A HISTOCHEMICAL ENZYME KINETIC SYSTEM APPLIED TO THE TRYPSIN-LIKE AMIDASE AND ESTERASE ACTIVITY IN HUMAN MAST CELLS

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ABSTRACT

A method for the determination of enzyme kinetic constants V_m, K_m, and K_i in a histochemical system has been devised. As a substitute for the reciprocal of the reaction velocity, the times necessary to reach a fixed amount of end product (the initial visible color) in a tissue site at various substrate concentrations are plotted, according to the method of Lineweaver and Burk, against the reciprocal of the substrate concentrations. The technique as applied to trypsin-like esterase and amidase activities in human mast cells indicates that a single enzyme or closely related enzymes in this site are responsible for the hydrolysis of both the amide and ester substrates and that typical trypsin substrates act as competitive inhibitors of their hydrolysis. Parallel biochemical studies were performed to evaluate the effect of certain aspects of the experimental histochemical method on a purified homospecific enzyme. The relative kinetic constants derived by the histochemical method afford a further means of characterizing enzymic activity in a histochemical system.

Enzymes are characterized biochemically primarily on the basis of their rates of reaction on specific substrates. Histochemically, enzyme characterization has been limited predominantly to a tabulation of the effects of modifiers (inhibitors and activators) on the enzyme-mediated change of specific substrates. Certain parameters, however, derived from the study of the rate of reaction of an enzyme on a specific substrate are known to be of especial value in enzyme characterization. The present report describes a histochemical technique for the study of the kinetics of an enzyme-catalyzed reaction, the derivation of kinetic constants from the data obtained, and the significance of these data in the characterization in human mast cells of an amidase and esterase activity having similarities to trypsin (1–3). Throughout this work, parallel biochemical studies were performed on crystalline trypsin for the purpose of evaluating the effect of certain aspects of the experimental histochemical method on a purified enzyme with similar substrate specificity.

HISTORICAL PERSPECTIVE

The first study of enzyme kinetics in a histochemical system was that of Barter, Danielli, and Davies (4). Graphical data were presented of β-glycerophosphate hydrolysis, as measured by micro-interferometry, relating initial rate of precipitation of calcium phosphate to substrate concentration. No attempt, however, was made to utilize these graphical data for the determination of numerical kinetic constants. Holt and O'Sullivan (5) investigated *in extensio* the theoretical parameters applicable to a study of enzyme kinetics in a histochemical system. In a further investigation of histochemical enzyme kinetics, Benditt and Arase (6) attempted the application

of the graphical method of Lineweaver and Burk (7) to a histochemical diazonium coupling system to obtain qualitative information as to the nature of enzyme activity. From the graphic data the nature of the inhibition could be evaluated. The authors concluded from these graphic representations that enzyme kinetic constants could be extracted, though no numerical values were given (6).

GENERAL THEORY

The most significant of enzyme parameters is the K_m or Michaelis-Menten constant, generally interpreted as signifying the substrate concentration at half maximum velocity. The Michaelis-Menten equation (8) for the determination of K_m is:

$$K_m = S \cdot \left[\frac{V_m}{v} - 1\right] \quad \text{or} \quad v = \frac{V_m \cdot S}{K_m + S}$$

The reciprocal form of this equation is

$$\frac{1}{v} = \frac{K_m}{V_m}\left[\frac{1}{S}\right] + \frac{1}{V_m}$$

as derived by Lineweaver and Burk (7). Since the equation is linear (*i.e.*, $y = ax + b$), there results a straight line when $1/v$ is plotted graphically against $1/S$. This line intersects the ordinate at $1/V_m$. The slope of this line is K_m/V_m and, since V_m can be determined from the intercept, K_m can be calculated, *i.e.* $K_m = V_m \cdot (sl)$. Also K_m can be determined by extending the line to the left of the ordinate until it intersects the abscissa. This point is $-1/K_m$ (9). In the presence of a competitive inhibitor the Michaelis-Menten equation, as modified by Lineweaver and Burk, gives

$$\frac{1}{v} = \frac{1}{V_m}\left[K_m + \frac{K_m \cdot I}{K_i}\right]\left[\frac{1}{S}\right] + \frac{1}{V_m}.$$

If $1/v$ is plotted against $1/S$, the slope of the resulting line is $K_m/V_m[1 + (I/K_i)]$ and the intercept is $1/V_m$. If K_m is determined in the absence of inhibitor, the K_i can be calculated, *i.e.*,

$$K_i = \frac{K_m \cdot I}{(sl) \cdot V_m - K_m}.$$

Any other graphical method for the determination of the kinetic constants, *e.g.* that of Dixon (9) or Eadie (10), is also applicable in checking the above method.

In their application of the graphical method to a histochemical diazonium coupling system, Benditt and Arase (6) utilized the fact that the reciprocal of the time necessary to change a certain amount of substrate is a direct measure of the enzymic activity (11), *i.e.* $v = Q/t$, and, if the quantity of substrate changed (end product) is made a constant, $1/v = k \cdot t$, then $1/v$ is directly proportional to t. In their example of the application of this method histochemically, a series of geometrically increasing time intervals and substrate concentrations were used and varying color intensities were given prescribed values, *i.e.*, A, B, and C. These authors then plotted the time necessary to obtain a fixed end product, *e.g.* the intensity of color represented by C at varying substrate concentrations. The same technique was applied in the presence of a single inhibitor concentration to plot inhibitor effect on the reaction rate.

RATIONALE FOR PRESENT METHOD

After numerous trials of the Benditt and Arase technique in several histochemical systems (1, 12), it became apparent that this method would have to be extensively revised if reproducible numerical kinetic data were to be derived from the graphical plots.

In the development of the present method certain aspects of enzymic reactions were taken into consideration. In order to approximate as closely as possible the real initial velocity of an enzymic reaction, the time of incubation should be kept as short as possible so as to minimize the effect of any inactivation of the enzyme. A short incubation

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v = velocity of the reaction
V_m = maximal velocity of the reaction
S = substrate concentration
I = inhibitor concentration
E = enzyme concentration
K_m = Michaelis constant, derived from equation

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} C \xrightarrow{k_3} P, K_m = \frac{k_2 + k_3}{k_1},$$

where diazonium coupling reaction is assumed to be complete and instantaneous.

t = time
K_i = inhibitor constant
sl = slope
K_{IES} = enzyme-substrate inhibition constant
Q = fixed amount of end product

time would also maintain the products of the reaction, which might cause a slowing of the reaction rate, at a low concentration relative to that of the substrate. Since the time necessary to reach a fixed end point is dependent upon substrate concentration, the time necessary to reach the fixed end point should be an unrestricted variable and not assigned a series of geometrically increasing values.

Specific features peculiar to the diazonium coupling reaction in histochemistry had also to be considered. One of the most important of these is the inhibitory effect of many diazonium salts or their adjuvants on enzymic activity. Another is the background coloration afforded to tissues by the diazonium coupling with proteins as well as the adsorption by proteins of decomposed diazonium salt products. Since these effects are a function of time, incubation time for the histochemical enzymic reaction should be kept to a minimum. Furthermore, diazonium salts, besides coupling with phenolic and indolic compounds, can react reversibly with primary aliphatic amines to form unstable triazene compounds. Whether this latter reaction has any effect on the enzymic hydrolysis of peptides and aliphatic amine substrates is unknown. However, the use of phenolic or indolic substrates or inhibitors in the kinetic system appears definitely contraindicated in view of the fact that azo products of these compounds, formed as the result of diazonium coupling, will reduce the effective substrate or inhibitor concentration at definite but unknown rates. The significance of the coupling rates of diazonium salts in determining kinetic constants will be dealt with in detail elsewhere in this report.

Certain factors relating to visual photometry also influenced the experimental approach. The point of "just perceptible luminance" is reproducible under optimal conditions to within 6 per cent on either side of the mean (13). Although the difference sensitivity for color is little affected by the general luminance level, a surrounding colored field greatly reduces the ability of the eye to sense color differences (14). A short incubation time would tend to reduce the distracting background coloration caused by diazonium salts.

In view of the above considerations it was decided to use, as a fixed end point in the histochemical kinetic system, that concentration of product necessary to produce microscopically in a tissue site a just visible color.

The assumption was made that the rate of dye production, as the result of the enzymic activity, is linear and that all just visible mast cells contain the same amount of dye product. Since no estimations of enzymic activity were based on integration from graded color intensities, only one intensity being used as reference point, it was not necessary to demonstrate linear stoichiometry over a wide dye intensity range, *i.e.* continuously graded intensities of color with increasing concentration of the colored product. In this way, visual threshold sensitivity was used to determine the end point and the incubation time was reduced to the absolute minimum for light microscopy. The time necessary to achieve the fixed end point was recorded, thereby becoming an unrestricted variable, and the use of substrates or inhibitors having phenolic or indolic moieties was avoided in kinetic studies.

REAGENTS

The substrates used in these experiments were the β-naphthylamide hydrochlorides of benzoyl-DL-arginine, benzoyl-D-arginine and benzoyl-DL-lysine, and the hydrobromides of Naphthol AS and AS-D ε-aminocaproate.[1] *Synthesis:* The synthesis of benzoyl-DL-arginine β-naphthylamide hydrochloride has been previously reported (1, 15) and the syntheses of the hydrobromides of the Naphthol AS and AS-D ε-aminocaproates will be described in a separate report (3). For permission to report the synthesis of benzoyl-DL-lysine β-naphthylamide HCl we are indebted to Dr. J. E. Folk, who first described the use of this compound (16).

BENZOYL-*D*-ARGININE β-NAPHTHYLAMIDE HYDROCHLORIDE was synthesized from benzoyl-DL-arginine β-naphthylamide following the general enzymic method described by Erlanger *et al.* (17), using trypsin to resolve the racemate. The compound was crystallized from ethanol-ethyl acetate, mp 219–220. Yield 40 per cent of theoretical. Calculated for $C_{23}H_{25}N_5O_2 \cdot HCl$: C, 62.8; H, 5.8; N, 15.9. Found: C, 62.8; H, 5.9; N, 15.8. $[\alpha]_D^{20}$ + 38.6° (*c*, 1.0 in pyridine). Difficultly soluble in water and alcohols;

[1] Abbreviations.

BANA	benzoyl arginine β-naphthylamide
BLyNA	benzoyl lysine β-naphthylamide
EACA-AS	Naphthol AS ε-aminocaproate
EACA-AS-D	Naphthol AS-D ε-aminocaproate
BAA	benzoyl-L-arginine amide
BAME	benzoyl-L-arginine methyl ester
Cbz	carbobenzyloxy
β-N-AS	Naphthol AS
β-NA	β-naphthylamine

soluble in pyridine, dimethylsulfoxide, and dimethylformamide. Mutarotation occurs in dimethylformamide.

BENZOYL-*DL*-LYSINE β-NAPHTHYLAMIDE HYDROCHLORIDE: The α-benzoyl-ε-Cbz-DL-lysine β-naphthylamide was synthesized from α-benzoyl-ε-Cbz-DL-lysine and β-naphthylamine by the mixed anhydride technique using isobutyl chloroformate in methylene chloride. Following washing and drying of the methylene chloride, the solution was concentrated and pentane added to a cloud. The compound crystallized on cooling and was recrystallized from 80 per cent methanol, mp 153–5. Calculated for $C_{31}H_{31}O_4N_3$: N, 8.25. Found: N, 8.24. Benzoyl-DL-lysine β-naphthylamide hydrochloride was obtained from the above Cbz derivative by hydrogenation in dilute hydrochloric acid in the presence of palladium black. The solvent was removed after filtration, the compound crystallized from ethanol by addition of ether, and recrystallized from hot methanol-ethyl acetate, mp 195. Calculated for $C_{23}H_{25}N_3O_2 \cdot HCl$: C, 67.1; H, 6.3; N, 10.2. Found: C, 67.0; H, 6.6; N, 10.0 $[\alpha]_D^{20}$ 0° (*c*, 1.0 in ethanol). Soluble in water and methyl and ethyl alcohol.

Benzoyl-L-arginine methyl ester and benzoyl-L-arginine amide were obtained from Mann Research Laboratories, New York; Fast Garnet GBC from Verona Dyestuffs, Union, New Jersey; trypsin 4× pancreatin from Nutritional Biochemicals, Cleveland, Ohio, and bovine trypsin (crystalline) from Worthington Biochemical Corp., Freehold, New Jersey, and Sigma Chemical Co., St. Louis, Missouri.

METHODS AND PRELIMINARY EXPERIMENTS

HISTOCHEMICAL: Sections of human and canine skin were obtained at necropsy and immediately frozen on dry ice and cut at 8 and 16 μ using an International Minot rotary microtome in a cryostat according to the method of Klionsky (18). Using continuously the same binocular Bausch and Lomb microscope with Abbe condenser, 100 × magnification and usual incandescent light source under Köhler illumination, we obtained the enzymic kinetic information needed in the following manner. Serial frozen skin sections mounted on slides were incubated at room temperature (25°C) in Coplin jar covers, each containing a constant quantity of buffer, diazonium salt, and varying concentrations of substrate (see specific studies for exact incubating solution compositions), and continuously agitated. The investigator took slides continuously from the incubating solutions at short time intervals for rapid microscopic examination and returned them to the solutions for continued incubation. At the moment when the observer first was able to visualize mast cells in a given slide, the time of observation for this slide was recorded. The time generally varied from 5 to 30 minutes. For the study of inhibitor effects, the incubating solution remained the same with the only addition being the presence of a constant concentration (determined by preliminary trials) of inhibitor (for Lineweaver-Burk plots). For the purpose of avoiding prejudice, the slides in solutions containing different substrate concentrations were given arbitrary letters by a second party who recorded the time from a stop watch when the investigator indicated his observation of just visible mast cells for each labeled slide.

In practice it was observed that sites with numerous mast cells, *e.g.* those surrounding hair follicles, tended to show cells earlier than those areas with a lower cell density. Therefore, the corresponding areas of the section were always studied. For this reason and also because definite differences in the enzymic activity in the mast cells of various skin sections were observed, it was necessary to use serial sections. Fig. 1 demonstrates that all the mast cells in a specific area of the section do not become visible at the same time, a fact expected on the basis of the random localization of the cells through the thickness of the section. Therefore, that moment when the investigator was able to see at least 4 mast cells was chosen as end point; *i.e.*, a group of mast cells rather than a single cell was used as the reference point. As soon as it was noted that the end point had been reached in the first of the incubating slides, this slide was taken into buffer solution to be used as reference to assure similarity in the end point estimations. In separate double-blind experiments calculation of the error in time needed for the appearance of the mast cells, other experimental conditions and the investigator being the same, consistently showed a coefficient of variation of less than 10 per cent. In Table I the results of two separate experiments are presented. The error of the over-all method when determining the kinetic constants was found to be less than 50 per cent.

Correction for the spontaneous hydrolysis of the substrate in the incubating solution (6) was made in those cases in which it was necessary.

BIOCHEMICAL: In the biochemical studies on the tryptic hydrolysis of the substrates used in the histochemical procedures, the source of the bovine trypsin was a 3× crystallized Worthington preparation. For comparison, several other commercial preparations were tested. When DL-BANA and DL-BLyNA were used as substrates, they were dissolved in distilled water. Because of the extreme aqueous insolubility of D-BANA, it was found necessary to dissolve it in dimethylsulfoxide which was then diluted with water to afford a solvent with little effect on the enzymic activity. EACA-AS was

dissolved in 0.1 mM hydrochloric acid to impede spontaneous hydrolysis. Buffer used throughout the study was 0.1 M phosphate, pH 7.2, and Fast Garnet GBC was used as the diazonium salt component. A solution of the salt (3.5 mg/ml) was freshly prepared, filtered, and kept in an ice cold flask before and during the experiments.

In simultaneous coupling experiments the diazonium salt was included in the incubation solution. The enzymic reaction was stopped by the addition of 0.7 ml of N acetate buffer (pH 4.2) in which Tween 20 (10 per cent) was incorporated to disperse the azo dye product.

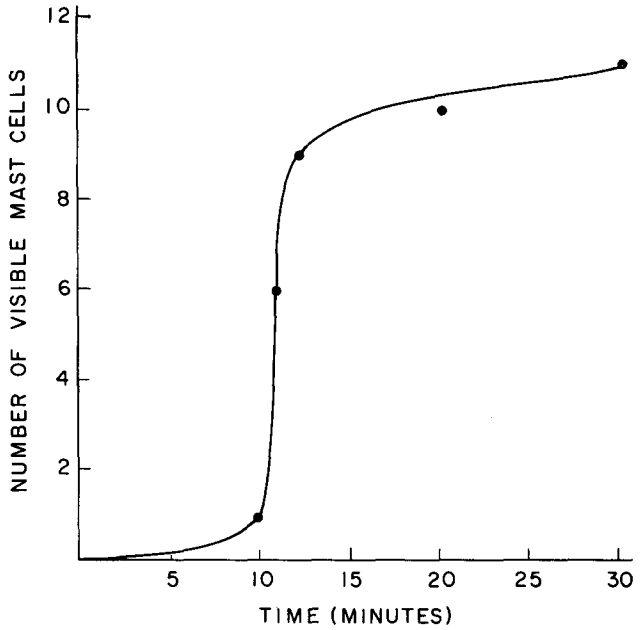

FIGURE 1

Time sequence for the appearance of visible mast cells in a specific microscopic field. Substrate, DL-BANA, 0.10 mM; Garnet GBC, 0.4 mg/ml.

In postcoupling experiments using procedures outlined previously (19), the enzymic reaction was stopped by the addition of 1.0 ml of N acetate buffer (pH 4.2) containing Garnet GBC and, to disperse the azo dye product, Tween 20 (10 per cent). In both the simultaneous and postcoupling methods the color absorptions were measured using a Beckman DU spectrophotometer at 525 mμ and 545 mμ for β-naphthylamine and Naphthol AS, respectively. Standard curves were prepared from an alcoholic solution of β-naphthylamine and Naphthol AS in conditions identical to those used experimentally.

A fluorimetric assay was developed to control part of the experiments. An Aminco-Bowman spectrophotofluorimeter was used, and the measurements of the enzymically liberated β-naphthylamine were obtained with the excitation wave length of 280 mμ and fluorescence at 410 mμ. The corresponding wave lengths in the assay of liberated Naphthol AS (20) were 310 mμ and 515 mμ, respectively. Essentially similar results were observed using the postcoupling and fluorimetric techniques. For further details of procedures, see the experimental section.

Effects of Diazonium Coupling Rate on Experimental Procedure

In the presentation of the method so far it has been assumed that the color formation in tissues from the enzymically liberated β-naphthylamine or Naphthol AS through coupling with the stabilized diazonium salt Garnet GBC occurred so rapidly that this step was not limiting in the determination of the reaction velocity. The coupling step is non-enzymatic, is dependent on the typical coupling characteristics of both the diazonium salt and the naphthylene compounds as well as on the concentration of these substances, and is a second order reaction.[2] In order to evaluate the effect of the diazonium coupling rate on kinetic constant determinations, the following experiments were performed.

The coupling velocity was studied in test tube experiments with a Beckman DU spectrophotom-

[2] Under optimal histochemical conditions where the concentration of diazonium salt is high in relation to the released naphthylene component, this could be considered as a first order reaction (5).

eter, using, as a substrate solvent, 1) an aqueous solution, 2) an aqueous solution with added albumin, 3) an aqueous solution with added Tween 20. The following solutions were prepared. β-naphthylamine (5.7 mg, 40 μM) was dissolved in 3 ml of absolute methanol and then diluted to 200 ml with distilled water to give a clear solution. Bovine albumin (Nutritional Biochemical Corporation) was used as a 1 per cent solution in 0.1 M phosphate buffer, pH 7.2. Tween 20 was used a a 10 per cent solution in 0.1 M buffer, pH 7.2. The addition of the albumin or Tween

TABLE I

Variation in the Time Required for the Appearance of Visible Mast Cells in Two Series of Double-Blind Experiments

Experiment No.	Individual observations	Mean	SD	SD/Mean
	(*Min.*)			%
1	6.5, 7.0, 6.0, 8.0, 7.0, 7.5, 7.0	7.0	0.65	9.3
2	16.0, 16.5, 18.0, 15.0, 17.0, 14.0, 15.0	15.9	1.37	8.6

In Experiment 1: substrate concentration of DL-BANA, 0.12 mM; in Experiment 2: 0.050 mM.

did not affect the pH of the solution. The test solution consisted of

Buffer, 0.1 M phosphate buffer, pH 7.2	1.7 ml
β-naphthylamine stock solution	1.0 ml
Garnet GBC stock solution (3.5 mg/ml)	0.3 ml

The β-naphthylamine solution and buffer were mixed at room temperature (27°C) in the spectrophotometric cuvette and the Garnet GBC solution was immediately added, mixed, and the cuvette placed in the spectrophotometer and readings taken instantly and at 15-second intervals until no further reaction occurred. In the remaining experiments 0.5 ml of buffer was replaced with 0.5 ml of the albumin buffer solution or the Tween 20 buffer solution. Half-reaction times were calculated on semilogarithmic graphs. Results of a typical experiment are presented in Fig. 2 and Table II, showing the half-reaction times measured. These experiments show that with these concentrations of coupling component (β-naphthylamine) and diazonium salt (Garnet GBC) relatively long half-reaction times in aqueous solution were observed. By doubling the concentration of diazonium salt, the half-reaction time was reduced 50 per cent of the original. Moreover, in the presence of albumin, the half-reaction time was lowered by 50 per cent, and, with the reaction solution containing Tween 20, the half-reaction time was less than 10 per cent that in water.

These experiments clearly show that the diazonium coupling rate is highly dependent upon the medium in which coupling occurs as well as the molecular proportions of the reactants. In tissue mast cells we have a known high concentration of proteins and lipoid materials which may act as surfactants to affect coupling rate. Furthermore, the naphthylene coupling component is liberated in monomolecular form (*status nascendi*) during the enzymic reaction, so that the actual coupling rate may be exceedingly rapid as compared to that in aqueous solution. Since we do not know either the actual local solvent situation in which the coupling reaction takes place or the actual concentration of diazonium salt during the steady state of the reaction, we cannot predict *from test tube experiments* the real coupling speed in mast cells during the histochemical reaction. It seems reasonable to expect that a delay caused by the coupling speed with Garnet GBC may cause a certain constant error in determinations of velocity in the present method; but this error may be insignificant, especially when viewed in terms of the scope of other experimental errors.

Effect of Concentration of Diazonian Salt on Histochemical Coupling Reaction

Essentially the same problem was studied in tissue sections, using as test object the mast cell, with varying concentrations of Garnet GBC in the incubating solution. As noted above, the coupling speed is directly proportional to the concentration of the diazonium salt. If the concentration of diazonium salt is a limiting factor in the coupling reaction, then at different concentrations of diazonium salt one would expect to observe the cells at different times. In Fig. 3 the effect of varying the diazonium salt concentration on the time needed to visualize mast cells is recorded. Al-

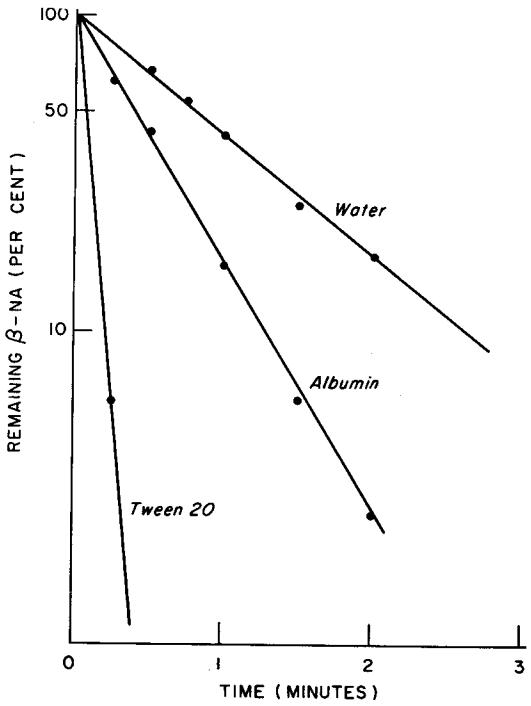

FIGURE 2

Determination of the half-reaction coupling times in various reaction media.

TABLE II

Dependence of the Coupling Speed of β-Naphthylamine with Garnet GBC on the Composition of the Reaction Mixture

Concentration of Garnet GBC	Half-reaction times		
	Water	Presence of albumin	Presence of Tween 20
(mg/ml)	*(sec.)*	*(sec.)*	*(sec.)*
0.70	93	48	7
1.40	42	23	4

though it would be expected that on increasing the concentration of diazonium salt the appearance of visible mast cells would be noted sooner, this is actually true only within a rather narrow concentration range. At too low a concentration, diffusion of the released coupling component occurs, as evidenced by poor localization of azo dye to the cell. At too high concentrations of diazonium salt, the background staining interferes with mast cell visualization, and inhibition of enzymic activity probably occurs, both factors combining to lengthen the time necessary for the first observation of mast cell staining. It is apparent that these latter effects are a limiting factor in attempts to shorten the incubation time by increasing the diazonium salt concentration above a certain point.

AMIDE SUBSTRATE EXPERIMENTS AND RESULTS

HISTOCHEMICAL STUDIES: With the racemic substrates DL-BANA and DL-BLyNA (each having a zero rotation in ethanol) the enantiomorphic-L-forms constituted 50 per cent of the substrate concentration. Final molar concentrations, therefore, were based only on the concentration of the L-form, though experiments are discussed in terms of the racemate. Preliminary studies showed that the shortest measurable time was obtained with final substrate concentrations of L-BANA of 0.11 mM or over. Substrate concentrations of under 11 μM increased the reaction time over 30 minutes. Therefore, the final graded series was adjusted between these limits. The following stock solutions were prepared: substrate solutions of DL-BANA and DL-BLyNA at a concentration of 1 mg/ml in water (L-BANA concentration of 1.1 mM and L-BLyNA concentration of 1.2 mM), a stock aqueous solution of Garnet GBC at 3.5 mg/ml, and stock aqueous inhibitor solutions of benzoyl-L-arginine amide

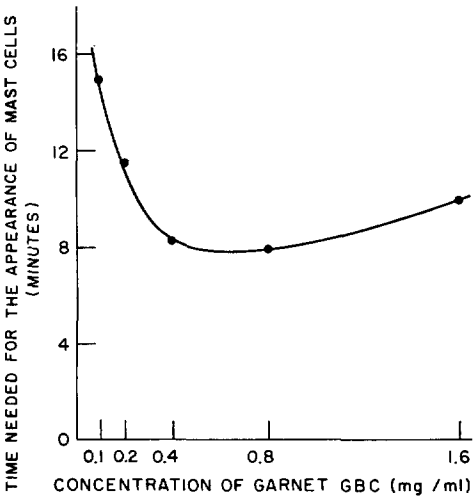

FIGURE 3

Dependence of the time needed for the appearance of visible mast cells on the concentration of Garnet GBC. Substrate concentration, 0.10 mM. Each point represents the mean of three separate experiments.

(BAA) and benzoyl-L-arginine methyl ester (BAME) at 20 mM concentrations. The incubation solutions had the following composition:

Substrate solution	L-BANA concentration	Phosph. buffer	Garnet GBC	Water
ml	mM	*ml*	*ml*	*ml*
1.00	0.11	5.0	1.0	3.0
0.50	0.06	5.0	1.0	3.50
0.35	0.04	5.0	1.0	3.65
0.25	0.03	5.0	1.0	3.75
0.20	0.02	5.0	1.0	3.80

The solutions were mixed just prior to use. In inhibitor studies using BAA as inhibitor, 2.5 ml of the inhibitor solution replaced an equal volume of water (final inhibitor concentration 5 mM). With BAME as inhibitor, 0.5 ml was used (final concentration, 1.0 mM).

RESULTS: Fig. 4 is a Lineweaver-Burk plot of the hydrolysis of DL-BANA in human mast cells

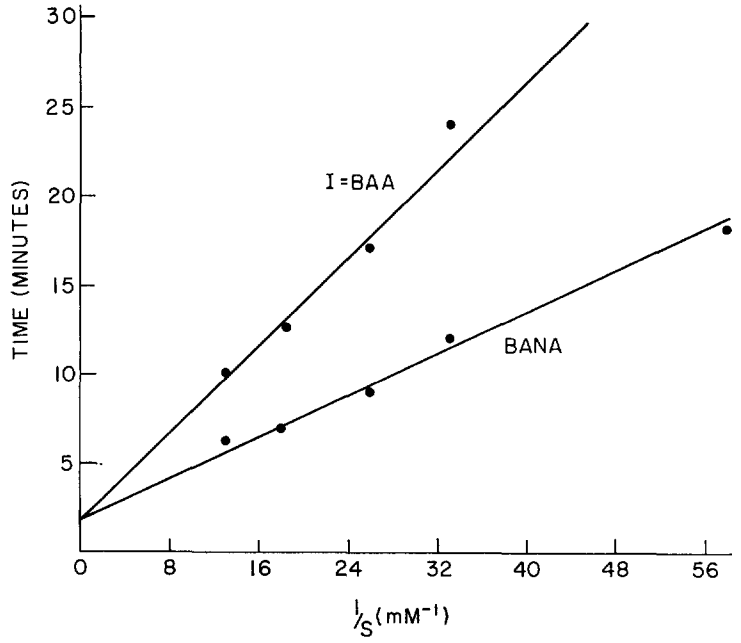

FIGURE 4

Lineweaver-Burk plot of the hydrolysis of DL-BANA by the mast cell enzyme(s). The lower line in the absence and the upper in the presence of BAA, 5.0 mM. Each point represents the mean of three separate experiments.

and reveals that the inhibition of that hydrolysis by BAA is competitive in nature. Another experiment with DL-BANA, but using BAME as inhibitor, is shown in Fig. 5 and reveals that this inhibition is also competitive. It was found that DL-BLyNA was hydrolyzed by the human mast cell enzyme at approximately the same rate as enzymic activity previously discussed became maximally operative. No reaction in canine mast cells was noted using DL-BLyNA as substrate during an incubation period of 1 hour.

The question arose whether the hydrolysis of these two substrates in human mast cells was catalyzed by the same or different enzymes. If both substrates were split by the same enzyme at the same rate, then, in the presence of the same competitive inhibitor, the hydrolysis of both substrates should be inhibited to the same extent. The K_i values determined for any competitive inhibitor are the K_m values of that inhibitor, if it acts as a substrate for the same enzyme. If the K_i values of the inhibitor are the same using two different substrates, then it can be generally assumed that the two substrates are split by the same enzyme or at least by similar enzymes. With BAA as inhibitor the inhibition was found to be competitive with both substrates and the K_i values similar (Table III). The problem was also studied from another viewpoint. If two different enzymes are acting on a mixture of both the naphthylamide substrates at identical concentrations in the same solution, the V_m observed would be expected to increase with the same slope being maintained. If, however, only one enzyme is involved, the V_m would remain the same but the slope would be decreased. Experiments revealed that the latter situation was the case. Therefore, these studies support the existence of only one

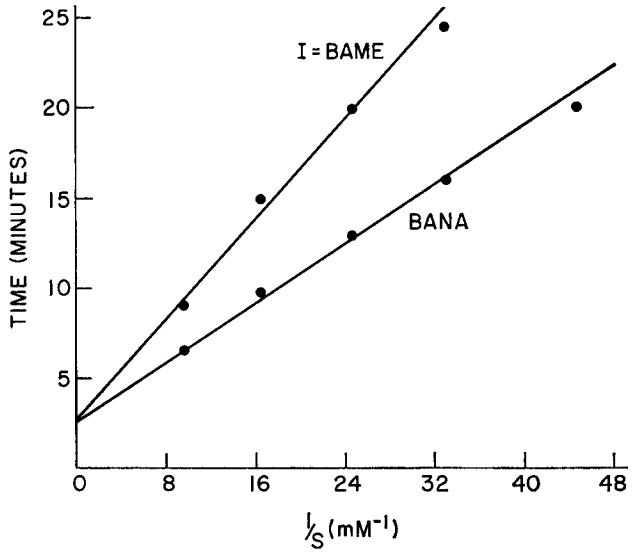

FIGURE 5

Lineweaver-Burk plot of the hydrolysis of DL-BANA by the mast cell enzyme(s). The lower in the absence and the upper in the presence of BAME, 1.0 mM. Each point represents the mean of three separate experiments.

TABLE III

Values of Kinetic Constants of the Hydrolysis of DL-BANA and DL-BLyNA by the Mast Cell Enzyme(s) with BAA as Competitive Inhibitor

Constant	Substrate	
	DL-BANA	DL-BLyNA
K_m	0.17×10^{-3} M	0.19×10^{-3} M
K_i (BAA)	5.0×10^{-3} M	5.8×10^{-3} M

DL-BANA, both requiring 5 to 6 minutes for the demonstration of mast cells. The theoretical V_m values determined from the Lineweaver-Burk plot were about the same and would give observable mast cells in 2 minutes. The K_m values given in Table III for both substrates are the same within the limits of experimental error. In dog tissues the reaction with optimal concentrations of DL-BANA was rather slow, with staining appearing in 20 to 25 minutes. Because of the slowness of the reaction in the mast cells of this species, no attempt to determine kinetic constants was made since the factors impairing evaluation of

enzyme or several similar enzymes acting on both DL-BANA and DL-BLyNA.

As earlier noted, substrate concentrations of the BANA and BLyNA racemates were calculated on the basis of the L-enantiomorph, since it was assumed that the D-form did not act as a substrate for the enzyme. To confirm this assumption experiments were carried out with D-BANA. It was found that D-BANA was not hydrolyzed in the mast cells and, when inhibition studies were performed, acted as a competitive inhibitor. It was, therefore, evident that in the racemic solution the D-enantiomorph acted as a competitive inhibitor to the L-form, but, since the concentration of these was always in a 1:1 ratio, the inhibitor effect was constant. However, these studies indicated that the kinetic data obtained for the rate constants of DL-BANA and DL-BLyNA referred only to the racemates and not to the effective L-enantiomorph.

STUDIES WITH CRYSTALLINE TRYPSIN: AMIDASE ACTIVITY

Comparative studies with trypsin appeared to be indicated since certain problems inherent in the histochemical system could best be evaluated using an enzyme having substrate specificities similar to those of the enzyme in human mast cells (1). Characteristics of the tryptic hydrolysis of DL-BANA have been described earlier by Riedel and Wünsch (15) and Blackwood and Mandl (21) and, though repeated and confirmed, are not reported here.

In our preliminary studies it was observed that the presence of a diazonium salt (the situation existing in the histochemical system) affects the hydrolysis of DL-BANA by trypsin and that this inhibitory effect is dependent on the diazonium salt concentration. Therefore, in order to attempt an evaluation of the histochemical hydrolysis of DL-BANA, a simultaneous coupling system was used to measure tryptic hydrolysis with the concentration of diazonium salt and pH the same as that in the histochemical system. Also, as earlier noted, it was assumed that only the L-enantiomorph of DL-BANA was acted upon by trypsin. In order to establish this as a fact and to determine whether the D-form present in the substrate affected the hydrolysis rate of the L-form, separate studies were made using pure D-BANA. For these studies the following stock solutions were used: enzyme stock solution consisted of 9 mg of bovine trypsin (Worthington) dissolved in 20 ml of 1 mM hydrochloric acid; the substrate stock solution was composed of 3.96 mg of DL-BANA dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.2; and the inhibitor stock solution consisted of 3.96 mg of D-BANA dissolved in 1 ml dimethylsulfoxide diluted to 20 ml with 0.1 M phosphate buffer, pH 7.2. Garnet GBC stock solution, consisting of 50 mg of the stabilized diazonium salt dissolved in 20 ml of distilled water, was prepared just prior to use and filtered. For the determination of the kinetics of DL-BANA in a simultaneous coupling system the following incubating solutions were used:

Stock substrate solution	Concentration of L-BANA	Buffer solution	Stock enzyme solution	Garnet GBC
ml	mM	*ml*	*ml*	*ml*
2.00	0.30	0.50	0.20	0.3
1.33	0.20	1.17	0.20	0.3
1.00	0.15	1.50	0.20	0.3
0.67	0.10	1.83	0.20	0.3

For study of the effects of D-BANA on the hydrolysis, an equal amount of buffer was replaced by the stock inhibitor solution to obtain two series of incubating solutions having constant concentrations of D-BANA of 0.30 and 0.60 mM. Controls used throughout consisted of similar solutions containing boiled trypsin. The incubation period was 5 minutes at 25°C, though preliminary studies revealed linear hydrolysis continuing at least 10 minutes. In postcoupling control experiments an equal quantity of buffer replaced the diazonium salt solution.

RESULTS: As mentioned earlier, the presence of Garnet GBC in the incubation mixture tended to inhibit the enzymic reaction. Table IV reveals the effect of varying low concentrations of diazonium salt on the enzymic reaction and demonstrates that this inhibition was greater with higher diazonium salt concentrations. However, a preincubation of 60 minutes prior to the addition of substrate affected the hydrolysis rate by no more than 15 per cent. Differences in the degree of inhibition were observed with different preparations of trypsin. Up to 75 per cent inhibition was found with 4× pancreatin (Nutritional Biochemical Corporation) (1).

Studies were undertaken to determine whether the inhibitory effect was due to stabilizers and/or surfactants present in the commercial preparations (about 50 per cent by weight) or to the diazonium salt itself. It was observed that *o*-aminoazotoluene had no effect; but, after diazotization according to the method of Knoevenagel (22), the inhibitory effect per unit weight was greater than that of the stabilized, commercial Garnet GBC. Therefore, it can be concluded that the inhibitory effect is due, at least in part, to the diazonium salt itself.

TABLE IV

Inhibition of the Tryptic Hydrolysis of DL-*BANA by Garnet GBC*

Concentration of Garnet GBC	Percentage inhibition 5 min.
(mg/ml)	
0.05	4.7
0.10	13.2
0.20	16.5
0.40	20.1
2.00	35.0

Substrate concentration of DL-BANA 0.60 mM; enzyme concentration, 10 μg/ml

In an attempt to determine the nature of this inhibition, data of corresponding experiments were plotted according to Lineweaver and Burk (Fig. 6). The presence of diazonium salt affected the reaction velocity; V_m in the absence of Garnet GBC was 50, and, in its presence, 30 mμM β-naphthylamine liberated per minute, representing an inhibition of about 40 per cent by the diazonium salt. This kind of plot is a typical representation of so called uncompetitive inhibition following the formulation

$$\frac{1}{v} = \frac{K_m}{V_m}\cdot\frac{1}{S} + \left(1 + \frac{I}{K_{IES}}\right)\frac{1}{V_m}.$$

The inhibition constant, K_{IES}, can be calculated and has been stated to represent the degree of association with the enzyme-substrate complex rather than with the enzyme. This type of plot, however, may only hold true for the concentration of Garnet GBC used in these experiments.

The K_m values for DL-BANA calculated from these plots are 5.8×10^{-4} M in the absence and 3.8×10^{-4} M in the presence of the diazonium salt. The latter value is more directly comparable to the values obtained in human mast cells in the histochemical system.

Studies with D-BANA showed that it was not a substrate for trypsin but did act as a competitive inhibitor to the tryptic hydrolysis of L-BANA

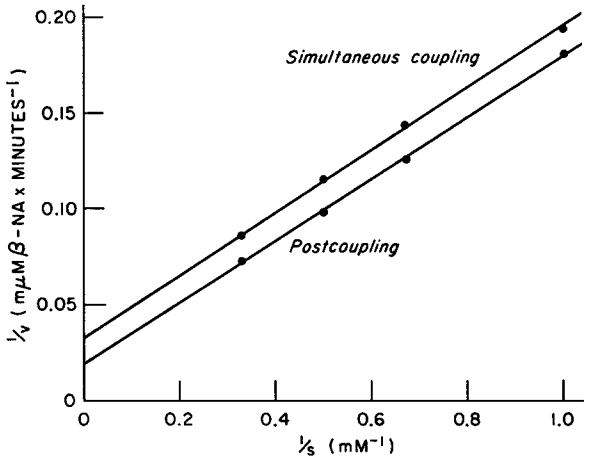

FIGURE 6

Lineweaver-Burk plot of the tryptic hydrolysis of DL-BANA in the presence (simultaneous coupling) and in the absence (postcoupling) of diazonium salt (Garnet GBC).

The K_i determined from the Lineweaver-Burk plot was 1.2×10^{-3} M.

ESTER SUBSTRATE

EXPERIMENTS AND RESULTS

HISTOCHEMICAL STUDIES: It was noted that the ester substrate, EACA-AS, underwent spontaneous hydrolysis in water, insignificant at pH 5–6, but more marked as higher pH values were reached. The time course of this spontaneous hydrolysis at pH 7.2 was exponential in character

and unaffected by the presence of diazonium salt. It was found that during the first 10 minutes the mean substrate concentration was 95 per cent; during 20 minutes, 92 per cent; and during 30 minutes, 90 per cent of the original concentration. Therefore, corrections for this spontaneous hydrolysis were made.

Preliminary studies revealed that the shortest measurable time to visualize mast cells was 2.0 to 2.5 minutes at a substrate concentration about 0.11 mM (0.05 mg/ml) (Fig. 7). Approximately half-maximal velocity was reached with a concentration of 7 μM. On the basis of these observations, the final graded substrate series was adjusted to correspond to the initial (sharply ascending) portion of the curve. The stock solution of EACA-AS consisted of an aqueous solution with a substrate concentration of 22 μM (0.01 mg/ml). The following graded substrate concentrations

Substrate solution	Substrate concentration
ml	μM
2.00	4.4
1.50	3.3
1.00	2.2
0.75	1.6
0.50	1.1

were used with the other components as listed in the study of the amide substrates.

The solutions were mixed just prior to use. In inhibitor studies 1.0 ml of a 20 mM solution of BAME replaced water.

RESULTS: Fig. 8 shows the results of studies using EACA-AS as substrate and BAME as inhibitor, demonstrating that BAME acts as a competitive inhibitor. The calculated K_m and K_i values are presented in Table V and indicate that the K_i value for BAME is of the same order of magnitude as that observed when DL-BANA was used as substrate.

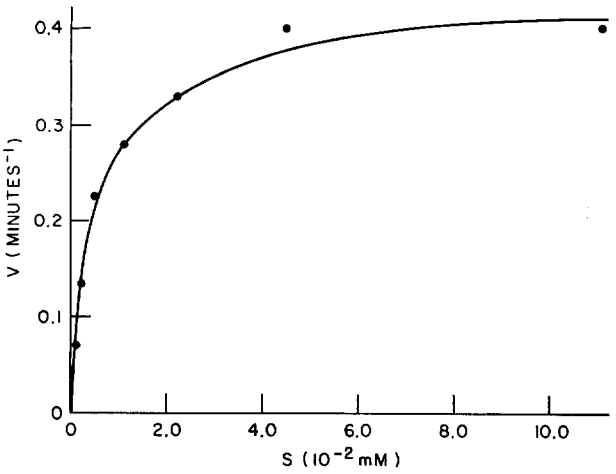

FIGURE 7

Dependence of the rate of the hydrolysis of EACA-AS by the mast cell enzyme(s) on the substrate concentration.

STUDIES WITH CRYSTALLINE TRYPSIN: ESTERASE ACTIVITY

As reported earlier (2), EACA-AS is split to a greater extent by bovine trypsin than by chymotrypsin, the hydrolysis by chymotrypsin being about 10 per cent that of trypsin at pH 7.2. A detailed kinetic study of the tryptic hydrolysis of the ester substrate was undertaken. In these studies the following stock solutions were used: enzyme stock solution consisted of 5 mg trypsin (Worthington) dissolved in 5 ml of 1 mM hydrochloric acid and diluted to 50 ml with 0.1 M phosphate buffer, pH 7.2; the substrate stock solution was composed of 4.6 mg of EACA-AS dissolved in 33 ml of 0.1 mM hydrochloric acid (0.33 mM). Garnet GBC stock solution, consisting of 50 mg of the stabilized diazonium salt dissolved in 20 ml of distilled water, was prepared just prior to use and filtered.

For the determination of the kinetics of EACA-

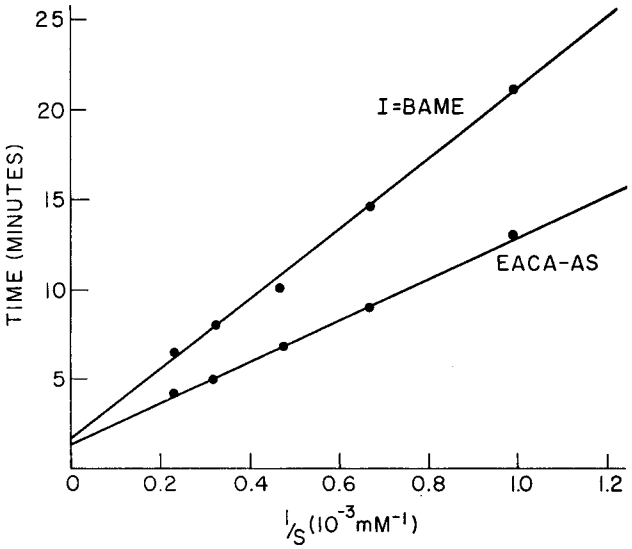

FIGURE 8

Lineweaver-Burk plot of the hydrolysis of EACA-AS by the mast cell enzyme(s). The lower line in the absence and the upper in the presence of BAME, 2.0 mM. Each point represents the mean of three separate experiments.

TABLE V

Values of Kinetic Constants of the Hydrolysis of DL-*BANA and EACA-AS by the Mast Cell Enzyme(s) and Trypsin with BAME as Competitive Inhibitor*

Constant	Amidase (DL-BANA)	Esterase (EACA-AS)
	Human mast cells	
K_m	0.17×10^{-3} M	0.73×10^{-6} M
K_i (BAME)	1.4×10^{-3} M	1.2×10^{-3} M
	Bovine trypsin	
K_m	0.38×10^{-3} M	1.4×10^{-5} M

AS hydrolysis in a simultaneous coupling system the following incubating solutions were used:

Substrate sol.	Substrate concentration	Buffer sol.	Enzyme	Garnet GBC	Water
ml	mM	*ml*	*ml*	*ml*	*ml*
1.00	0.110	1.00	0.5	0.5	—
0.80	0.088	1.00	0.5	0.5	0.20
0.60	0.066	1.00	0.5	0.5	0.40
0.40	0.044	1.00	0.5	0.5	0.60
0.30	0.033	1.00	0.5	0.5	0.70
0.20	0.022	1.00	0.5	0.5	0.80

The incubation period was 1 minute at 27°C. Controls used throughout consisted of similar solutions containing boiled trypsin. In post-coupling experiments the diazonium salt solution was replaced with an equal volume of buffer.

RESULTS: As shown in Fig. 9, tryptic hydrolysis of EACA-AS at a concentration of 0.11 mM is directly proportional to the trypsin concentration over at least a fivefold range up to 20 μg/ml. The rate of reaction, as shown in Fig. 10, using the same range of substrate and trypsin concentrations is linear over a 4-minute period. The rate of enzymic hydrolysis of EACA-AS by trypsin is proportional to the substrate concentration over a relatively narrow range (Fig. 11), with approximately 50 per cent of the reaction velocity calculated from the Lineweaver-Burk plot being reached with a substrate concentration of 20 μM. The K_m for EACA-AS with trypsin is presented in Table V. Again, the same observation made using DL-BANA as substrate was noted with EACA-AS, *i.e.*, the diazonium salt Garnet GBC noticeably inhibited the hydrolytic reaction, 20 to 40 per cent depending on the experimental conditions. EACA-AS-D when used as substrate gave essentially the same results as EACA-AS in both biochemical and histochemical studies.

DISCUSSION

The method used by us for determining enzyme kinetic constants in a histochemical system is derived from that suggested by Benditt and Arase (6), but extends and modifies their method, at-

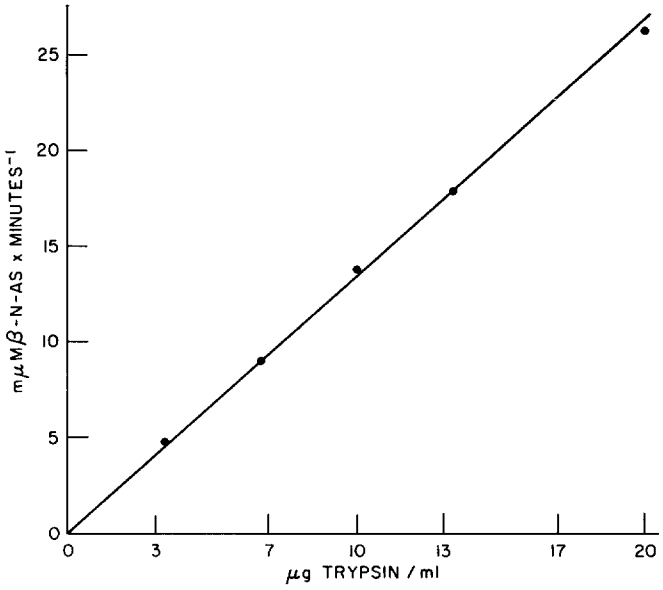

FIGURE 9

Rate of the hydrolysis of EACA-AS by trypsin as a function of enzyme concentration.

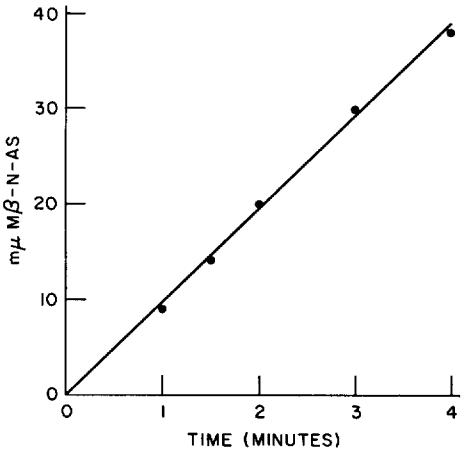

FIGURE 10

Time dependence of the hydrolysis of EACA-AS by trypsin.

tempting to account for some of the factors affecting enzyme kinetics in a biochemical system, the restrictions of visual photometry and some of the effects of diazonium salts on substrates and inhibitors. These factors were considered when the end point of the reaction was taken as the first visible color produced in the histochemical system; reaction time was recorded as an unrestricted variable and only substrates and inhibitors that do not form irreversible azo compounds in the presence of diazonium salts were used. The result of our method, when compared to the method mentioned above (6), has been to increase the number of determinations possible with a similar number of tissue sections, to decrease the incubation time to the practical minimum in order to approximate as nearly as possible the real initial reaction velocity, and to decrease the interference with accurate visual photometry resulting from background coloration caused by the diazonium salt. Inherent in the method is the requirement for low substrate concentrations.

Any quantitative method in which subjective steps are incorporated tends to lack the same accuracy and reproducibility which characterize purely instrumental techniques. However, when the necessary precautions are taken, the limits of variation are in close accord with the known sensitivity of the eye as a visual photometer (13). In practice, the only clearly subjective step in the histochemical procedure is the determination of the reaction end point, *i.e.*, determination of the moment of observation of mast cell staining. Some investigators will undoubtedly observe staining sooner than others and the same investigator will observe staining earlier when he becomes more accustomed to the shape and localization of the cells being stained. By taking as an end point the time necessary to visualize 4 cells (about ½ total mast cells in the microscopic field), we observed that the coefficient of variation in this step was always less than 10 per cent. To obtain reliable values, therefore, the procedure should be re-

peated several times at different substrate concentrations. In order to perform studies of this type the enzymic activity in a site must be great enough to produce a visible reaction in a relatively short period before background coloration, enzyme inhibition, and other deleterious effects adversely influence the visual observations. It is also imperative that the diazonium salt concentration be optimal (see Preliminary Experiments) and that the enzymic activity be great enough to be demonstrable under less optimal conditions, *i.e.*, when using low substrate concentrations and inhibitors.

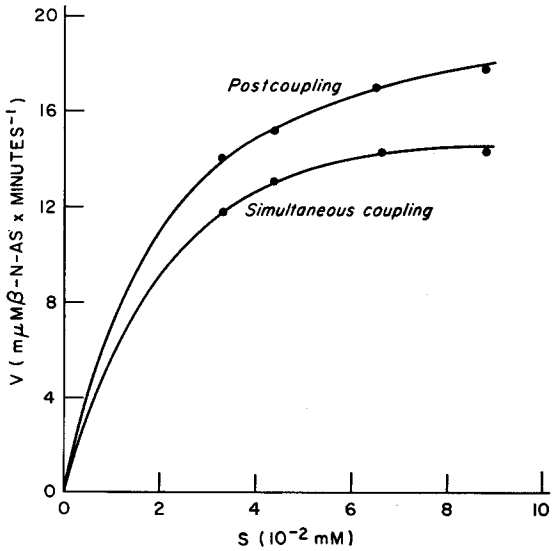

FIGURE 11

Dependence of the rate of the hydrolysis of EACA-AS by trypsin on the substrate concentration in the presence (simultaneous coupling) and the absence (postcoupling) of diazonium salt (Garnet GBC).

From studies made to determine whether the velocity estimates in the present method are real velocities of the enzymic reactions or are affected by the velocity limitations of other reactions involved (especially by the speed of diazonium coupling to the product of the enzymic reaction to form a colored precipitate), it was difficult to reach a definite conclusion. This difficulty was predicated upon our inability to estimate the actual conditions in the cells where the reaction is occurring. We do not know the actual concentration of diazonium salt, and, at the time when both the biochemical and chemical reactions are in a steady state, we do not know the real intracellular concentrations of substrates or inhibitors. Although in an 8 μ section the cytoplasm of the majority of mast cells is exposed to the incubating solution, diffusion of reactant into the section and into enzyme-containing organelles (*e.g.*, lysosomes or mitochondria) within the cell may represent rate-limiting factors (23). The high local concentration of bound enzyme surrounded by cell cytoplasm may also affect the reaction by causing spatial limitations and impairment of substrate and/or inhibitor penetration to the active enzyme center. In the present work, however, all calculations were based on the assumption that the concentration of substrates and inhibitors in or on the exposed surface of the mast cells is the same as that in the incubating solution.

The diazonium coupling reaction does not appear to be the rate determinant in the esterase studies, since initial first order reactions could be obtained using the esterase substrate, EACA-AS. This would certainly not be the case if the coupling reaction were the rate determinant in the velocity estimations. If it were the rate determinant, zero order reactions would be observed, since constant concentrations of Garnet GBC were used. The question arises as to what effect the coupling reaction has on the observed reaction velocities (see Preliminary Experiments for data referable to this question). If Fig. 3 is inverted, *i.e.*, the time (t) becomes $1/t = k \cdot v$, the observed reaction velocity is found to be dependent on the concentration of Garnet GBC at a constant substrate concentration. At low concentrations, the time required to reach the end point is long and the diffuse histochemical localization of the reaction product is

evidence that the coupling has not been effective. The observed velocities, therefore, do not reflect the speed of the enzymic reaction. An increase in the diazonium concentration above certain limits, though giving good histochemical localization, does not increase the observed reaction velocity beyond a certain value, however. On the contrary, it tends to decrease it. At concentrations between these limits, the reaction time is the shortest obtainable; and that portion of the total reaction time caused by non-instantaneous diazonium coupling is the smallest possible. However, a certain time is required to attain a steady state concentration of β-naphthylamine, and this time would result in a constant increment in the measured times (t). This increment in time causes an error which becomes more significant at shorter reaction times. This error, therefore, tends to reduce the slopes of the lines and gives an erroneous V_m in the Lineweaver and Burk plot. The calculated K_m values must be affected by these factors. The effect caused by the speed of coupling on the K_i values is perhaps less significant, since K_i is a function of the difference in the slopes found in the presence and absence of a single inhibitor concentration, *i.e.*

$$K_i = \frac{(sl_1)}{(sl_2) - (sl_1)} \cdot I.$$

The same methodological error is included in both slopes and does not influence the difference of the slope values.

From the evidence presently available, we feel that the kinetic data derived in the histochemical system represent *relative* values and are referable only to the incubating conditions, substrate, inhibitor, and diazotate concentrations, and visual photometric techniques employed in this system. These data may not be comparable to those obtained using pure enzymes in test tube experiments because of the differences in the methods used for determining them and the inherent difference in the state of the two preparations. Our comparative studies with crystalline trypsin lead to few definite conclusions in respect to the mast cell enzyme; moreover, in spite of many similarities, we have no reason to suspect that the enzyme in mast cells is identical to trypsin. Of interest, however, is the fact that the order of magnitude of the K_m values for the three different substrates used is about the same for both the mast cell enzyme and trypsin. On the assumption that some comparison can be made between the histochemically and biochemically determined kinetic constants, these observations tend to show similarities in the characteristic kinetic constants between the human mast cell enzyme and trypsin. Certainly, if any comparisons between histochemical and biochemical kinetic values are to be made, the biochemical enzyme system should be comparable to the histochemical system; *i.e.*, the incubating system in biochemical studies should contain diazonium salt, since this has been shown to affect the values of the kinetic constants. The actual relationship of the histochemical and biochemical kinetic data must be determined by extensive comparative studies upon those enzymes demonstrated in a histochemical system which can also be studied biochemically. Such studies with several enzyme systems are presently in progress.

It is possible with the present histochemical kinetic method to determine the relative K_m values of one or more enzyme substrates and, thereby, determine the possible preference of the enzyme(s) for these compounds in a given tissue site. By the use of various inhibitors it is also possible to determine the nature of their inhibition, *i.e.*, whether competitive, uncompetitive, or non-competitive. From this, one can determine whether the compound chosen might itself possibly serve as a substrate for the enzyme studied and, thereby, obtain some knowledge as to the possible "physiological" substrates for the enzyme. This technique was used to characterize the enzyme activities in the present work, and it was concluded from these studies that trypsin substrates compete with the histochemical substrates at the active center of the human mast cell enzyme. In our study a further interesting observation was made. It was found that D-BANA was not hydrolyzed by either the mast cell enzyme or trypsin, yet acted as a competitive inhibitor to both these enzymes. This extends the finding of Erlanger *et al.* (17) that the D-enantiomorph of benzoyl arginine *p*-nitroanilide is a competitive trypsin inhibitor and emphasizes certain considerations that must be taken into account when evaluating the constants for racemic substrates.

As noted earlier, more than one enzyme in the same tissue site may act on the same substrate and, thereby, cause difficulties in the interpretation of histochemical findings. The same problem

exists in evaluating kinetic studies. Theoretically, if more than one enzyme is involved in the hydrolysis of a single substrate, then curved lines should be observed on the Lineweaver-Burk plots. For example, in the case of hydrolysis of a single substrate by two enzymes the initial slope should be

$$\frac{K_{m1} V_{m1} + K_{m2} V_{m2}}{(V_{m1} + V_{m2})^2}$$

and the final slope

$$\frac{K_{m1} K_{m2}}{V_{m1} K_{m2} + K_{m1} V_{m2}}.$$

From these formulae it is apparent, however, that if either K_{m1} is similar to K_{m2} or if either V_{m1} or V_{m2} is small compared to the other, the slope of the resulting function is so close to a straight line that differentiation is not possible, especially when experimental errors may obscure the results. Therefore, with these possible exceptions acknowledged, a straight line obtained using the present method would signify that a single substrate is acted upon by a single enzyme or a group of almost identical enzymes.

When two or more histochemical substrates are hydrolyzed in the same tissue site, it should be theoretically possible, by comparing V_m and slope values, to determine whether the substrates are acted upon by one and the same enzyme or by different enzymes. In this manner, it was concluded that DL-BANA and DL-BLyNA were split by the same enzyme or enzymes. The same problem was also studied in another manner. Since the K_i values for several competitive inhibitors are actually the K_m values for the compounds if they act as substrates for the enzyme, a comparison of the K_i values obtained using a competitive inhibitor with several histochemical substrates should indicate whether the competitive inhibitors are inhibiting one and the same enzyme or enzymes. We found that the K_i values were similar when BAME was used as a competitive inhibitor of the hydrolysis of DL-BANA, DL-BLyNA, and EACA-AS, and the same situation existed when L-BAA was used as a competitive inhibitor of the hydrolysis of DL-BANA and DL-BLyNA. From these findings, it could be concluded that both L-BAA and L-BAME are possible substrates for a single enzyme, or two or more enzymes characterized by the same Michaelis constant. Stated in another way, it could be concluded that all the enzymes were inhibited competitively by the same inhibitors with the same inhibitor characteristics. It would appear unlikely that human mast cells contain more than one enzyme hydrolyzing the three different histochemical substrates with similar inhibitor constants for a single inhibitor compound. However, this assumption can only be verified following biochemical enzyme isolation. These findings support the original hypothesis (1, 2) that the histochemical substrates studied are acted upon predominantly by one and the same enzyme or group of almost identical enzymes having both amidase and esterase activity.

A question arises as to whether a comparison can be made of kinetic data obtained from a single site in different species or different sites in the same animal. Mast cells vary in size from species to species and also from site to site in the same animal. It would be easier to observe mast cell staining first in a species or site in which the cells were of a greater diameter, since in those cells the reacting enzyme layer would be consistently thicker and wider, and thus attract the eye of the observer. The error caused would not change the K_m values but rather would affect the velocity estimates (V_m) and, therefore, enzyme activity comparisons. Also, it is evident that, if, in a tissue site or in a species, an enzyme inhibitor which cannot be freely dissolved in the incubation medium is present (24), the kinetic constants do not represent the relative amounts of enzyme, but only that portion of the enzyme activity remaining. In our study, it was observed that DL-BANA and DL-BLyNA were split in human mast cells at the same rate, but DL-BANA was hydrolyzed to a far greater extent than DL-BLyNA in canine mast cells and by trypsin. From this, it can be concluded either that human and dog mast cells as well as trypsin have some differences in their activity toward these substrates, or that the differences are dependent upon other (perhaps cytoplasmic) effects and unrelated to the enzyme itself. When any comparative study is being made, the above considerations should be taken into account. For example, we have observed a decrease in reaction velocity for the hydrolysis of DL-BANA and EACA-AS in the numerous cutaneous mast cells in a case of urticaria pigmentosa. Yet, in view of the previously mentioned possible causes of changes in kinetic data, we were able to conclude

only that the activity of the enzyme was decreased, but not that there was an absolute decrease in the amount of enzyme present. The feasibility of using the present kinetic techniques in determining the presence of changes in the activity of an enzyme in pathologic states is apparent. The present method has been recently modified by us and experimentally applied in another laboratory for this purpose, with the results being verified biochemically (25).

It would appear from our studies that, when the necessary precautions are taken, the quite reproducible, relative enzyme kinetic data obtained from the histochemical system are of value in characterization of enzyme activity. It would be desirable to adapt this histochemical technique for studying enzyme kinetics to an instrumental method, *e.g.*, microspectrophotometry or microdensitometry, in order to obtain totally objective data. A report on the application of these methods to histochemical enzyme kinetic studies and the effect of other factors on enzyme characteristics obtained in a histochemical system will be presented in detail in future communications.

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REFERENCES

1. GLENNER, G. G., and COHEN, L. A., *Nature*, 1960, **185,** 846.
2. GLENNER, G. G., HOPSU, V. K., and COHEN, L. A., *J. Histochem. and Cytochem.*, 1962, **10,** 109.
3. HOPSU, V. K., and GLENNER, G. G., *J. Histochem. and Cytochem.*, 1963, **11,** in press.
4. BARTER, R., DANIELLI, J. F., and DAVIES, H. G., *Proc. Roy. Soc. London, Series B*, 1955, **144,** 412.
5. HOLT, S. J., and O'SULLIVAN, D. G., *Proc. Roy. Soc. London, Series B*, 1958, **148,** 465.
6. BENDITT, E. P., and ARASE, M., *J. Histochem. and Cytochem.*, 1958, **8,** 431.
7. LINEWEAVER, H., and BURK, D., *J. Am. Chem. Soc.*, 1934, **56,** 658.
8. MICHAELIS, L., and MENTEN, M. L., *Biochem. Z.*, 1913, **49,** 333.
9. DIXON, M., *Biochem. J.*, 1953, **55,** 170.
10. EADIE, G. S., *J. Biol. Chem.*, 1942, **146,** 85.
11. BODANSKY, O., *J. Biol. Chem.*, 1937, **120,** 555.
12. GLENNER, G. G., FOLK, J. E., and MCMILLAN, P. J., *J. Histochem. and Cytochem.*, 1962, **10,** 481.
13. LE GRAND, Y., Light, Colour and Vision, New York, J. Wiley and Sons, Inc., 1957.
14. BROWN, W. R. J., *J. Opt. Soc. America*, 1952, **42,** 837.
15. RIEDEL, A., and WÜNSCH, E., *Z. physiol. Chem.*, 1959, **316,** 61.
16. FOLK, J. E., *Arch. Biochem. and Biophys.*, 1956, **64,** 6.
17. ERLANGER, B. F., KOKOWSKY, N., and COHEN, W., *Arch. Biochem. and Biophys.*, 1961, **95,** 271.
18. KLIONSKY, B., *Am. J. Clin. Path.*, 1961, **36,** 235.
19. FOLK, J. E., and BURSTONE, M. S., *Proc. Soc. Exp. Biol. and Med.*, 1955, **89,** 473.
20. LAGUNOFF, D., and BENDITT, E. P., *Nature*, 1961, **192,** 1198.
21. BLACKWOOD, C., and MANDL, I., *Anal. Biochem.*, 1961, **2,** 370.
22. KNOEVENAGEL, E., *Ber. deutsch. chem. Gesell.*, 1895, **28,** 2048.
23. SINGER, T. P., and LUSTY, C. J., *Biochem. and Biophys. Res. Comm.*, 1960, **2,** 276.
24. BLACKWOOD, C., and MANDL, I., *Fed. Proc.*, 1961, **20,** 216d, Abstract.
25. ENGEL, W. K., EYERMAN, E. L., AND WILLIAMS, H. E., *New Eng. J. Med.*, 1963, **268,** 135.