs below pH 4.0. However, the exceedingly slow initial rate of ester hydrolysis by the butyrylated enzyme followed by a more rapid phase is consistent with the formation of a labile transient derivative of the type described by Houston and Walsh (1970). This similarity is further supported by the partial reactivation of butyrylthrombin in the presence of dilute imidazole. It therefore seems likely that *N*-butyrylimidazole reacts with a histidyl and/or a seryl residue at the active site of thrombin. In our earlier work on the reaction of *N*-acetylimidazole with thrombin we did not observe this phenomenon. This was somewhat surprising considering the similar enzymatic specificity of

Table V: The Extent of Tyrosine Modification in Thrombin by *N*-Acylimidazoles.

| Reaction Conditions[a] | Moles of Tyr Modified/Mole of Thrombin[b] | % Inactivation[c] |
|---|---|---|
| *N*-Acetylimidazole ($9.6 \times 10^{-4}$ M) | 2.40 | 64 |
| *N*-Acetylimidazole ($1.9 \times 10^{-3}$ M) | 4.6 | 79 |
| *N*-Butyrylimidazole ($3.6 \times 10^{-4}$ M) | 1.57 | 94 |
| *N*-Butyrylimidazole ($7.2 \times 10^{-4}$ M) | 1.47 | 95 |

[a] The reactions were performed as described under Figure 1. [b] The extent of tyrosine modification was determined by the decrease in absorbance at 278 nm as described in the text. [c] The per cent inactivation was determined by reference to a preparation of thrombin subjected to identical manipulation in the absence of *N*-acylimidazole.

Table VI: Effect of Inhibitor and Substrate on *N*-Butyrylimidazole Inhibition of Thrombin.

| Reaction Conditions[a] | % Inactivation[b] |
|---|---|
| + *N*-Butyrylimidazole[c] | 70, 85 |
| + *N*-Butyrylimidazole,[c] + benzamidine[d] | 40, 44 |
| + *N*-Butyrylimidazole,[c] + Tos-L-ArgOMe[e] | 46, 39 |

[a] The reactions were performed as described under Figure 1 with the indicated additions. [b] Taken from initial rate measurements with respect to fibrinogen-clotting activity. [c] $1.8 \times 10^{-4}$ M. [d] 1 mM. [e] 2 mM.

these two enzymes as well as the suggested homology of these two proteins (Hartley, 1969). Trypsin is considerably less specific than thrombin. For example, while the proteolysis of fibrinogen by thrombin is associated with cleavage of only four peptide bonds (Laki and Gladner, 1964), that by trypsin is considerably more extensive (Mihalyi and Godfrey, 1963). The importance of hydrophobic interactions in the determination of thrombin specificity has been previously suggested by several workers (Cole *et al.*, 1966; Liem and Scheraga, 1973; Lundblad, 1973a). Thus, it is reasonable that reaction at the active site is observed with the butyryl and not the acetyl derivative.

It is abundantly clear that the extent of tyrosine modification with *N*-butyrylimidazole in bovine thrombin is far less than that observed with *N*-acetylimidazole. The difference in the effectiveness of inhibition between *N*-acetylimidazole and *N*-butyrylimidazole cannot be explained on the basis that the butyryl derivative is a more effective acylating agent. Indeed, the acetyl derivative is clearly a more effective acylating agent as shown either by reaction with thrombin or the Glu-Tyr copolymer. The more rapid and specific reaction of *N*-butyrylimidazole with thrombin as contrasted with *N*-acetylimidazole is therefore thought to be a direct reflection of the hydrophobic environment of the reactive tyrosyl residue or residues. This concept is supported by the suggestion of Myers and Glazer (1971) that tyrosine residues in an hydrophobic environment would be more susceptible to modification by either *N*-acetylimidazole or tetranitromethane.

The changes in activity observed upon tyrosine modification in thrombin by *N*-butyrylimidazole can be explained by changes in substrate binding. This concept is supported by both the differential nature of the inactivation (fibrinogen-clotting activity is inhibited to a greater extent than esterase activity under our standard assay conditions) as well as the observed changes in affinity for an ester substrate, Tos-ArgOMe, upon modification with *N*-butyrylimidazole. The $K_m$ of the butyrylated enzyme for Tos-ArgOMe is approximately four times larger than that observed for the native enzyme in these experiments. We see no changes in the observed value for $V_{max}$ in any of these enzyme preparations. It is noted that the $K_m$ value reported here for the acetylated enzyme ($4.1 \times 10^{-4}$ M) compares favorably with the value ($4.8 \times 10^{-4}$ M) reported for acetylthrombin (Seegers *et al.*, 1968). The higher $K_m$ value observed for the butyryl enzyme as that compared to the acetyl enzyme is likely a reflection of the greater bulk of the butyryl moiety.

Acknowledgments

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