

AN ENZYME IN MAST CELLS WITH PROPERTIES LIKE CHYMOTRYPSIN*

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Because of their heparin, histamine, and, in some species, 5-hydroxytryptamine content, mast cells have excited the curiosity of many people. Gomori (1) observed in mast cells of sectioned tissues the presence of an enzyme capable of hydrolyzing 3-chloroacetoxy-2-naphthoic acid anilide. This ester bears no obvious relationship to substances known to be present in tissues. To understand the mast cell better a more precise definition of the enzyme is necessary. The first clue to its substrate specificity came from an observation (2) that chymotrypsin was weakly capable of hydrolyzing the related histochemical substrate 3-acetoxy-2-naphthoic acid anilide and that mast cells showed weak histochemical activity with this substrate. The evidence which follows shows the striking similarity between the histochemically demonstrable enzyme activity in rat mast cells and bovine alpha chymotrypsin.

Methods and Materials

Isolation of Mast Cells.—Isolation of mast cells was performed using a modification of the method of Padawar and Gordon (3). Rats weighing 180 to 300 gm. of the Sprague-Dawley strain were the cell source. Each animal was anesthetized with ether, the chest cavity opened above the diaphragm, and the animal exsanguinated from the heart or aorta. The abdominal skin was then incised in the midline from symphysis to xiphoid and reflected laterally. In the muscle of the midabdomen a 2 cm. midline incision was made. Small hemostats were attached to the edges of this, and these were suspended by their handles from burette clamps on a ring stand. This procedure minimized bloody contamination of the peritoneal cavity.

The peritoneal cavity was washed by introducing 8 to 10 ml. of the lavage fluid, gently agitating the fluid by tapping the sides of the abdomen for 1 minute, then withdrawing as much fluid as possible with a polyethylene pipette.

In the majority of the early experiments described here cells were harvested in 0.9 per cent NaCl containing 1 unit of heparin (Abbott Laboratories) per ml. Later experience showed that 5 to 10 units of heparin per ml. was superior in preventing coagulation of the fluid. Ethyl-

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enediaminetetraacetic acid (versene), 0.15 per cent, was also tried. Using this agent we encountered much more hemolysis.

Cell separation was achieved as follows: The lavage fluid was transferred to a 25 mm. diameter plastic centrifuge cup. Yield averaged 7.0 ml. fluid containing 1.5×10^8 mast cells comprising 5 per cent of the total cell population. A high density sucrose solution was prepared according to the directions of Padawar and Gordon (3), omitting the gelatin. Twelve ml. of this was layered under the peritoneal fluid by carefully introducing it from a 14 gauge 4 inch needle with a flat tip and syringe so that the sharpest possible interface was formed. After layering the tube was centrifuged in an SB 2 (International) centrifuge at 125 G (maximum, calculated to the fluid interfaces) for 10 minutes from contact to cut-off.

After removal from the centrifuge the tube was carefully transferred to a burette clamp,

TABLE I

Effect of Varying Specific Gravity of Sucrose Solution on Purity and Yield of Mast Cells After first centrifugation (127 G for 10 minutes).*

Proportions stock sucrose: H ₂ O	Specific gravity 32°C./25°C.	Total cells/c.mm.	Mast cells/c.mm.	Mast cell <i>per cent</i>
1:0.2	1.265	68	40	59
1:0.25	1.255	69	64	90
1:0.3	1.245	92	69	75
1:0.4	1.230	134	55	41
1:0.5	1.212	454	50	11
1:0.6	1.119	286	36	13

*G, relative centrifugal force calculated from centrifuge speed and radius of revolution measured to interface of fluid in cup.

and, with a fresh 14 gauge needle introduced to the tube bottom, as much of the sucrose layer was gently aspirated as possible without disturbing the interface. The needle was removed, and the sucrose then was transferred to another tube.

In this operation the specific gravity of the sucrose solution was found to be of great importance. Table I illustrates the effect of varying the sucrose density by varying the amount of water added to the sucrose stock solution. The greatest purity and the best yield of mast cells were obtained between specific gravity (32°C./25°C.) 1.255 and 1.245. The proportions of water to sucrose stock shown did not always provide these specific gravities because of varying amounts of water in the sucrose. Hence each batch of stock sucrose was tested.

Further purification of mast cells was obtained by centrifuging a second time for 20 minutes at 1000 G. All but 1 to 2 ml. of the sucrose was then removed by aspirating from the fluid surface. Care was taken not to aspirate the cells from the bottom. The residual sucrose regularly contained mast cells of a purity of 95 ± 5 per cent. The yields of purified mast cells ranged from 20 to 50 per cent and averaged 35 per cent. The bulk of the loss occurred in the first separation. The cells were packed by centrifuging the cell concentrate in the tube for 1 hour at 1000 G. The residual sucrose was removed and the cells were stored in the frozen state for several days to several months prior to use in the enzyme studies. The number and purity of cells in each batch were determined by counting aliquots removed from the well mixed final concentrate prior to final packing. Counting was performed using 1:11 dilutions in white blood cell counting pipettes with a fluid containing 0.05 per cent toluidine blue O, 20 per cent ethanol V/V, and 4 per cent formaldehyde. Three pipettes were filled, mixed,

and the mixture from each pipette introduced into one side of a Neubauer counting chamber. The cells in nine large squares equivalent to 0.9 mm.³ were counted. The average of the three values so obtained was used.

Substrates.—3-Chloroacetoxy-2-naphthoic acid anilide was prepared by Gomori (1). Acetyl-L-tyrosine ethyl ester, acetyl-L-tryptophan ethyl ester, and acetyl-L-phenylalanine ethyl ester were obtained from both Mann Research Laboratories, Inc., New York, and H. M. Chemical Co., Ltd., Santa Monica, California. *P*-toluenesulfonyl-L-arginine methyl ester was obtained from the last named source.

Enzymes.—Alpha chymotrypsin and trypsin (bovine) were crystallized salt-free preparations obtained from Armour and Company, Chicago.

Fast Violet BN.—The stabilized diazonium salt of 6-benzoylamino-4-methoxy-*m*-toluidene was produced from General Dyestuffs Corporation, Chicago.

TABLE II

The pH of Bicarbonate Solutions in 30% (V/V) Propylene Glycol and 3.3% (V/V) acetone in equilibrium with 5 per cent CO₂ gas phase at 37°C.

HCO ₃ concentration	pH
<i>m</i> /liter	
0.001	6.45
0.0033	6.96
0.010	7.51
0.025	7.60
0.033	7.80
0.050	7.90
0.10	8.32
0.33	8.78

Measurement of Enzyme Activity.—Hydrolysis of 3-chloroacetoxy-2-naphthoic acid anilide was followed colorimetrically in a modification of the original histochemical substrate system. The composition of the initial substrate mixture was 0.2 ml. of an 18.2 μM CANAS¹ solution in acetone, 3.0 ml. propylene glycol, N.F., 2.0 ml. 0.2 M sodium phosphate buffer pH 6.4, 4.8 ml. distilled H₂O and 10 mg. of fast violet BN salt. The 3-chloroacetoxy-2-naphthoic acid anilide in acetone was mixed with the propylene glycol, the buffer added, then water, and finally the diazonium salt. This was mixed and then quickly filtered. To 2.0 ml. of this mixture 0.2 ml. of enzyme solution in 0.2 M phosphate buffer pH 6.4 was added and incubated at 25°C. The color produced was measured at appropriate time intervals at 540 mμ in 12 x 75 mm. cuvettes in a Coleman, Jr., spectrophotometer.

3-chloroacetoxy-2-naphthoic acid anilide hydrolyzes spontaneously at a rapid rate (4). Hence substrate blanks were run in each assay substituting 0.2 M phosphate buffer for the enzyme or cell preparation.

Hydrolysis of amino acid esters was followed by observing liberation of CO₂ from a CO₂-bicarbonate buffer system in Warburg manometers. The aromatic amino acid esters were made up as 0.5 M stock solutions in absolute acetone. The test system was composed of 0.10 ml. of substrate stock solution, 0.90 ml. of propylene glycol, sufficient bicarbonate solution (0.5 or 0.1 M) to neutralize the HCl of the ester preparations if present and to give a final

¹ CANAS, 3-chloroacetoxy-2-naphthoic acid anilide.

residual concentration which in combination with 5.0 per cent CO_2 in the gas phase would at 37°C . give the desired pH. Water made up the fluid volume to 3.00 ml. Substrate mixtures were placed in the vessel and enzyme solutions in the side arm of 15 ml. Warburg flasks.

The presence of organic solvent made it necessary to ascertain the concentration of bicarbonate required to give a specified pH. This was done as follows: mixtures of acetone, propylene glycol, water, and NaHCO_3 were made. Equilibration at 37°C . with 95 per cent N_2 -5 per cent CO_2 was achieved by bubbling the gas through the solutions. The pH was measured by a glass electrode inserted in the solution. In Table II the pH's of a series of bicarbonate concentrations in this mixture at equilibrium with the gas phase are recorded.

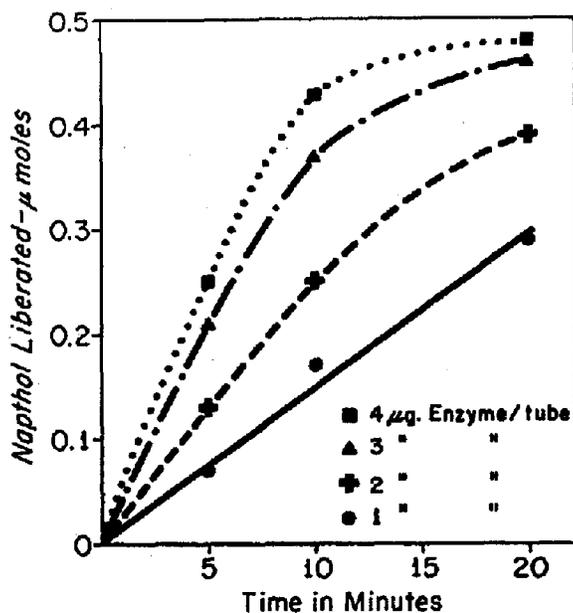


FIG. 1. The rate of hydrolysis of 3-chloroacetyl-2-naphthoic acid anilide by chymotrypsin.

EXPERIMENTAL OBSERVATIONS

The Hydrolysis of 3-Chloroacetoxy-2-naphthoic Acid Anilide by Chymotrypsin, Trypsin, and Mast Cells.—The capacity of chymotrypsin to hydrolyze 3-chloroacetoxy-2-naphthoic acid anilide was demonstrated by preliminary trials of the enzyme in the modified histochemical substrate system. Following this a series of experiments designed to ascertain appropriate conditions of time and enzyme concentration were performed. The results of a representative experiment are shown in Fig. 1. The curves sufficiently approximate linearity for the first 5 minutes to be used for assay purposes.

The activity of isolated mast cells was assayed in the same system. A preparation of mast cells having a purity of 95 per cent was utilized. Preliminary

trials allowed choice of the appropriate quantities of cells. In Table III are shown the data obtained from one of several such experiments. Here it is evident that there is hydrolysis and that the rate is proportional to the number of cells. Similar trials with trypsin demonstrated it to have little activity (3 per cent) compared with chymotrypsin.

The Inhibition of Chymotrypsin and Mast Cell Activity by Diisopropylfluorophosphate (DFP)².—

Chymotrypsin in a concentration of 20 micrograms per ml. in 0.1 M phosphate buffer, pH 6.37, was incubated with several concentrations of DFP for 35 minutes at 25°C. The same

TABLE III

The Rate of Hydrolysis of 3-Chloroacetoxy-2-naphthoic Acid Anilide by Purified Rat Mast Cells

No. of mast cells per tube	Micromoles of naphthol liberated in 5 min.
1×10^5	0.06
2×10^5	0.12
4×10^5	0.22

TABLE IV

The Parallel Inhibition of Chymotrypsin and Mast Cell Enzyme by Diisopropylfluorophosphate

DFP concentration in preliminary incubation with enzyme	Naphthol liberated in 5 min.	
	By chymotrypsin	By mast cell enzyme
	μM	μM
10^{-4} M	0.01	0.02
10^{-5} M	0.08	0.07
None	0.20	0.23

3-chloroacetyl-2-naphthoic acid anilide substrate.

was done with a suspension of mast cells in the same buffer. The enzymatic activity of the mast cell preparations was adjusted to be equivalent to that of the chymotrypsin. A 0.2 ml. aliquot of the enzyme mixture incubated with DFP was then added to 2.0 ml. of substrate mixture and assayed.

Table IV shows the results of such an experiment. The parallel inhibition of the two enzyme systems by DFP is clearly evident.

The Hydrolysis of N-Acetyl-L-tryptophan Ethyl Ester by Mast Cells: Its pH Optimum Compared with That of Chymotrypsin.—

² DFP, diisopropylfluorophosphate.

N-acetyl-L-tryptophan ethyl ester was incorporated into the 30 per cent propylene glycol substrate medium at a concentration of 0.0167 M and at pH's ranging from 6.5 to 8.8. In one experiment 0.32×10^6 and in another experiment 5×10^6 mast cells per flask was used. Similarly 5 or 15 micrograms of chymotrypsin was employed. Since the maximum activity of the enzyme was exhibited from the 6th to 12th minute of the runs, this interval was used for assay of enzymatic activity. Fig. 2 shows the mean of the data derived from the two experiments. The pH optimum of the mast cell preparation was 8.4 and that of the chymotrypsin under these conditions was 7.9.

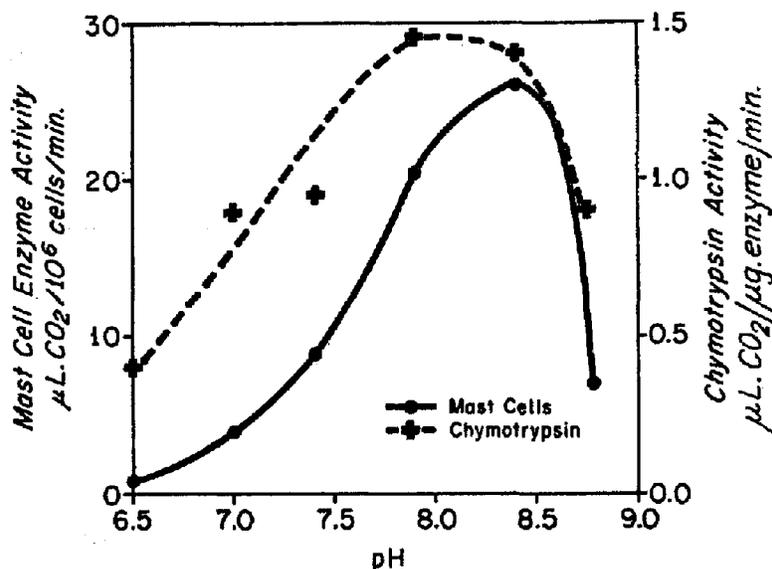


FIG. 2. Comparison of pH vs. activity of mast cells and chymotrypsin on *N*-acetyl-tryptophan ethyl ester.

Analysis of the data showed the difference to be within the limits of the experimental error of the observations.

Comparison of the Action of Mast Cells and Chymotrypsin on Several Ester Substrates.—

The two enzyme preparations were assayed in the same test system used with acetyl-L-tryptophan ethyl ester. Sodium bicarbonate was present in a final concentration of 0.033 M to give a pH, with 95 per cent N₂ and 5 per cent CO₂, of 7.8. The experiments were repeated with concentrations of 5 and 10 micrograms of chymotrypsin and 2.5 and 5.0 $\times 10^6$ mast cells. Since the reaction rates tended to decrease after 5 to 10 minutes, particularly with the phenylalanine substrate and more rapidly with chymotrypsin than with the mast cells, activities were computed for the first 5 minutes.

Table V shows the activities of the two enzyme preparations on the three substrates. The ratio of tyrosine to tryptophan activities was similar for the

two enzymes, 2.2 for chymotrypsin and 2.4 for mast cells. The ratio of tyrosine to phenylalanine activity was 14 for chymotrypsin and 2 for mast cells.

The Lack of Activity of the Mast Cell Enzyme on p-Toluenesulfonyl Arginine Methyl Ester.—

p-Toluenesulfonyl arginine methyl ester was incorporated at a concentration of 0.0167 M into the medium with a bicarbonate concentration of 0.072 M to give a pH of 8.1 (5). Trypsin, 5 micrograms, chymotrypsin, 20 micrograms, and 1.5×10^6 mast cells were placed in separate flasks containing the substrate. Gas evolution with trypsin was active and very nearly constant for the first 6 to 12 minutes. The rate with trypsin was 2.22 μ l. CO₂/ μ g of enzyme/minute. Chymotrypsin produced only 0.27 μ l. CO₂/ μ g. minute and mast cells produced no detectable hydrolysis.

TABLE V
The Activities of Mast Cells and Chymotrypsin on Several Aromatic Amino Acid Ester Substrates

Substrate	Chymotrypsin	Mast cells
	μ l. CO ₂ / μ g. enzyme/min.	μ l. CO ₂ 10 ⁶ cells/min.
Acetyl-L-tryptophane ethyl ester.....	0.7	26
Acetyl-L-tyrosine ethyl ester.....	1.5	62
Acetyl-L-phenylalanine ethyl ester.....	0.11	30

System: Substrate 0.0167 M in solvent containing 30 per cent propylene glycol, 0.033 M NaHCO₃, gassed with 95 per cent N₂-5 per cent CO₂ and temperature 37°C.

DISCUSSION

Using the synthetic substrate 3-chloroacetoxy-2-naphthoic acid anilide Gomori (1) demonstrated a hydrolytic enzyme in mast cells. In the present study we found that this enzymatic activity showed a striking resemblance to that of alpha chymotrypsin. The resemblances are as follows: Chymotrypsin is able to split 3-chloroacetoxy-2-naphthoic acid anilide, the histochemical substrate. Both chymotrypsin and the mast cell enzyme activity are inhibited to the same extent by several different concentrations of diisopropylfluorophosphate. Both the mast cell enzyme and chymotrypsin hydrolyzed the *N*-acetylated ethyl esters of tryptophan and tyrosine, which are typical chymotrypsin substrates (6), at the same relative rates. However, the mast cells in our system split phenylalanine ethyl ester much faster than chymotrypsin; this discrepancy cannot be explained at present. *p*-toluenesulfonyl-arginine methyl ester, a substrate split by trypsin and minimally acted upon by chymotrypsin, was not acted upon by the mast cell enzyme. The pH optimum of activity of the mast cell enzyme was similar to that of the activity of chymotrypsin on acetyl-L-tryptophan ethyl ester.

In addition to these similarities, it is known that formaldehyde does not destroy the activity of either chymotrypsin (6) or of the histochemically demonstrated activity of mast cells in tissue sections. *N*-acetyl-L-tryptophan ethyl ester can act as competitive inhibitor of the 3-chloroacetoxy-2-naphthoic acid anilide in the histochemical system (4), and the enzymatic activity is noncompetitively inhibited by DFP (7). This is further evidence that the enzyme in the mast cells observed histochemically is the same as that of the isolated cells acting upon the aromatic amino acid esters.

From the data presented above one can make an estimate of the concentration of enzyme activity in the mast cells of the rat. Thus in the experiments utilizing the 3-chloroacetyl-2-naphthoic acid anilide as substrate, 1 μ g. of salt-free chymotrypsin provided an activity equivalent to 10^6 mast cells; 10^6 mast cells have a volume of 1.3 c.mm. (8). From these data one calculates a concentration of 77 μ g. per c.mm. or 0.7 per cent *W/V* for the enzyme activity in chymotrypsin equivalent. On the other hand the data shown in Table V, summarizing experiments with tryptophan and tyrosine esters, indicate equivalent activities of 2.9 and 3.2 per cent *W/V*. The reason for the difference in the two estimates is not apparent at this time, but in either case it is clear that the enzyme represents a considerable amount of the cell substance.

Evidence suggesting location of the enzyme in the mast cell granule is as follows: Chymotrypsin is known to be present in pancreas in the inactive form of chymotrypsinogen which must be activated before it can catalyze hydrolysis. In the instance of the mast cell enzyme this does not seem to be the case. Enzymatic activity can be observed in fixed or fresh tissue sections without preliminary activation and in isolated mast cells without further activation. Either the enzyme has no precursor or else it is closely associated with a second enzyme capable of activating it. If the enzyme does not need activation then presumably it is inhibited *in situ*. Our best histochemical preparations suggest that the enzyme is associated with the granules of the mast cell. This information is consistent with the hypothesis that the enzyme is associated with the heparin present in the granules. Supporting this conception is the fact that heparin and chymotrypsin precipitate out of aqueous solution when present in concentrations of magnitude estimated to be present in mast cells (8, 9).

The functional activities of this enzyme *in vivo* are not yet known. One attractive possibility is the liberation of secretory granule substance by the intracellular activation of the enzyme. Another possibility is that the enzyme is released from the cells along with heparin and histamine under various conditions. This we have observed in tissue preparations (9). The chymotrypsin-like enzyme would then be free to operate upon certain connective tissue components as, for example, the protein attached to chondroitin sulfate.

Chymotrypsin, we have observed (10), has a relatively specific action in releasing chondroitin sulfate from its protein attachment in cartilage.

The specificity of the enzymes hydrolyzing the histochemical substrate, 3-chloroacetoxy-2-naphthoic acid anilide, is not entirely clear. Mast cells, as Gomori observed (1), are the only elements in formaldehyde-fixed tissue sections which stain after 1 to 2 minutes' incubation. Myeloid elements begin to stain after 10 minutes' incubation; and other tissues such as liver, after 20 minutes. The observed difference in staining time could be either a matter of enzyme activity or of substrate specificity. It remains to define in each tissue location the nature of the enzyme demonstrated by these unnatural histochemical substrates. We have recently described one method of doing this (4); the present work provides another means.

The chymotrypsin-like enzyme which we have characterized has been demonstrated histochemically in the mast cells of the rat, mouse, rabbit, dog, and man (1). This constituent of mast cells must rank in functional importance with heparin, histamine, and the other potent agents now identified in these cells.

SUMMARY

Mast cells contain an enzyme which hydrolyzes 3-chloroacetoxy-2-naphthoic acid anilide. By using highly purified mast cells isolated by differential centrifugation in high density sucrose solutions we have been able to study this enzymatic activity in more detail.

The enzyme has properties similar to those of chymotrypsin: Chymotrypsin will hydrolyze the histochemical substrate, and the chymotrypsin and mast cell activities with this substrate are similarly inhibited by diisopropylfluorophosphate. The mast cell enzyme is capable of hydrolyzing the *N*-acetyl esters of tryptophan, tyrosine, and phenylalanine, the relative rates of hydrolysis being similar to those seen with chymotrypsin. A characteristic trypsin substrate, *p*-toluenesulfonyl arginine methyl ester, is not acted upon by the mast cell enzyme or chymotrypsin. The pH activity curve of the new cell enzyme is similar to that of chymotrypsin as determined with *N*-acetyl-L-tryptophan ethyl ester as substrate.

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