Benzosalicylanilide Ester Substrates of Proteolytic Enzymes. Kinetic and Histochemical Studies. I. Chymotrypsin*

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A series of benzosalicylanilide esters has been synthesized and tested for histochemical applicability. Kinetic constants were determined for the hydrolysis of the esters by α-chymotrypsin and rates of hydrolysis were determined for chymotrypsin and trypsin. The specificity evident among the substrates in histochemical studies of chymotrypsinlike enzymes is explicable on the basis of differences in k_0. The significance of the uniformly low K_0 values for these substrates is considered.

Hydrolytic enzymes have proven highly amenable to histochemical analysis in the 24 years since the first *in situ* demonstration of alkaline phosphatase (Gomori, 1939; Takamatsu, 1939). Along with the phosphatases, the esterases were early and extensively studied by histochemical procedures (Nachlas and Seligman, 1949; Pearse, 1960). The fact that enzymes which split proteins also act on small molecular compounds was first demonstrated by Bergmann and his collaborators (Bergmann and Fruton, 1942). Subsequent investigations have indicated that a number of the proteolytic enzymes are not only esterases as well as amidases but that ester substrates are split more rapidly than amides (Neurath and Schwert, 1950). The esterolytic activities of chymotrypsin, trypsin, cathepsin B, and cathepsin C provide a convenient approach to the histochemical demonstration of these proteases through the use of naphtholic esters.

Ravin and Seligman (1954) synthesized the β-naphthol ester of *N*-benzoylphenylalanine but were unsuccessful in their attempts to use this compound as a histochemical reagent. Naphthol AS chloracetate[1] was synthesized in 1953 by Gomori as a histochemical reagent. Although initially the character of the enzyme which split this substrate in tissues was unknown, the compound proved to be the first histochemically applicable substrate for an endoprotease. The work of Benditt and Arase (1959) established that chymotrypsin split the chloracetate and that the enzyme Gomori (1953) had discovered in the mast cell histochemically was quite closely related to α-chymotrypsin (Benditt and Arase, 1958, 1959). Further work has extended the knowledge of the mast-cell enzyme (Lagunoff and Benditt, 1963). In the present study, a series of benzosalicylanilide esters has been investigated as substrates for chymotrypsin and as histochemical reagents.

EXPERIMENTAL

Benzosalicylanilide Esters.—The esters of benzosalicylanilide were all prepared by direct esterification using the appropriate acyl chloride. Benzosalicylanilide[2] (0.02 mole) was suspended in 50 ml 10% dry pyridine in acetone. Acyl chloride (0.025 mole) was slowly added, directly or in an acetone solution, to the benzosalicylanilide suspension with constant mixing. After completion of the addition of the acyl chloride, the solution was allowed to stand at room temperature for 30 minutes to 1 hour or, in the case of the acetate, gently heated on a water bath. The product was obtained by the addition of water to the reaction mixture or by pouring the reaction mixture into several volumes of water at 0°. The precipitate was collected by filtration; the esters were dried and recrystallized several times from hot methanol. Final drying was carried out *in vacuo* over P_2O_5. The melting points and analytical results obtained for the series of compounds are presented in Table I.[3] The formulas for the series of esters are given in Figure 1.

Spectra.—Absorption spectra were obtained with a Beckman DB spectrophotometer equipped with either a Varicord or Beckman DB recorder. Fluorescent measurements were made with a Farrand spectrofluorimeter equipped with an A-c xenon arc lamp and an RCA 1P21 photomultiplier tube. Spectral values have not been corrected for lamp output or photomultiplier response.

Fluorescence of Benzosalicylanilide.—The principal fluorescence of benzosalicylanilide has an excitation maximum at 310 mμ with an emission maximum at 515 mμ (Fig. 2). The intensity of the fluorescence is linearly related to concentration over the range 0.1–10 μM (Fig. 3). The variation of fluorescence with *p*H (Fig. 4) suggests that the ionized naphthol is the principal fluorescent species in the alkali *p*H range.[4] The significance of the naphtholate ion for fluorescence is also attested to by the low fluorescence in absolute methanol and the fact that the esters of benzosalicylanilide have one-twentieth or less the fluorescence of the free benzosalicylanilide (Fig. 2). This low fluorescence of the esters of benzosalicylanilide has been exploited to assay the scission of these esters by measuring the increase in fluorescence that occurs on the appearance of free benzosalicylanilide.

The fluorescence-excitation maximum of benzosalicylanilide at 310 mμ corresponds to an absorption maximum which is abolished on esterification. Over the range of *p*H 6.0–9.0, hydrogen-ion concentration has relatively little effect on the absorption of benzosalicylanilide at 305 mμ (Fig. 4).

Enzymes.—Crystalline α-chymotrypsin and trypsin were obtained from Worthington Biochemical Corp., Freehold, N. J. The respective extinction coefficients,

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[1] The naming of the naphthol AS esters has proved difficult: Gomori (1953) called the first of the series chloracetyl Naphthol As and Benditt and Arase (1958) used 2-chloroacetoxy-naphthoic acid anilide, for the chloracetate ester of benzosalicylanilide.

[2] The benzosalicylanilide (Naphthol AS) was a generous gift from Mr. O. Stallman, E. I. du Pont de Nemours & Co., Wilmington, Del.

[3] We are indebted to Dr. R. Watts for her repeated crystallizations of these compounds and the determinations of melting points.

[4] For a comparison of these results with the effect of *p*H on the fluorescence of other related compounds, see the review by Van Duren (1963).

TABLE I
ELEMENTAL ANALYSIS AND MELTING POINTS OF THE SERIES OF ESTERS OF BENZOSALICYLANILIDE

	I		II		III				IV		V		VI	
Ester[a]	C	H	C	H	C	H	O	N	C	H	C	H	C	H
Calculated[b]	78.46	4.66	78.72	5.02	78.90	5.30	12.2	3.50	79.20	5.66	75.22	5.37	67.90	4.56
Found[b,c]	78.53	4.60	78.89	4.93	78.83	5.29	12.2	3.61	79.27	5.58	75.37	5.39	67.76	4.52
Mp (uncorr)	203–204°		151–152°		141–142° (corr)				134–135°		165–167°		156–159°	

[a] See Fig. 1 for the identification of esters I–IV. [b] Per cent. [c] Determinations made by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

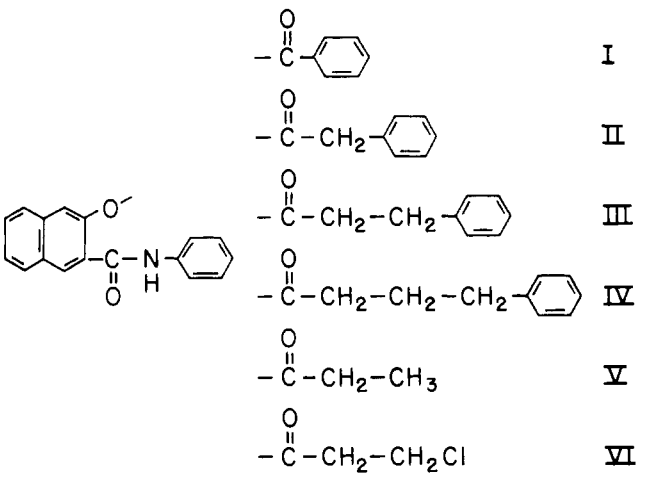

FIG. 1.—Structural formulae of esters.

$E^{1\%}_{280}$ 20.6 and $E^{1\%}_{280}$ 14.4 (Dreyer *et al.*, 1955), were used to calculate concentrations of the enzymes.

Kinetic Studies.—Stock solutions (0.1 mM) of the esters were prepared in the absolute methanol and stored at −15°. For the measurement of hydrolysis rates the stock solutions were diluted immediately prior to use in a medium consisting of 70 parts 0.1 M Tris buffer *p*H 8.0, 10 parts 0.3 M $CaCl_2$, and 20 parts methanol. Insolubility of the esters dictated a maximum concentration of 0.01 mM (except for compounds III and VI, for which the maximum concentrations were 0.006 and 0.05 mM, respectively). Enzyme solution (5–20 μl; 1–20 mg/ml) was added to 4.0 ml of substrate solution and mixed by inversion, and the change in fluorescence was recorded. Spontaneous hydrolysis was negligible except in the case of compound VI. At *p*H 8.0 the spontaneous hydrolysis of compound VI was 0.065%/min at an initial concentration of 0.05 mM. This rate was equal to 0.12% of the rate of enzymic hydrolysis by chymotrypsin. Temperature was not rigorously controlled but did not vary more than ±2° from 25°.

Histochemical Procedures.—Tissues were fixed in neutral buffered formalin for 24 hours at 4° and then placed in gum acacia–sucrose for 24 hours at 4°. Small tissue blocks were frozen in isopentane chilled in liquid N_2; sections were cut in a cryostat, placed immediately in methanol at 4° for 15 minutes, and washed briefly in distilled water at 4° before incubation in substrate–diazonium salt solution at room temperature. The following solution was used routinely: Benzosalicylanilide ester (0.2 ml, 1 mM) in methanol was diluted in 10 ml buffer (0.2 or 0.05 M Tris–absolute methanol, 70:30, v/v) at *p*H 8.0. Twenty mg of diazonium salt (usually Fast Garnet GBC) was added, mixed, and rapidly filtered into Columbia staining dishes. Incubation was carried out at room temperature for up to 30 minutes.

Hexazotized pararosaniline (Davis, 1959) was also used as a coupling agent and found to give a spurious brown color with mast cells in the absence of substrate. This color was distinguishable from the red hexazopararosaniline-benzosalicylanilide product when the latter was sufficiently intense.

Sections were washed with water, examined, and finally mounted in polyvinylpyrrolidone (Burstone, 1959). Very brief counterstaining with hematoxylin was used on occasion. Other nuclear stains had too great an affinity for mast-cell granules to be useful.

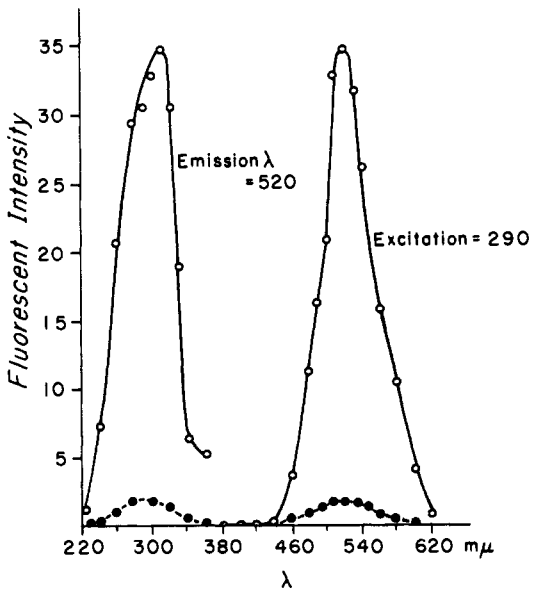

FIG. 2.—Fluorescence excitation and emission spectra of benzosalicylanilide and benzosalicylanilide β-phenylpropionate, 0.01 mM, *p*H 8.0, 0.05 M Tris-methanol, 60:40. Benzosalicylanilide, ○—○; benzosalicylanilide β-phenylpropionate, ●—●.

RESULTS

Values of K_0 and k_0 for hydrolysis by chymotrypsin were calculated as the regression coefficient and ordinate intercept, respectively, of Eadie plots of the kinetic data. The results are compiled in Table II.

TABLE II
KINETIC CONSTANTS FOR CHYMOTRYPSIN HYDROLYSIS OF BENZOSALICYLANILIDE ESTERS[a]

Ester[b]	K_0[c] (× 10^3 M)	k_0[d] (sec^{-1})
I	0.0031	0.0097
II	0.0090	0.0315
III	0.0063	1.2
IV	0.0076	0.077
V	0.0043	0.0083
VI	0.060	0.078

[a] 25° ± 2°, in 20% methanol, 80% Tris buffer, *p*H 8.0, 0.0875 M, 0.03 M $CaCl_2$. [b] See Fig. 1. [c] Determined as the regression coefficients of Eadie plots of the initial reaction rates. [d] Determined as the ordinate intercepts of Eadie plots.

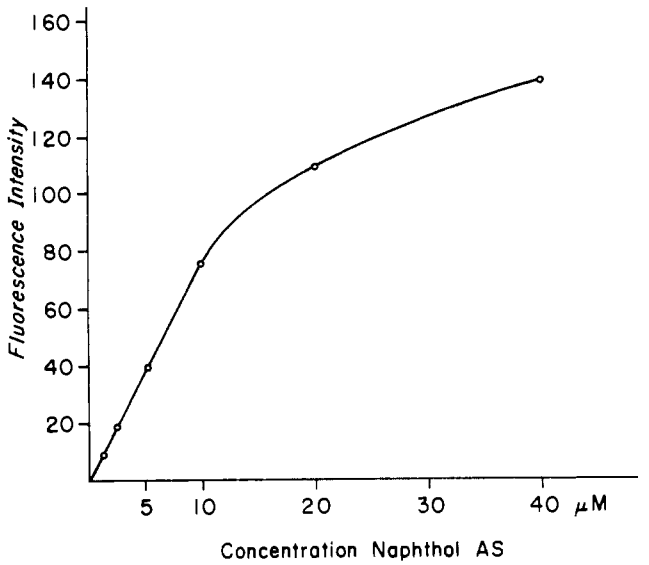

FIG. 3.—Effect of concentration of benzosalicylanilide on the intensity of fluorescence, *p*H 8.0, 0.05 M Tris-methanol, 60:40. Excitation, 310 mμ; emission, 515 mμ.

Complete kinetic data were not obtained for trypsin but a comparison of the reaction rates with trypsin and chymotrypsin for the various substrates is provided in Table III.

TABLE III
RATES OF HYDROLYSIS OF THE BENZOSALICYLANILIDE ESTERS BY α-CHYMOTRYPSIN AND TRYPSIN[a]

Ester[b]	C[c] (mM)	Rate of Hydrolysis: Trypsin (sec^{-1})	Rate of Hydrolysis: Chymotrypsin (sec^{-1})
I	0.01	0.00083	0.0082
II	0.01	0.00043	0.015
III	0.006	0.0016	0.60
IV	0.01	0.00067	0.045
V	0.01	0.0018	0.0068
VI	0.02	0.00096	0.048

[a] 25° ± 2°, in 20% methanol, 80% Tris buffer, *p*H 8.0, 0.0875 M, 0.03 M $CaCl_2$. [b] See Fig. 1. [c] Initial concentration of substrates.

The hydrolysis of ethyl β-chloropropionate by chymotrypsin and trypsin was tested at a maximum concentration of 0.05 M in 0.2 M KCl buffered to *p*H 8.0 with 4×10^{-4} M phosphate buffer. Hydrolysis was measured with a *p*H-stat. Final concentrations of the enzymes as high as 4 mg/ml did not cause measurable hydrolysis.

The histochemical studies demonstrated that strong activity with benzosalicylanilide β-phenylpropionate and benzosalicylanilide β-chloropropionate was present in mast cells of the several species (dog, man, cat, rat) investigated. No activity was evident using the phenylacetate or benzoate. Weak activity was observed using the γ-phenylbutyrate. The dye precipitate appeared to be localized to the granules of the cells. Activity was also present in cells in the intestine of the rat which did not have the typical staining characteristics of the usual connective-tissue mast cells. These cells which were present in the mucosa did not stain as intensely metachromatically as typical mast cells at *p*H 3.5 and did not exhibit distinct granules, although weak metachromasia was evident. The cells have been tentatively considered as a late stage in the maturation of the mast cell.

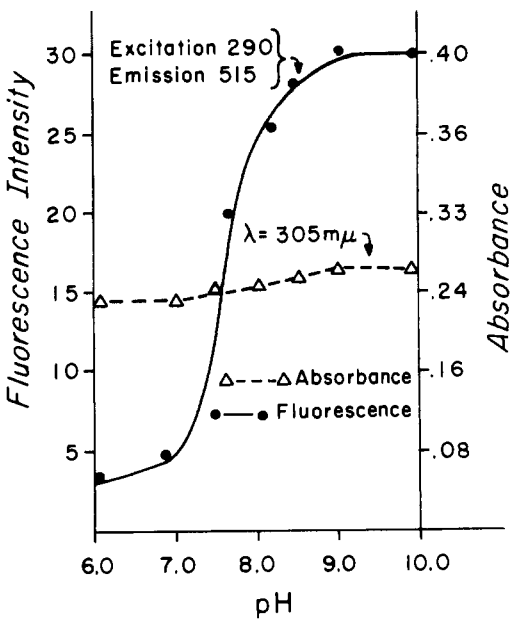

FIG. 4.—Effect of *p*H on absorption and fluorescence of benzosalicylanilide, 0.01 mM, 0.05 Tris-methanol, 60:40.

In sections of rat pancreas prepared in the standard fashion, weak activity was evident in the larger ducts using the β-phenylpropionate derivative of benzosalicylanilide as substrate. When the sections were preincubated 15–30 minutes at room temperature in 0.1% trypsin in 0.1 M $CaCl_2$, activity was observed in the zymogen granules. This activity was not as intense as that of the mast cells. Activity was also evident in material in the lumens of ducts of all sizes. No other tissue elements manifested any hydrolytic activity with these substrates under the conditions employed. Basophils in methanol-fixed smears of rat bone marrow did not exhibit any chymotrypsinlike activity, nor did any of the other blood elements.

DISCUSSION

The assay of rates of hydrolysis of naphtholic esters by fluorescence provides a number of significant advantages over diazo coupling. Measurements can be made continuously on one sample without the introduction of inhibitory diazonium salts. The sensitivity compares favorably with diazo-coupling procedures, and any error introduced in the measurement of the velocity of the enzymic reaction by the rate of the coupling reaction (Lagunoff, 1962) is obviated. Moss (1960) has previously reported briefly on the measurement of phosphatase activity utilizing the difference in the fluorescence of free α-naphthol and α-naphthol phosphate, and esters of several other intensely fluorescent aromatic substances have been exploited for sensitive enzyme assays (Mead *et al.*, 1963; Robinson, 1956, Rotman, 1963).

The dangers of interpreting the value obtained for $1/K_0$ as an affinity constant have been emphasized by Neurath and Hartley (1959), Sturtevant (1959), and Hein and Niemann (1961). A further complication in the analysis of the data obtained with the esters of benzosalicylanilide is the presence of a relatively high concentration (20%) of methanol in the assay system so that methanolysis as well as hydrolysis is likely (McDonald and Balls, 1956; Bender and Glasson, 1960). However, assuming for the sake of analysis the mechanism

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^1 \xrightarrow{k_3} E + P$$

$$K_0 = \left(\frac{k_{-1} + k_2}{k_1}\right)\left(\frac{k_3}{k_3 + k_2}\right)$$

$$K_m = \frac{k_{-1}}{k_1} \quad \text{and} \quad \frac{1}{k_0} = \frac{1}{k_2} + \frac{1}{k_3}$$

then $K_0 \cong K_m$ when $k_3 \gg k_2$ and $k_1 \gg k_2$. These two conditions appear to obtain for some but not all small molecular substrates of chymotrypsin (Neurath and Hartley, 1959). The lack of any consistent significant variation in K_0 for the series of benzosalicylanilide esters I–IV when k_0 varies over a 100-fold range suggests that K_0 may be independent of k_0 for these substrates so that k_3, the rate constant for deacylation, would not be limiting and k_0 would approximate k_2, the rate constant for acylation. Since K_0 is little affected by k_0, K_0 would according to this reasoning be a good approximation of K_m. However it is not possible to prove rigorously from the available data that k_3 is in fact not rate limiting; direct measurement of k_2 and/or k_3 for the various substrates under the appropriate conditions is required. Caplow and Jencks (1962) have determined the rate constant for the deacylation of benzoylchymotrypsin. This value should of course correspond to k_3 for compound I if the conditions are the same. The value obtained by Caplow and Jencks is approximately 0.08 min^{-1} or 0.0013 sec^{-1} (corrected for 0.1 M Tris buffer at *p*H 8.0), which is significantly smaller than the k_0 of 0.0097 $sec.^{-1}$ which we have determined; the presence of 20% methanol (McDonald and Balls, 1956) and/or the $CaCl_2$ may be responsible for this discrepancy. If deacylation is the limiting step it should be possible to demonstrate a burst phenomenon with the fluorogenic substrates analogous to that observed with the esters of *p*-nitrophenol.

The K_0 values for the esters of benzosalicylanilide are unusually low in comparison to other substrates of chymotrypsin (Neurath and Hartley, 1959; Hein and Neimann, 1961). For example, the K_0 of methyl β-phenylpropionate (methylhydrocinnamate) is 3.84×10^{-3} M (Laidler, 1958), about 600 times higher than that of benzosalicylanilide β-phenylpropionate. If the low K_0 of the latter resulted solely from $k_2 \gg k_3$, then k_2 would have to be 600×1.2 sec^{-1} or 720 sec^{-1}, which is 3.0×10^4 greater than the k_2 (0.025 sec^{-1} (Laidler, 1958) for methyl β-phenylpropionate. The contrast between the lack of measurable hydrolysis of methyl β-chloropropionate and the hydrolysis of benzosalicylanilide chloropropionate by chymotrypsin provides further evidence of the effect of the benzosalicylanilide moiety. Evidence of the binding of α-naphthol to α-chymotrypsin has been presented by Wallace *et al.* (1963). They found that α-naphthol competitively inhibited the enzyme and determined a value of 0.2 mM for K_I.

If the analysis by Hein and Niemann (1962) of substrate binding by chymotrypsin is applied to the benzosalicylanilide esters, then binding can be considered to be determined perhaps principally but at least in part by the benzosalicylanilide group at R_3 (Fig. 5). The low k_0 values obtained are predictable on the basis of the absence of an orienting R_1 group leading to a high proportion of "nonproductive binding." According to this line of reasoning, the higher k_0 of the β-phenylpropionate would be ascribed to a more appropriate orienting effect than is provided by phenylalkoxy esters with shorter or longer alkyl chains. This effect is impressive when the small differences in length of the alkyl chains are considered. The propionate ester, V, lacking an aromatic ring at R_2, has a K_0 comparable to that of the phenylalkoxy esters, and the smallest k_0 of the series of esters; this compound may represent a pure R_{3-p} substrate in Hein and Niemann's (1962) terminology; that is, the binding to the enzyme is determined only by the benzosalicylanilide group in position R_3. The greater k_0 for the β-chloropropionate in comparison with the propionate may be explicable in terms of an inductive effect of the chlorine leading to increased rate of acylation and/or deacylation. The 10-fold increase in K_0 for the chloropropionate over the propionate suggests the possibility of an antibinding effect of chlorine; however, if k_3 is limiting, the ratio of K_0 values could merely reflect the higher k_3 value.

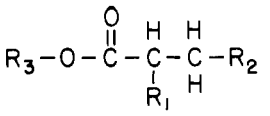

FIG. 5.—Structural formula for typical ester substrate of α-chymotrypsin.

The presence of the acylanilide group in benzosalicylanilide raises the possibility that this group may function as an R_1 substituent. If the aniline ring is coplanar with the naphtholic system (Speigler, 1953) steric correspondence with an R_1 group is impossible. However, since it is possible to construct a noncoplanar model which yields a reasonable correspondence, investigations of the hydrolysis of analogous esters of β-naphthol are necessary to determine the significance of the anilide portion of the molecule.

The results obtained demonstrate a significant modification of the specificity of chymotrypsin for esters of benzosalicylanilide. The blurring of substrate specificity observed for chymotrypsin with benzosalicylanilide esters is even more prominent in the case of putative trypsin substrates which exhibit similar K_0 values for both chymotrypsin and trypsin (Lagunoff *et al.*, 1962). However the influence of the benzosalicylanilide group should not obscure the considerable specificity, dependent on differences in k_0, that is still demonstrable for enzymes *in situ* with histochemical substrates; the effect of varying the length of the alkyl chain separating the phenyl ring and the ester bond in the benzosalicylanilide substrates is a clear case in point.

With the possible exception of the unusual cells in the epithelium of the gastrointestinal tract, only two cell types appear to contain demonstrable levels of an enzyme with the substrate specificity of chymotrypsin. It is not surprising to find that the pancreatic zymogen granules contain an activable chymotrypsin precursor, chymotrypsinogen. This localization has been convincingly demonstrated previously by the technique of immunofluorescence (Marshall, 1954) and the isolation of zymogen granules from homogenates of pancreas (Siekevitz and Palade, 1958). The occurrence of an active, chymotrypsinlike enzyme in mast cells was a histochemical discovery which has been confirmed by the demonstration of the enzyme in isolated mast cells (Benditt and Arase, 1959) and more recently in preparations of mast-cell granules (Lagunoff and Benditt, 1963). It would have been difficult to discover by other than *in situ* histochemical means the cellular location of this alkaline protease. Despite its high concentration in the mast cell, its concentration in tissues is quite low because of the relatively small number of this cell type in most tissues. The specification and localization of other proteases is necessary for an accurate and complete description of tissues of complex animals, and *in situ* histochemistry utilizing sufficiently

specific substrate provides a potent tool for this type of analysis.

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Studies on Structure and Enzymatic Activity of Myosin
Relationship between Conformations and Adenosine Triphosphatase Activity of Myosin and H-Meromyosin in Urea*

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To understand the nature of the enzymatic action of myosin on the basis of its structural specificity, the relationship between ATPase activity and conformational changes induced by urea treatment of myosin A and H- and L-meromyosin was investigated. Conformational changes were studied by means of optical rotation, viscosity determinations, and ultracentrifugation. L-Meromyosin, which has a high percentage of α-helix content, rapidly transforms to a coiled form in urea and regains its original structure upon removal of urea. The H fragment of myosin A, on which ATPase activity is localized, has a less-ordered structure and responds to urea treatment more slowly, although the conformational change finally induced cannot be reversed. These rapid and slow, reversible and irreversible responses of L- and H-meromyosins to urea exist as a complex in myosin A. Loss of ATPase activity of myosin A caused by urea may be owing to (1) oxidation of SH groups at the active site in the presence of metal ion, (2) disintegration of myosin A initiated by the unfolding and subsequent dissociation of L component, and (3) irreversible changes in the secondary structure of the H component. The partial recovery of ATPase activity by urea-treated myosin after removal of urea might be attributed to the refolding ability of L-meromyosin.

Dissociation of the myosin A molecule into three apparently identical subunit chains, in concentrated urea (Small *et al.*, 1961) and guanidine-HCl (Kielley and Harrington, 1960; Young *et al.*, 1962; Woods *et al.*, 1963), has been demonstrated. Upon dilution or removal of these denaturing agents by dialysis, re-formation of the original multistranded structure of myosin A occurred to an appreciable degree; however, no Ca^{2+}-activated ATPase activity was found. With enzymes, such as RNAase (White, 1961) and lysozyme (Imai *et al.*, 1963), a clearer relationship was found between their primary and secondary structures and their enzymatic activities; i.e., these enzymes, denatured in 8 M urea, regain both their structure and activities upon the removal of urea. The mechanism of enzymatic action of

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