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Aromatic Esters Which Inhibit Plasmin or Thrombin by Formation of Relatively Stable Acyl Enzymes

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Plasmin and thrombin were inhibited by several substituted benzoic acid esters due to the formation of a moderately stable, inactive, covalent intermediate (acyl enzyme). Although both of these enzymes are serine proteinases of trypsin-like specificity, selective inhibition is shown to be possible through the use of active esters (nitrophenyl) of benzoic acid carrying positively charged substituents such as sulfonium, isothiouronium, and pyridinium. Para substitution favored the selective inhibition of plasmin which appeared to be more susceptible to this type of inactivation than thrombin by the compounds studied thus far. However, thrombin was more extensively converted to the acyl enzyme form than plasmin by certain meta-substituted derivatives.

Thrombin and plasmin are proteolytic enzymes of the serine proteinase class for which there is a common mechanism of hydrolysis involving a covalent intermediate between the enzyme and part of the substrate.[1] The carboxyl group participating in the bond of the substrate that is cleaved becomes transferred to a serine group of the active center of the enzyme. This intermediate, an acyl enzyme, is subsequently split hydrolytically to liberate the carboxylic acid and regenerate the enzyme, completing a catalytic cycle. With typical substrates, the kinetic properties of the acylation and deacylation steps are such that in steady-state function little of the enzyme is present as the acyl enzyme, judging from the well-studied example of chymotrypsin.[2] This is true even in the case of esters for which the rate of acylation is greater than that of deacylation (in contrast to amides). For example, in the case of *N*-acetyl-L-phenylalanine ethyl ester for which the kinetic constants at pH 5 are available,[2] the steady-state level of acyl enzyme can be calculated (using eq 9 of ref 3) to be 0.55% at a substrate concentration of 10^{-5} *M* or 5.1% at 10^{-4} *M*. This is the concentration range of interest in the present investigation. On the other hand, there have been found some synthetic esters for which the deacylation step is very slow. As a consequence these "poor" substrates lead to a steady state in which much or all of the enzymes is tied up as an acyl enzyme and is therefore not available for function. For example, trypsin is inactivated, even at a pH optimal for its function, by the ethyl ester[4] or *p*-nitrophenyl ester (NPGB)[5] of *p*-guanidinobenzoic acid. In view of the fact that an increasing number of serine proteases of physiological importance are being discovered whose inhibition may be of therapeutic value, it was proposed that ester substrates might be synthesized that would provide a means for their selective inactivation.[3,6]

Since NPBG was found to convert thrombin and plasmin completely to an acyl enzyme form, although with different stabilities,[3] a number of other substituted benzoates have been synthesized and examined for their ability to provide this type of inactivation. These contained positive charges since earlier work with trypsin[4,7] as well as in the action of esters of *p*-guanidinobenzoic acid with thrombin and plasmin[3] indicated that substrate behavior was encountered with aromatic esters containing an amidino or guanidino group and having an overall geometry comparable to lysine or arginine.

With respect to the alcohol portion of the esters, the present study was confined to nitrophenyl esters which provided a twofold advantage. Enzymatic cleavage could be readily followed by spectrophotometry and the extent of conversion of thrombin or plasmin to an acyl enzyme could be determined by the subsequent addition of NPGB as a titrant[8] to the test mixture during the same analytical procedure. In addition, nitrophenyl esters are activated with respect to enzyme acylation and thus kinetically favor the accumulation of acyl enzyme. However, the deacylation rate for acyl enzyme hydrolysis is generally rate limiting[2,9] and therefore the extent of inactivation by activated or nonactivated (*viz.* ethyl) esters may be the same. The nature of the alcohol group is a structural variable that may be usefully manipulated to modulate the inhibitory properties of an ester whose main characteristics are determined by the structure of the acyl component. The esters synthesized in the present work structurally complement a group available from earlier work with trypsin[10] and are now examined for their action on thrombin and plasmin.

Chemistry. The new esters described in this study were prepared from the nitrophenyl esters of *p*- and *m*-bromomethylbenzoic acid by reaction with nucleophiles which displaced the bromine without disrupting the ester bond (Table I). The remaining compounds were obtained as described earlier.[10]

Results

The nitrophenyl esters examined were derived from benzoic acid which had a meta or para substituent, either uncharged or positively charged, and were tested at pH 8.3 in the concentration range of 10^{-5}–10^{-4} *M*. As expected, esters with uncharged substituents such as *p*-hydroxy-, methyl-, amino-, chloroacetamido-, as well as nitrophenyl benzoate itself, gave evidence of being very poor substrates for thrombin and plasmin. On the other hand, positively charged substituents such as isothiouronium, sulfonium, or pyridinium

Table I. *p*-Nitrophenyl Esters of Ring-Substituted Benzoic Acids

No.	Position	Substituent	Mp, °C	Formula	Analyses	Yield, %
1	m	$(CH_3)_2S^+CH_2$-	110-113	$C_{16}H_{16}BrNO_4S$	C, H, N	37
2	p	$CH_3(C_2H_5)S^+CH_2$-	139-141	$C_{17}H_{18}BrNO_4S$	C, H, N	38
3	m	$CH_3(C_2H_5)S^+CH_2$-	115-117	$C_{17}H_{18}BrNO_4S$	H, N; C[a]	17
4	m	$HN{=}C(NH_2)SCH_2$-	193-195	$C_{15}H_{14}BrN_3O_4S$	C, H, N	60
5	m	$HN{=}C(NHCH_3)SCH_2$-	156-158	$C_{16}H_{16}BrN_3O_4S$	C, H, N	72
6	m	$CH_3N{=}C(NHCH_3)SCH_2$-	196-198	$C_{17}H_{18}BrN_3O_4S$	H, N; C[b]	56
7	m	$HN{=}C(NHC_3H_7)SCH_2$-	146-149	$C_{18}H_{18}BrN_3O_4S$	C, H, N	81
8	p	$C_5H_5N^+CH_2$-	235-238	$C_{19}H_{15}BrN_2O_4$	H, N; C[c]	43
9	m	$C_5H_5N^+CH_2$-	215-218	$C_{19}H_{15}BrN_2O_4$	C, H, N	15
10	p	$4\text{-}C_6H_5\text{-}CH_2\text{-}C_5H_5N^+CH_2$-	234-236	$C_{26}H_{21}BrN_2O_4$	C, H, N	68
11	m	$4\text{-}C_6H_5\text{-}CH_2\text{-}C_5H_5N^+CH_2$-	239-241	$C_{26}H_{21}BrN_2O_4$	C, H, N	40
12	m	$2\text{-}NH_2C_5H_4N\text{-}CH_2$-	241-242	$C_{19}H_{16}BrN_3O_4$	C, H, N	5

[a] C: calcd, 49.52; found, 49.98. [b] C: calcd, 46.37; found, 46.92. [c] C: calcd, 54.95; found, 54.44.

were generally substrates and, in addition, some of these led to the accumulation of an acyl enzyme intermediate.

Isothiouronium Derivatives. Addition of nitrophenyl *p*-amidinothiomethylbenzoate to a solution of thrombin led to a very rapid release of nitrophenol followed by a slower steady-state formation (Figure 1). This biphasic response is characteristic of acyl enzyme accumulation, the extent of which was determined by addition of NPGB to the mixture (Figure 1, arrow). Since the free thrombin measured by this titrant was 34% of control titrations, it was evident that 66% of the thrombin was temporarily converted to the acyl enzyme. The effect on plasmin was similar with respect to speed of acylation, but the extent of inactivation (91%) was greater (Figure 1). In this and subsequent figures, the incomplete formation of acyl enzyme after the steady state has apparently been reached may be due to relatively poor initial binding and/or to the fact that acylation is not considerably faster than deacylation.[3] Further kinetic analysis will be necessary to evaluate these possibilities. With some reagents, the effect of an increased concentration was determined and the results are reported without interpretation pending further analysis. In this paper we have been concerned chiefly with finding some evidence of selectivity of action based on ester structure. When the *p*-isothiouronium ester under discussion (Figure 1) was tested at a higher concentration (10^{-4} *M*), the results obtained with respect to acyl enzyme formation were quite similar.

The *p*-mono-*N*-methyl derivative was acted on more slowly by both enzymes (Figure 2, note change of time scale relative to Figure 1); however, it was considerably more selective as a plasmin inhibitor (79%) than a thrombin inhibitor (7%)

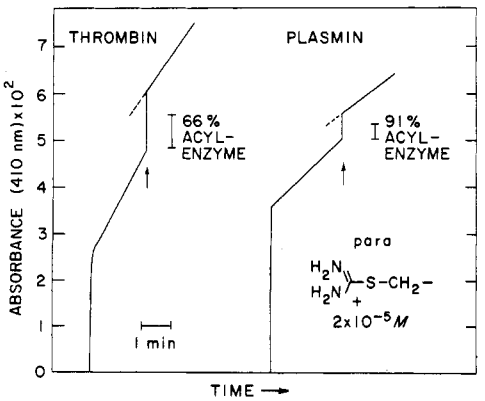

Figure 1. Comparison of the action of nitrophenyl *p*-amidinothiomethylbenzoate on thrombin (bovine) and plasmin (human). Arrows indicate addition of the enzyme titrant, NPGB.[5] In all figures, the time of substrate addition is the point of initial absorbance change.

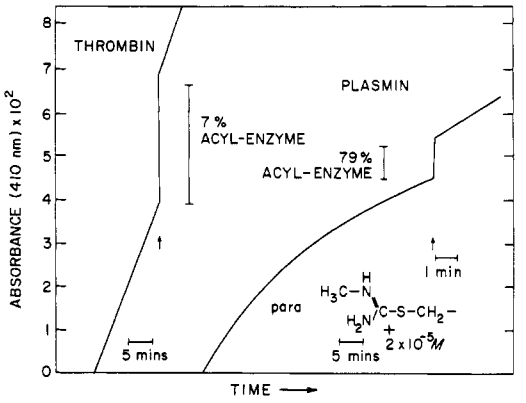

Figure 2. Comparison of the action of nitrophenyl *p*-(*N*-methylamidinothiomethyl)benzoate on thrombin (bovine) and plasmin (human). Arrows indicate addition of NPGB.

at 2×10^{-5} *M*. In addition, the post-burst turnover (Figure 2, final portion of each curve) is lower for plasmin than for thrombin, indicating a more durable acyl enzyme. The *p*-*N,N'*-dimethyl derivative, on the other hand, was a poor substrate for both enzymes, even at 10^{-4} *M*, and did not lead to acyl enzyme accumulation.

The meta isomers of these esters provided an interesting contrast. The unsubstituted derivative was a good substrate for both thrombin and plasmin at 2×10^{-5} *M*, but only thrombin was converted to a measurable proportion of acyl enzyme (20%). The *m*-*N*-methyl derivative, at the same concentration, was even more effective (41% acyl thrombin), whereas its action on plasmin, even at 10^{-4} *M*, was very limited (Figure 3). The *m*-*N,N'*-dimethyl derivative was cleaved by both enzymes without net acyl enzyme formation at 10^{-4} *M*.

In view of the properties of the monomethyl, another monoalkyl derivative was examined, the *m*-*N*-allyl ester; at 2×10^{-5} *M* both thrombin and plasmin provided 35-40% acyl enzyme; the main difference with the methyl derivative was the slower apparent deacylation rate.

Sulfonium Derivatives. The *p*-(dimethylsulfoniomethyl)-benzoate was hydrolyzed by thrombin without accumulation of acyl enzyme (Figure 4). However, during the action of plasmin, the rate continually decreased suggesting inactivation. As shown in Figure 4, 2×10^{-5} *M* ester produced essentially complete inactivation rather slowly. At 10^{-4} *M*, this process reached completion in 4 min. Results with the *p*-(methylethylsulfoniomethyl) ester were comparable in that only plasmin was acylated (74%) at 2×10^{-5} *M*, whereas at 10^{-4} *M* there was less selectivity; 20% formation of acyl thrombin was observed.

In this series, the meta isomers were not conducive to

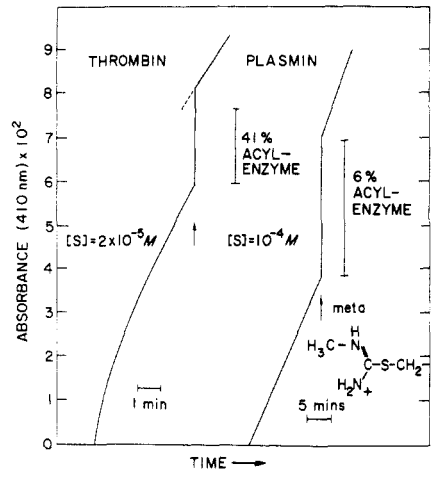

Figure 3. Comparison of the action of nitrophenyl *m*-(*N*-methylamidinothiomethyl)benzoate on thrombin (bovine) and plasmin (human). Arrows indicate addition of NPGB.

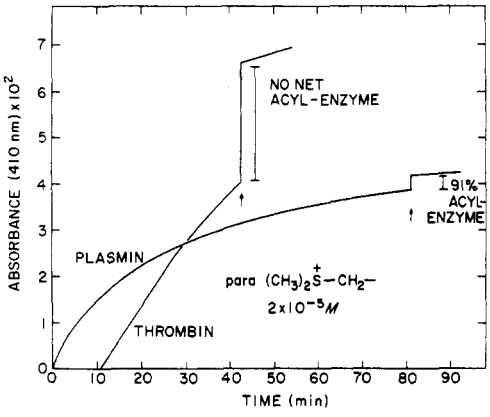

Figure 4. Comparison of the action of nitrophenyl *p*-(dimethylsulfoniomethyl)benzoate on thrombin (bovine) and plasmin (human). Arrows indicate addition of NPGB.

acyl enzyme accumulation. The *m*-dimethylsulfonium isomer at 10^{-4} *M* was merely a substrate for both plasmin and thrombin; the *m*-methylethylsulfonium derivative, at 10^{-4} *M*, was slightly more effective, leading to 15% net acylation with both enzymes.

Pyridinium Derivatives. This group represented an additional class of positively charged esters that were hopefully similar enough to trypsin substrates to undergo cleavage with possible arrest at an intermediate stage. The *p*-pyridinium ester showed promising selectivity as a plasmin inactivator at 2×10^{-5} *M* (Figure 5). Complete conversion of plasmin to the acyl enzyme was observed at 10^{-4} *M*; however, at this level 75% formation of the acyl thrombin was also observed. On the other hand, the *p*-(4-benzylpyridinium) derivative was even more selective; at 2×10^{-5} *M* (Figure 6), or even at 10^{-4} *M*, it was only effective as a plasmin inactivator.

The meta isomers of these esters, as in some other cases, showed a greater propensity to arrest thrombin as the acyl enzyme (Figure 7). Results with the *m*-(4-benzylpyridinium) ester were comparable to the *m*-pyridinium derivative shown in regard to lack of selectivity. It was more rapidly acting, however. A small yield of pyridinium salt was obtained from the reaction of the nitrophenyl ester of *m*-bromomethylbenzoate with 2-aminopyridine, and this derivative showed more selectivity as a thrombin inhibitor (Figure 8).

The inhibition of thrombin by acyl enzyme formation was

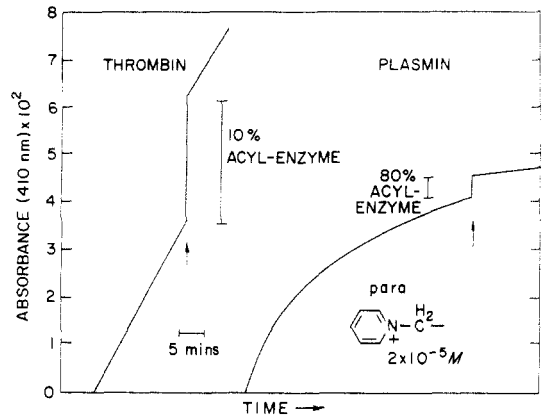

Figure 5. Comparison of the action of nitrophenyl *p*-(*N*-pyridinomethyl)benzoate on thrombin (bovine) and plasmin (human). Arrows indicate addition of NPGB.

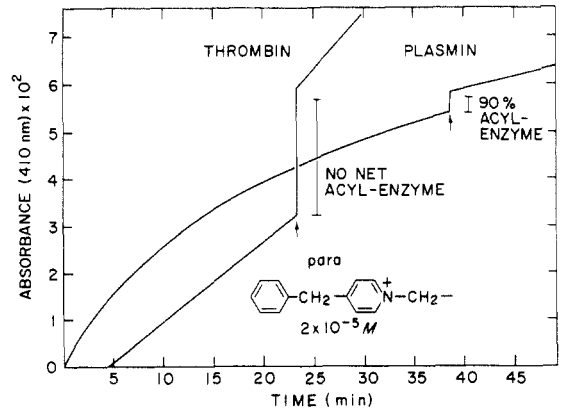

Figure 6. Comparison of the action of nitrophenyl *p*-(4-benzyl-*N*-pyridinomethyl)benzoate on thrombin (bovine) and plasmin (human). Arrows indicate addition of NPGB.

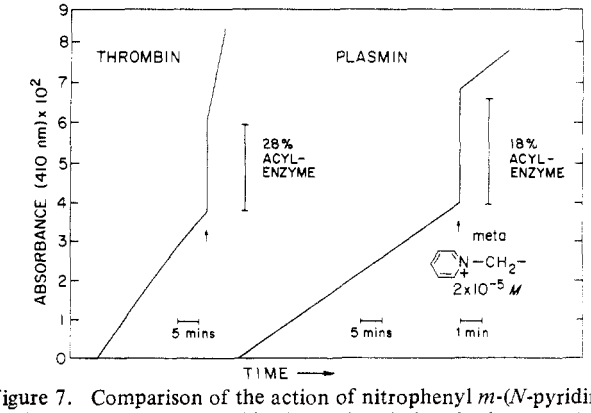

Figure 7. Comparison of the action of nitrophenyl *m*-(*N*-pyridinomethyl)benzoate on thrombin (bovine) and plasmin (human). Arrows indicate addition of NPGB.

also demonstrated by a coagulation assay. Reaction mixtures of thrombin and pyridinium esters shown to form acyl enzyme by spectrophotometric assay were prepared and aliquots were tested in a standard fibrinogen assay. As shown in Figure 9, there was a progressive loss of active thrombin to a steady-state level followed by a gradual recovery of activity due to exhaustion of reagent and deacylation.

Discussion

The inhibition of a target enzyme by covalent modification has appeal as a chemotherapeutic approach because

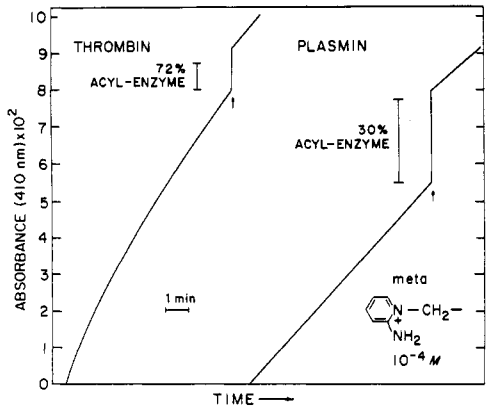

Figure 8. Comparison of the action of nitrophenyl *m*-(2-amino-*N*-pyridinomethyl)benzoate on thrombin (bovine) and plasmin (human). Arrows indicate addition of NPGB.

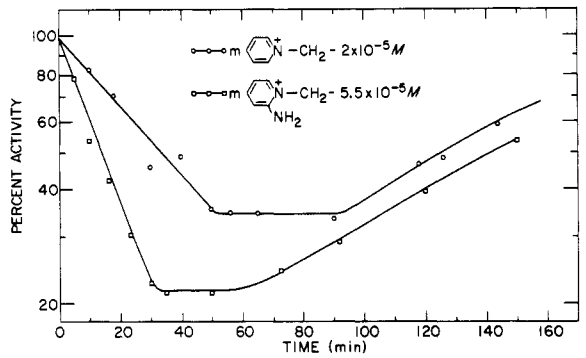

Figure 9. Inhibition of coagulation by action of nitrophenyl esters on thrombin.

such changes are generally irreversible and the inhibition is lasting.[6,11] Inhibition of serine proteinases by acyl enzyme formation as studied in this paper does not lead to irreversible inactivation but may provide an intermediate of such long life the enzyme is significantly inactivated from a functional point of view. Thus, the half time for regeneration of plasmin from *p*-guanidinobenzoylplasmin is over 35 hr at pH 8.3.[3]

In the work described a survey was made of benzoic acid derivatives containing positively charged substituents in the hope of extending observations made with nitrophenyl *p*-guanidinobenzoate. Thrombin and plasmin were studied simultaneously since they are serine proteinases occurring in plasma and offer a challenge in selective enzyme inhibition. Furthermore, thrombin and plasmin are at the opposite ends of the coagulation-fibrinolytic system since thrombin leads to the deposition of a thrombus while plasmin clears the thrombosed area. It has been proposed by Baker[12] that because of their mechanistic similarity selective inactivation of serine proteinases would be possible only by modification outside of their active center regions (*i.e.*, exoalkylation[11]), an approach pursued in his laboratory chiefly in the complement series of serine proteinases.[13]

The results obtained in the present work support the point of view that selective inhibition of serine proteinases is possible by means of ester substrates which exhibit quite different kinetic properties even with closely related enzymes. Although thrombin and plasmin are trypsin-like with respect to their substrate preferences among simple esters, considerable differences were observed in their behavior toward nitrophenyl *p*-guanidinobenzoate.[3] Several compounds described in the present study are even more selective. The sulfonium ester (*i.e.*, nitrophenyl ester of *p*-carboxyphenylmethyldimethylsulfonium bromide, **11**) as well as the *p*-pyridinium **8** and *p*-benzylpyridinium **10** analogs are essentially selective inactivators of plasmin. On the other hand, meta-substituted derivatives such as **5** or **12** show a greater effectiveness with thrombin. A more extensive kinetic study will be necessary to evaluate the differences in binding and the relative acylation and deacylation rates that determine the extent and duration of the inhibition.[3] The current results offer promising leads. Reference to many other reports of synthetic inhibitors for plasmin and thrombin may be found in the recent paper by Okano, *et al.*[14] In most cases competitive inhibitors have been studied;† occasionally these have been esters and it is not clear whether or not acyl enzyme formation has played a part in the observed inhibition.

Chloromethyl ketones derived from amino acids have provided a number of enzyme specific inactivators which irreversibly alkylate trypsin and related enzymes.[6,16] However, it is considered unlikely that these could be used systemically without side reactions. Consequently, the alternate approach utilizing esters was undertaken and, as the present results indicate, shows promise as a source of selective inhibitors.

The nitrophenyl ester of guanidinobenzoic acid (NPGB) has proved to be a useful reagent in enzymological studies of trypsin,[3] thrombin,[3] plasmin,[3] and the mechanism of plasminogen activation.[17] Even closely related forms of trypsin can be kinetically distinguished.[18] It may, therefore, be expected that some of the esters described in this paper will also be of value as analytical reagents due to increased selectivity or favorable kinetic properties.

Experimental Section

p-Nitrophenyl *m*-Bromomethylbenzoate. *m*-Bromomethylbenzoic acid was prepared by acid hydrolysis of the nitrile.[19] The acid (5.4 g), *p*-nitrophenol (4.2 g), and dicyclohexylcarbodiimide (5.3 g) were stirred in acetonitrile (100 ml) at room temperature for 24 hr. The filtrate was taken to dryness and the residue obtained was recrystallized from absolute ethanol yielding 5.4 g (64%), mp 123–127°. *Anal.* ($C_{14}H_{10}NO_4Br$) C, H, N.

Synthesis of Reagents. The nitrophenyl *m*- or *p*-bromomethylbenzoate[10] (0.50 g) in ethyl acetate (10 ml) was stirred with 1–2 equiv (as solubility permitted) of a given nucleophile at room temperature from 2 to 7 days. During this period the product precipitated. It was recrystallized from alcohol with addition of ether if necessary. The new esters are described in Table I. Melting points were determined with a Fisher-Johns apparatus. Nmr spectra obtained with a Varian T-60 spectrometer were consistent with the assigned structures. Diethyl sulfide and methyl allyl sulfide resisted alkylation.

Materials and Methods. Bovine thrombin was purified by chromatography[20] and was 95% active. Purified human plasmin was kindly provided by Dr. Alan Johnson, Department of Medicine, New York University School of Medicine, and was 60% active. Stock solutions of both enzymes were prepared at comparable concentrations, as measured by titration with NPGB,[9] so that 2.5-μl aliquots provided an enzyme concentration of 2×10^{-6} *M* in the 2-ml assay volume. The accuracy of the assay is ±5%.

Stock solutions of the reagents to be tested were prepared in dimethylformamide at 10^{-2} *M* and, in some cases, at 2×10^{-3} *M*. Aliquots (20 μl) were added to a 2-ml cell containing the enzyme and 0.1 *M* sodium Veronal buffer, pH 8.3, at a final volume of 1.98 ml. This provided a final reagent concentration of 10^{-4} or 2×10^{-5} *M*, depending on the stock solution of reagent used, and represented a 50- or 10-fold molar ratio to enzyme. For extended observation of plasmin activity, 0.02 *M* lysine was included in the Veronal buffer for stabilization.[3] If the plasmin stock solution contained glycerol, a compensatory amount was added to the reference cell.

Spectrophotometric Assays. These were carried out at room temperature in a Beckman DB spectrophotometer equipped with an amplifier and recorder providing scale expansion. The molar amount

†Geratz has studied an irreversible inhibitor of the sulfonyl fluoride class.[15]

of enzyme used in the individual assays was established by measuring the "burst" obtained at 410 nm with NPGB.[9] Following this, the ability of a new nitrophenyl ester to tie up the enzyme in an acyl enzyme form could be assessed, in a separate mixture containing the same aliquot of enzyme, by a subsequent addition of NPGB to the mixture of the ester and enzyme. A diminution of the burst indicated net acyl enzyme formation produced by the ester under test.

The following procedure was found to give the most reproducible results. Two-ml cells containing 1.98 ml of buffer (reference cell) or buffer plus enzyme (sample cell) were balanced. With the recorder turned off, the inhibitor to be tested was added to the reference cell with mixing and then to the sample cell, mixing being completed within 10 sec, after which time the recorder was turned on. If nitrophenol was released, time was allowed to elapse until 1–2 molar equiv of color was produced. The free enzyme concentration was then determined. For this purpose, 20-μl pipettes loaded with stock NPGB were prepared. Pipettes with a constriction retained their load of reagent while in a horizontal position, permitting rapid manipulation. The recorder was turned off, and NPGB was added to the sample cell first and then to the reference cell, with inversion of both for mixing. The maneuver could be completed within 30 sec at which time the recorder was turned on. The observed burst was corrected for the change occurring during the elapsed time by extrapolation of the post-burst rate back 30 sec (as shown in Figure 1).

In some cases, due to the low solubility of the reagent, 100 μl or more of DMF was carefully layered over the enzyme solution. The reagent was added to this layer prior to mixing. DMF in the amounts used (under 10% v/v) did not affect the stability of the enzymes nor the extent of acyl enzyme formation although it appeared to slow the acylation rate somewhat.

Inhibition of Coagulation. A reaction mixture composed of concentrated thrombin solution (20 μl), the ester to be tested (0.10 ml of a 10^{-3} *M* solution in DMF), and 1.70 ml of 0.05 *M* imidazole buffer, pH 7.38, 0.15 *M* in sodium chloride was prepared. The final thrombin concentration was 2.4×10^{-6} *M*. Aliquots removed for coagulation assay ranged from 20 to 75 μl (as required to maintain a clotting time of 13–19 sec in the Kline assay[21]) and were added to 0.0139 *M* imidazole buffer, pH 7.38, 0.15 *M* in sodium chloride to achieve a final volume of 0.5 ml for assay.

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Absolute Configuration of α-Methyldopamine Formed Metabolically from α-Methyldopa in Man†

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The absolute configuration of metabolic α-methyldopamine [1-(3,4-dihydroxyphenyl)-2-aminopropane, **2a**] has been unambiguously established. Urine obtained from a hypertensive patient on α-methyldopa [(*S*)-(−)-3-(3,4-dihydroxyphenyl)-2-methylalanine, **1**] therapy was acidified, lyophylized, and extracted with methanol. The methanol extracts were methylated with diazomethane and subsequently purified by acid–base partitioning. The basic fraction, which was shown to contain 1-(3,4-dimethoxyphenyl)-2-aminopropane (**7**), was derivatized with the chiral reagent (*S*)-(−)-*N*-pentafluorobenzoylprolyl 1-imidazolide (**6**). The resulting amide was shown to have a glpc retention time corresponding exactly to the amide formed from authentic (*S*)-(+)-amine **7a**.

The mode of action and metabolic fate of the antihypertensive agent α-methyldopa [(*S*)-(−)-3-(3,4-dihydroxyphenyl)-2-methylalanine, **1**] have been studied by numerous groups.[1] Since it is generally held that the pharmacologic effects of this compound are dependent on its conversion to active metabolites, a more detailed description of the relative stereochemistries of the metabolites should be of value in understanding the mode of action of the drug. As part of our studies on the metabolic fate of α-methyldopa, we have determined and report here the absolute configuration of α-methyldopamine [1-(3,4-dihydroxyphenyl)-2-aminopropane, **2a**] formed *in vivo* by a patient on α-methyldopa therapy.

Sjoerdsma and Udenfriend[2] have shown that the pharmacological activity of **1** is found only in the levorotatory enantiomer, the absolute configuration of which has been independently established by Terashima, *et al.*,[3] and Tristram, *et al.*,[4] to be *S*.

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