ml of xylene, and charged into a reaction flask. To this stirred mixture, 51.06 g (0.60 mole) of 2-pyrrolidinone in 125 ml of xylene was added dropwise at 60–80°. The initial vigorous evolution of hydrogen abated after 1 hr. The mixture was heated to 140–150°, and 53.98 g (0.25 mole) of 1,4-dibromobutane in 100 ml of xylene was added dropwise.

The mixture was then heated to reflux and refluxed for 17 hr. After cooling to 60°, 8.6 ml of acetic acid was added, and stirring was continued for 10 min. The mixture was filtered using glass filter paper, and the solids were washed with 150 ml of benzene. The filtrate was concentrated under reduced pressure (water pump). Vacuum distillation (bath temperature of 140–190°, 0.4 mm Hg) removed unreacted starting materials. Upon cooling, the product solidified in the distillation flask. Recrystallization from benzene–cyclohexane afforded 35.1 g (63%) of product, mp 69–77°. A second recrystallization gave analytically pure material, mp 82.5–85°; IR (potassium bromide): 6.0 μm (C=O).

Anal.—Calc. for $C_{12}H_{20}N_2O_2$: C, 64.26; H, 8.99; N, 12.49. Found: C, 64.28; H, 8.91; N, 12.61.

1,3-Bis-[*N*-(2-oxopyrrolidino)]propane (VII*a*)—This compound was obtained from 21.3 g (0.25 mole) of 2-pyrrolidinone, 20.2 g (0.10 mole) of trimethylene bromide, and 16.8 g (0.40 mole) of a 57% mineral oil dispersion of sodium hydride according to the procedure for VII*b*. Workup and distillation gave 16.5 g (79%) of liquid, bp 177°/0.36 mm; IR (film): 5.97 μm (C=O).

Anal.—Calc. for $C_{11}H_{18}N_2O_2$: C, 62.83; H, 8.63; N, 13.32. Found: C, 62.95; H, 8.72; N, 13.28.

1,3-Bis-[*N*-(3-ethoxalyl-2-oxopyrrolidino)]propane (VIII*a*)—A suspension of 16.8 g (0.40 mole) of a 57% mineral oil dispersion of sodium hydride in 140 ml of dry tetrahydrofuran was heated to reflux. To it was added a solution of 58.5 g (0.40 mole) of diethyl oxalate and 21.0 g (0.10 mole) of VII*a* in 260 ml of dry tetrahydrofuran dropwise with stirring. Hydrogen evolution was monitored with a mercury bubbler.

After completion of the addition, the mixture was refluxed for 15 hr. After cooling to room temperature, 23 ml of acetic acid was added, and stirring was continued for 15 min. The mixture was poured into 1500 ml of ice water with stirring, and the precipitate was filtered and dried in a vacuum desiccator. Trituration with 50 ml of petroleum ether (bp 30–60°) and filtration gave 19.8 g (48%) of product, mp 121–123°. Recrystallization from 95% ethanol led to the pure compound, mp 124.5–126°.

Anal.—Calc. for $C_{19}H_{26}N_2O_8$: C, 55.60; H, 6.39; N, 6.83. Found: C, 55.63; H, 6.42; N, 6.58.

1,4-Bis-[*N*-(3-ethoxalyl-2-oxopyrrolidino)]butane (VIII*b*)—This compound was obtained from 11.2 g (0.05 mole) of VII*b*, 29.2 g (0.20 mole) of diethyl oxalate, and 8.42 g (0.20 mole) of a 57% mineral oil dispersion of sodium hydride according to the procedure for VIII*a*. Workup gave 13.0 g (61%) of solid, mp 142–147°. Recrystallization produced an analytically pure product, mp 146–148°.

Anal.—Calc. for $C_{20}H_{28}N_2O_8$: C, 56.60; H, 6.65; N, 6.60. Found: C, 56.42; H, 7.14; N, 6.69.

Pharmacological Testing—*P-388 Assay*—Ascitic fluid containing about 6×10^6 cells was inoculated intraperitoneally into male CDF_1 mice. In this assay, median survival times of %T/C ≥ 125 are considered significant. The compounds were administered by the intraperitoneal route in a saline plus alcohol vehicle. Nine daily doses were given, starting 24 hr after tumor inoculation.

B16 Melanocarcinoma Assay—B16 melanocarcinoma homogenate (dilution 1:10) was inoculated intraperitoneally into male BDF_1 mice. In this assay, median survival times of %T/C ≥ 125 are considered significant. Administration and dosing of compounds were the same as for the P-388 testing.

REFERENCES

(1) K.-H. Lee, E.-S. Huang, C. Piantadosi, J. S. Pagano, and T. A. Geissman, *Cancer Res.*, **31**, 1649 (1971).

(2) S. M. Kupchan, M. A. Eakin, and A. M. Thomas, *J. Med. Chem.*, **14**, 1147 (1971).

(3) P. A. Grieco, J. A. Noguez, Y. Masaki, K. Hiroi, M. Nishizawa, A. Rosowsky, S. Oppenheim, and H. Lazarus, *ibid.*, **20**, 71 (1977).

(4) S. M. Kupchan, D. C. Fessler, M. A. Eakin, and T. J. Giacobbe, *Science*, **168**, 376 (1970).

(5) A. Rosowsky, N. Papathanasopoulos, H. Lazarus, G. E. Foley, and E. J. Modest, *J. Med. Chem.*, **17**, 672 (1974).

(6) K.-H. Lee, T. Ibuka, S.-H. Kim, B. R. Vestal, I. H. Hall, and E.-S. Huang, *ibid.*, **18**, 812 (1975).

(7) P. A. Grieco, J. A. Noguez, Y. Masaki, K. Hiroi, M. Nishizawa, A. Rosowsky, S. Oppenheim, and H. Lazarus, *ibid.*, **20**, 71 (1977).

(8) I. K. Stamos, S. Evans, A. McKenzie, S. R. Byrn, and J. M. Cassady, Abstracts of 174th ACS Meeting, Medi 56, Chicago, Ill., 1977.

(9) K.-H. Lee, S.-H. Kim, C. Piantadosi, E.-S. Huang, and T. A. Geissman, *J. Pharm. Sci.*, **63**, 1162 (1974).

(10) M. C. Seidel and R. S. Cook, *J. Heterocycl. Chem.*, **3**, 311 (1966).

(11) H. W. Moore, *Science*, **197**, 527 (1977).

(12) M. J. Kornet, A. M. Crider, and E. O. Magarian, *J. Med. Chem.*, **20**, 1210 (1977).

(13) R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep., Part 3*, **3** (2), 1 (1972).

ACKNOWLEDGMENTS

The author is indebted to Mr. Marshall Davis, Mr. George Piltz, and Mr. James Swencki for the syntheses of some of the compounds.

Facile *In Vitro* Method for Screening Inhibitors of IgE Binding to Mast Cells

STEPHEN M. COUTTS ˣ, DOROTHY M. REID, and IRA WEINRYB

Received July 6, 1978, from the *Department of Biochemistry and Drug Disposition, USV Pharmaceutical Corporation, Tuckahoe, NY 10707.* Accepted for publication August 28, 1978.

Abstract □ A method for rapidly testing large numbers of chemical structures as potential modulators of the interaction between immunoglobulin E (IgE) and its specific receptors on rat peritoneal mast cells is described. IgE, isolated from the ascitic fluid of a transplantable rat IgE immunocytoma, is labeled with iodine-125 under mild conditions employing the Bolton–Hunter reagent. The antibody is incubated with mixed peritoneal cells at 37°, and the cell-bound IgE is separated from unbound label by sedimentation through an 8% sucrose–polymer solution in microsediment tubes. Optimal conditions for the interaction of 3 n*M* IgE with 3×10^5 mast cells in 150 μl are: incubation time, 2 hr; pH, 6.5–7.0; and ionic strength, equivalent to 150 m*M* NaCl. Mixed peritoneal cells bind IgE with an affinity equal to that of purified mast cells. Human IgE pentapeptide III and several antiallergic agents do not compete with rat IgE in this assay.

Keyphrases □ Immunoglobulin E—binding to mast cells, *in vitro* method for screening inhibitors □ Binding—immunoglobulin E to mast cells, *in vitro* method for screening inhibitors □ Inhibitors—immunoglobulin E to rat peritoneal mast cells, *in vitro* screening method

The immunologically induced secretion of mediators of anaphylaxis by sensitized rat or human mast cells exposed to the appropriate antigen triggers a series of intracellular events that require Ca^{2+}, involve an activatable

0022-3549/79/0300-0353$01.00/0

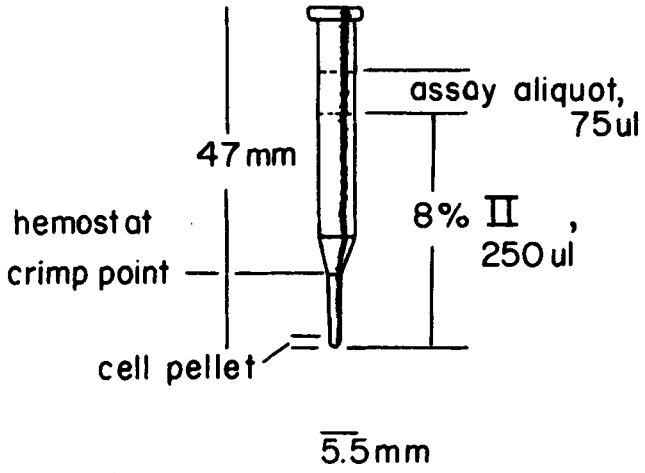

Figure 1—*Diagram of the polyethylene microsediment tube. The capillary is cut between the crimp point and the cell pellet.*

serine protease, can be blocked by metabolic inhibitors, and are modulated by cyclic nucleotides (1). Sensitization of the mast cells is accomplished by the binding of immunoglobulin E (IgE) to specific receptors on the cell's surface and is long lived, a reflection of the high affinity of the process (2).

Little attention has been paid to the possibility of altering the IgE–mast cell interaction, although this approach is potentially one of the most specific ways of interfering with the release of mediators. This paper describes the development and optimization of an assay to measure directly IgE binding to mast cells. This procedure is a modification of previously reported assays (3, 4) and permits the rapid study of large numbers of potential modulators. Also included is an evaluation of the putative inhibitor, human myeloma IgE pentapeptide III (5), and several antiallergic agents.

EXPERIMENTAL

Materials—Ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (I), a copolymer of sucrose and epichlorohydrin (II, mol. wt. ~40,000), and zwitterionic buffers 2-(*N*-morpholino)ethanesulfonic acid (III), piperazine-*N,N'*-bis(2-ethanesulfonic acid) (IV), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (V) were used as obtained[1]. Calfskin gelatin[2] was warmed to aid in its dissolution in buffers.

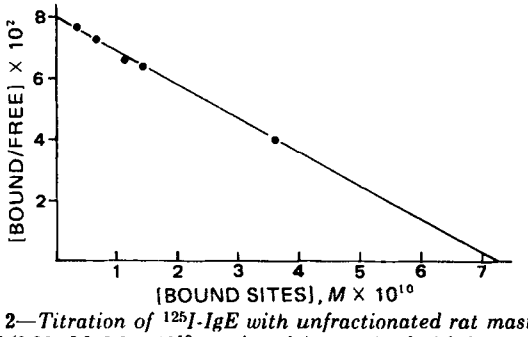

Figure 2—*Titration of ^{125}I-IgE with unfractionated rat mast cells. ^{125}I-IgE (0.89 nM, 3.0 × 10^{10} cpm/μmole) was mixed with from 2 × 10^5 to 4 × 10^6 mast cells in a total volume of 210 μl. Blank values, determined with a 100-fold excess of unlabeled IgE, were subtracted from test values. The least-squares line, having a correlation coefficient of 0.99, extrapolated to a value of 0.72 nM sites bound at infinite cell concentration. A value of 3 × 10^5 sites/cell was assumed in the calculation, although the actual number of sites per cell influenced only the slope of the line calculated* (i.e., K_a) *and not the value of the concentration of bound sites at infinite cell concentration (x-intercept). The molecular weight of IgE was assumed to be 183,000 (7).*

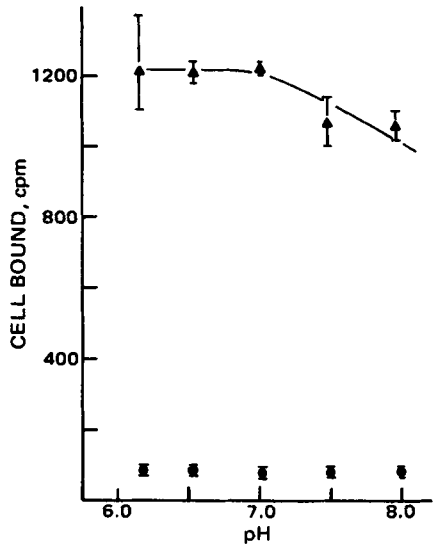

Figure 3—*pH-binding profile of ^{125}I-IgE with unfractionated mast cells. Assays were run in triplicate, with the standard error of the mean indicated by vertical bars. Cell-bound counts of 1000 are equivalent to 2 × 10^{-8} μmole of ^{125}I-IgE. Key: ▲, ^{125}I-IgE; and ●, ^{125}I-IgE plus a 100-fold excess of unlabeled IgE.*

The following antiallergic compounds were tested: theophylline[1], 1-methyl-3-isobutylxanthine[3], 7-(2-hydroxyethoxy)-9-oxoxanthene-2-carboxylic acid sodium salt (VI)[4], 5,5-dimethyl-11-oxo-5*H*,11*H*-(2)-benzpyrano[4,3-*g*](1)benzopyran-9-carboxylic acid ethanolamine salt (VII)[5], doxantrazole[6], disodium cromoglycate[7], etazolate hydrochloride[8], and the prostaglandins[1] E_1, A_1, and $F_{2\alpha}$. Pentapeptide III (aspartyl-seryl-aspartyl-prolyl-arginine[9]) was subjected to amino acid analysis after a 22-hr hydrolysis and yielded the following molar ratios of aminio acid to proline: aspartic acid, 2.08; serine, 0.91; arginine, 1.01; proline, 1.0; and lysine, 0.01. Less than 10 nmoles/mg of peptide was detected for the amino acids.

Bolton-Hunter reagent[10] was purchased in vials of 500 μCi with a specific activity of 1500 Ci/mmole. Polyethylene capillary microsediment tubes[11] were selected to fit the adapted rotor holes of a small centrifuge[12]. Collodion bags for protein dialysis[13] had an exclusion limit of 25,000 daltons. Dimethyl sulfoxide[14] (grade 1) and other solvents[1] were ACS grade.

Buffers—The buffer generally employed in isolating and washing cells (cell isolation buffer) had the following composition: 150 m*M* NaCl, 2.7 m*M* KCl, 0.1 m*M* I, 0.1% gelatin, and 1 m*M* NaH_2PO_4; it was adjusted to pH 7.0 with sodium hydroxide. The buffer utilized in the assay solution (assay buffer) consisted of 500 m*M* NaCl, 9 m*M* KCl, 0.3 m*M* I, 0.32% gelatin, and 50 m*M* IV; it also was adjusted to pH 7.0 with sodium hydroxide.

The assay contained 50 μl of cells in cell isolation buffer, 30 μl of assay buffer, test compound, and labeled and unlabeled IgE in a total of 150 μl. The final salt concentrations were 150 m*M* NaCl, 2.7 m*M* KCl, 0.1 m*M* I, 0.1% gelatin, and 10 m*M* IV (pH 7.0).

Cells—Mast cells were obtained by peritoneal lavage of male Sprague-Dawley rats[15] (250–350 g). The animals were anesthetized with

[1] Sigma Chemical Co., St. Louis, Mo. The trade name of II is Ficoll; supplier's designations of other compounds are: I, EGTA; III, MES; IV, PIPES; and V, HEPES.
[2] Eastman Chemical Corp., Rochester, N.Y.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] AH 7725, Allen and Hanburys Research Ltd., Hertfordshire, England.
[5] PR-D92-EA, Pharma-Research Canada Ltd., Pointe Claire, Quebec, Canada.
[6] Wellcome Research Laboratories, Triangle Park, N.C.
[7] Fisons Corp., Bedford, Mass.
[8] SQ 20,009, Squibb, Princeton, N.J.
[9] Peninsula Laboratories, San Carlos, Calif.
[10] New England Nuclear, Boston, Mass.
[11] No. 47/7, W. Sarstedt, Inc., Princeton, N.J.
[12] Eppendorf 3200 microfuge, Bio-Rad Laboratories, Richmond, Calif.
[13] Schleicher and Schuell, Keene, N.H.
[14] Fisher Scientific, Springfield, N.J.
[15] Charles River Laboratories, Cambridge, Mass.

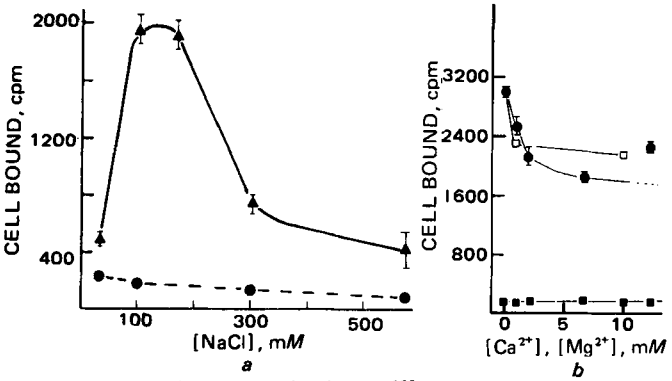

Figure 4—*Dependence of the binding of ^{125}I-IgE to mast cells on ionic strength and divalent metal ions. (a) Each assay tube contained, in a total volume of 150 µl, 1.5 µmoles of III (pH 7.0), 0.4 µmole of potassium chloride, 0.015 µmole of I, 150 µg of gelatin, 5.4–86 µmoles of sodium chloride, 3 × 10^5 unfractionated mast cells, and 6.4 × 10^{-7} µmole of ^{125}I-IgE (specific activity of 2.8 × 10^5 cpm/µg). Cell-bound counts of 1000 are equivalent to 2 × 10^{-8} µmole of ^{125}I-IgE. Contributions from III and I to the sodium-ion concentration were calculated according to the Henderson–Hasselbach equation. Key: ▲, ^{125}I-IgE; and ●, ^{125}I-IgE plus a 100-fold excess of unlabeled IgE (blanks). Vertical bars represent error of the means. (b) Each assay tube contained, in a total volume of 150 µl, 1.5 µmoles of III (pH 7.0), 0.4 µmole of potassium chloride, 22.5 µmoles of sodium chloride, 0.015 µmole of I, 150 µg of gelatin, 3 × 10^5 unfractionated mast cells, and 6.7 × 10^{-7} µmole of ^{125}I-IgE (specific activity of 2.8 × 10^5 cpm/µg). Calcium chloride (0.15–2.0 µmoles) or magnesium chloride (0.3–1.5 µmoles) was added to the various tubes. Key: ●, calcium; □, magnesium; and ■, blanks.*

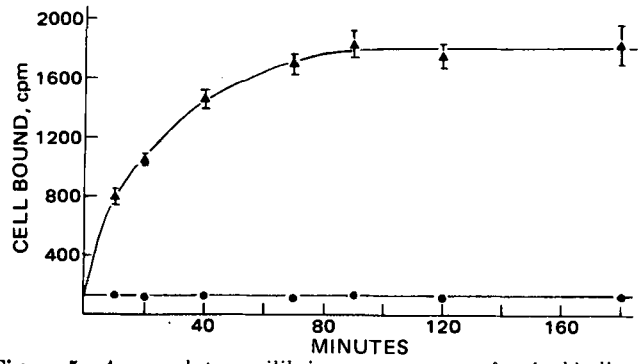

Figure 5—*Approach to equilibrium measurement for the binding of ^{125}I-IgE to unfractionated rat mast cells at 37°. Each assay tube contained, in a total volume of 150 µl, 3 × 10^5 unfractionated mast cells, 6.7 × 10^{-7} µmole of ^{125}I-IgE (specific activity of 2.7 × 10^5 cpm/µg; 1000 cpm is equivalent to 2 × 10^{-8} µmole of antibody), 1.5 µmoles of III (pH 7.0), 22.5 µmoles of sodium chloride, 0.4 µmole of potassium chloride, 0.015 µmole of I, and 150 µg of gelatin. Key: ▲, ^{125}I-IgE; and ●, ^{125}I-IgE plus a 100-fold excess of unlabeled IgE.*

ether, decapitated, and exsanguinated before the injection of cold cell isolation buffer (~7 ml/animal) into the peritoneal cavity. Combined peritoneal washes were harvested by sedimentation at 160×*g* for 10 min, and the cell pellet was washed three times with cell isolation buffer (mixed cells). The final pellet was suspended in 1 ml of cell isolation buffer, an aliquot was stained with 0.1% toluidine blue in phosphate-buffered saline, and the mast cell concentration was determined with a hemocytometer.

Purified mast cells were obtained by the method of Ishizaka *et al.* (6), except that cell isolation buffer was employed. The cells were kept at 0° except during centrifugation, which was done at room temperature. Mixed peritoneal cells generally contained 5–10% mast cells, with a yield of 1–2 × 10^6 mast cells/rat. Cells fractionated over II were generally 50–80% mast cells, with a yield of 0.5–1 × 10^6 mast cells/rat. Cells were diluted with cell isolation buffer such that 50 µl contained 2–3 × 10^5 mast cells.

IgE—Pure IgE was isolated from the ascitic fluid of a transplantable IgE immunocytoma (IR-162) grown in rats (7). The IgE-containing ascites[16] was treated according to Kulczycki and Metzger (3), with certain modifications[17], to yield a preparation of IgE that showed just two bands on 10% polyacrylamide gels subjected to electrophoresis under denaturing conditions (sodium lauryl sulfate and β-mercaptoethanol) (8). The purified IgE was stored as a dry lyophilized powder[17] at −80° after dialysis against 15 m*M* Na_2HPO_4.

^{125}I-Labeling of IgE—The Bolton–Hunter reagent was used according to the manufacturer's directions (*cf.*, 9); the charcoal filter was employed to trap volatile iodine compounds. The combination vial containing the dried reagent (500 µCi, 0.33 nmole) was chilled at −18° for 2 hr and then placed in ice for 30 min to ensure that the insulated inner vessel was at 0°. A solution of 500 µg of IgE (3.3 nmoles) in 100–200 µl of ice-cold 0.1 *M* sodium borate buffer, pH 8.5, was injected into the vial, which was then mixed, centrifuged briefly, and set aside at 0° overnight.

The vial's contents were transferred with two rinses of 100 µl of 0.1 *M* sodium borate buffer (pH 8.0) into a collodion bag, and the combined solutions were dialyzed against the same buffer (for a total dilution factor of 10^6) at 4°. The dialyzed solution was diluted to 2.0 ml with the pH 8.0 borate buffer, and the specific activity was determined, using a value of 1.36 for $E^{0.1\%}_{280\,nm}$ (3). Specific activities ranged between 4 and 5 × 10^5 cpm/µg of protein, 95–97% of which could be precipitated by 10% trichloroacetic acid in the presence of a 40-fold excess of bovine serum albumin. These values corresponded to ~60% of radiochemical incorporation of iodine-125 into protein.

The solution of ^{125}I-IgE was stored at 4° and was stable in borate buffer for at least 3 months.

Binding Assay—Rat peritoneal mast cells (2–3 × 10^5 cells/tube) were incubated with assay buffer. If desired, the test compound was added at this time to preincubate at 37° for 5 min prior to the addition of ^{125}I-IgE. The labeled antibody was then added; incubation was continued for 2 hr in a shaking water bath, which was adjusted to 150 cpm to keep the cells evenly suspended. The total volume of the assay was 150 µl, and the final concentrations of ^{125}I-IgE and the test compound were generally 3 n*M* and 100 µ*M*, respectively. Other components present were 150 m*M* NaCl, 2.7 m*M* KCl, 0.1 m*M* I, 0.1% (w/v) gelatin, and 10 m*M* IV (pH 7.0).

After the incubation was completed, 75 µl was withdrawn from each tube, after first evenly distributing the cells, and layered over 250 µl of an 8% solution of II (made up in cell isolation buffer, with a final refractive index of ~1.3455) in a polyethylene microsediment tube. Cell-bound radioactivity was separated from unbound ^{125}I-IgE by centrifuging the cells at 8000×*g* for 2 min. The cell pellet then resided in the capillary tip of the microsediment tube and was collected by cutting the tip off with a hot wire after first crimping, with a hemostat, the top of the capillary beneath the cell cushion of II (Fig. 1).

The tip was dropped into a plastic vial and was counted in a γ-spectrometer. If the effect of a test compound was measured, the control consisted of adding solvent (in which the test compound was added) alone to the mast cells and ^{125}I-IgE and treating the tubes in the same manner (*e.g.*, with 5-min preincubation). The blank was obtained by adding a 100-fold excess of unlabeled IgE to an additional three tubes to check for nonspecific binding, entrainment of labeled antibody in the cell pellet, and background radiation.

Many test compounds of interest were not soluble in aqueous buffers. Then the choice was to disperse them in a more or less homogeneous suspension by physical means (*e.g.*, sonication) or to dissolve them in an organic solvent. It was convenient to dissolve most compounds in dimethyl sulfoxide, to dilute them with buffer, and to add them to the assay mixture such that the final dimethyl sulfoxide concentration was 0.17%. This low concentration of dimethyl sulfoxide did not affect the binding parameters of IgE to mast cells, nor did it affect the spontaneous release of histamine from the same cells. Cell viability after the assay under these conditions was about 60–80% in control samples, as measured by trypan blue exclusion.

pH Profile—Labeled antibody was incubated with mast cells under standard ionic strength conditions (150 m*M* NaCl, 2.7 m*M* KCl, 0.1 m*M* I, and 0.1% gelatin) in the presence of different buffers at 10 m*M*. Buffers utilized for the different pH values were III (pH 6.0), IV (pH 6.5 and 7.0), and V (pH 7.5 and 8). The buffers were made up in stock solutions of 100 m*M*, and the pH was checked for each sample after the buffer had been diluted to the same concentration and ionic strength as used in the assay.

[16] Gift of Dr. H. Metzger, National Institutes of Health.

[17] Dr. H. Metzger, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD 20014, personal communication.

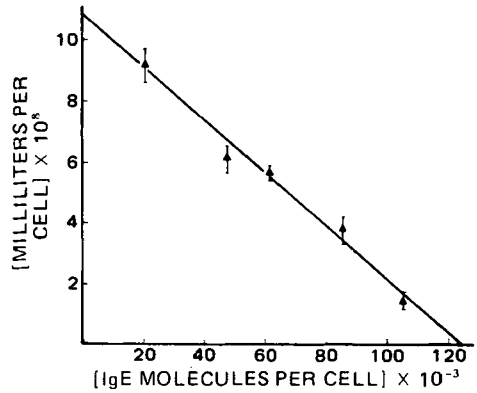

Figure 6—*Scatchard plot of the binding of ^{125}I-IgE to unpurified rat mast cells. Each assay tube contained 3 × 10^5 mast cells and 0.4–13 n*M *^{125}I-IgE (specific activity of 1.65 × 10^5 cpm/μg) in a total volume of 150 μl. The points were run in triplicate, with a blank (100-fold excess unlabeled IgE added to labeled IgE) subtracted from each point. The standard error of the mean is indicated by bars. The least-squares line yielded values of 125,000 IgE receptor sites/cell with an affinity constant of 5 × 10^8 M^{-1}. The correlation coefficient for the plot is 0.99.*

RESULTS

Binding Assay—The IgE isolated from IR-162 ascites had a high degree of purity as judged by sodium lauryl sulfate gel electrophoresis. Autoradiography of the IgE labeled with Bolton-Hunter reagent after electrophoresis yielded a pattern identical to the stained bands. Over 95% of the labeled antibody could be precipitated with trichloroacetic acid, and approximately 80% of the iodinated IgE could be bound by mast cells as assessed from the intercept value of a Scatchard analysis of the binding data (Fig. 2) in which bound and free *receptor sites* were the variables.

The interaction of ^{125}I-IgE with rat peritoneal mast cells was measured in an assay utilizing a facile, one-step separation of cell-bound labeled IgE from unbound antibody. In general, 6–12% of the total counts was bound to cells under the assay conditions. Optimal conditions for the interaction between IgE and its mast cell receptors on both purified and unfractionated mast cells were pH 6.5–7.0 (Fig. 3), about 150 m*M* NaCl (in the presence of 2.7 m*M* KCl, 0.1 m*M* I, and 10 m*M* buffer) (Fig. 4*a*), no magnesium or calcium (Fig. 4*b*), and 37°. Kinetic experiments at 37° under the optimized conditions indicated that, at the IgE and mast cell concentrations employed in the assay (3–5 n*M* and 1–2 × 10^6 cells/ml, respectively), less than 2 hr was sufficient to establish equilibrium (Fig. 5).

A number of experimental observations with this system supported the conclusion that the binding equilibrium of ^{125}I-labeled IgE was to a finite number of receptors on mast cells, *i.e.*, was specific:

1. A 100-fold excess of unlabeled IgE blocked the binding of labeled IgE by greater than 95%.

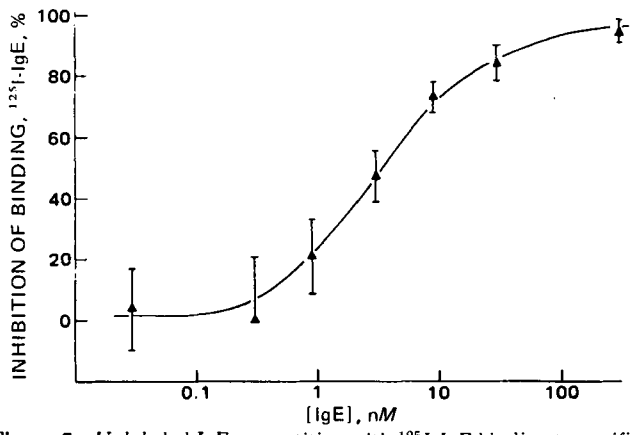

Figure 7—*Unlabeled IgE competition with ^{125}I-IgE binding to purified rat mast cells. Each assay tube contained 2 × 10^5 mast cells and 3 n*M *^{125}I-IgE (specific activity of 2.7 × 10^5 cpm/μg); 9.7% of the total counts was bound to cells in the control. The I_{50} for unlabeled IgE obtained from this plot is 3.5 n*M.

Table I—Effects of Standard Antiallergic Agents on ^{125}I-IgE Binding to Rat Mast Cells [a]

Agent	Cell Bound, cpm ± *SEM*		Inhibition of IgE Binding, % ± *SEM*
	Control	100 μ*M* of Compound	
Theophylline (10)	1843 ± 47	1717 ± 17	7 ± 3
Methylisobutylxanthine (11)	1843 ± 47	1746 ± 98	5 ± 8
Doxantrazole (10)	1843 ± 47	1589 ± 156	15 ± 11
VI (10)	1843 ± 47	1886 ± 41	0 ± 5
VII (12)	1843 ± 47	1743 ± 78	6 ± 7
Etazolate hydrochloride (10)	1843 ± 47	1982 ± 180	−5 ± 8
Disodium cromoglycate (10)	1703 ± 35	1622 ± 108	5 ± 12

[a] These assays were performed using unfractionated cells. The final dimethyl sulfoxide concentration was 0.67%, and a 100-fold excess of unlabeled IgE inhibited labeled IgE binding by 93%. Greater than 25% inhibition is regarded as significant.

2. Greater than 98% of the binding activity of ^{125}I-IgE could be eliminated by heating the antibody preparation at 56° for 4 hr.

3. Scatchard plots, according to Conrad *et al.* (4) (Fig. 6), on both purified mast cells and mixed peritoneal cells yielded the same binding parameters for both cell preparations. An average of eight experiments gave the number of binding sites per cell ± *SEM* as 2 ± 0.4 × 10^5, the affinity constant K_a ± *SEM* as 6.4 ± 0.9 × 10^8 M^{-1}, and the correlation coefficient ± *SEM* as 0.96 ± 0.01. These data were *not* corrected for the apparent inability of 20% of the labeled IgE to bind to mast cells. Conrad *et al.* (4) obtained values of 3 × 10^5 for sites per cell and about 10^9 M^{-1} for K_a.

4. A dose-response inhibition curve obtained by adding variable amounts of unlabeled IgE to a constant amount of ^{125}I-IgE (Fig. 7) gave a normal dose-response with an I_{50} value equal to the concentration of labeled antibody.

The extent of antibody binding was not affected by 10 m*M* NaN_3, suggesting that cellular ATP is not required for receptor-antibody interaction.

Anaphylactic Agents—Several compounds with demonstrated antiallergic activities were assayed at 100 μ*M* as inhibitors of IgE binding. However, none demonstrated significant inhibition (Table I). Since prostaglandins are known to interact with mast cell membranes (13), three representatives (A_1, E_1, and $F_{2\alpha}$) were tested at 10 μ*M* for their ability to inhibit the antibody receptor formation. All were inactive.

Pentapeptide—Experiments to test the inhibition of binding of ^{125}I-IgE to mast cells by unlabeled peptide were performed (Table II). The data demonstrate that a 100-fold excess of unlabeled IgE inhibited labeled IgE binding by 95% and that, within experimental error, a large excess of pentapeptide did not affect antibody binding to the receptor.

DISCUSSION

Desirable features for an assay for measuring the binding of ^{125}I-labeled IgE to rat peritoneal mast cells are the availability of a pure antibody preparation and a gentle labeling procedure that yields a native product of high specific activity. The first condition was fulfilled by the existence of rodent immunocytoma lines from which IgE could be readily isolated (7). The preparation reported here showed two major bands on sodium lauryl sulfate and β-mercaptoethanol gel electrophoresis, with apparent molecular weights corresponding to the heavy and light chains of IgE (~76,000 and 20,000 daltons, respectively).

Table II—Ability of Pentapeptide to Compete for IgE Binding Sites on Rat Mast Cells

Inhibitor	Inhibitor-^{125}I-IgE Ratio[a]	cpm_{bound}^{I}/cpm_{bound}^{C} [b]
IgE	100	0.05 ± 0.02
Pentapeptide	1.2 × 10^4	0.93 ± 0.22
	1.2 × 10^5	1.10 ± 0.16
	4.8 × 10^5	1.07 ± 0.29
	1.2 × 10^6	1.12 ± 0.28

[a] Inhibitor-^{125}I-IgE is the molar ratio of the particular inhibitor investigated to ^{125}I-labeled IgE, whose concentration in these experiments was between 0.1 and 1 n*M*. There were about 2 × 10^5 rat mast cells/aliquot counted. [b] cpm_{bound}^{I} and cpm_{bound}^{C} are cell-bound counts in the presence (*I*) and absence (*C*) of inhibitor, respectively.

The second condition can be fulfilled by one of several methods. For example, ^{14}C-phenylisothiocyanate can be used under nondenaturing conditions to label antibodies without loss of activity (14). However, because of its high specific activity, iodine-125 is still most often the isotope of choice, although the labeling procedures frequently utilize oxidizing agents such as chloramine-T or hydrogen peroxide. Conrad *et al.* (4) found that less than 50% of the IgE labeled with chloramine-T oxidation could be bound to rat mast cells. However, Kulczycki and Metzger (3) found that up to 80% of the IgE labeled by the same method bound to rat basophilic leukemia cells. The findings reported here indicate that 80% of the IgE reacted with Bolton–Hunter reagent could be bound to mixed peritoneal cells (Fig. 2), even after 3 months of storage at 4° in borate buffer. The fact that unlabeled IgE inhibited with an I_{50} equal to the concentration of ^{125}I-IgE (Fig. 7) provided additional support that the labeled antibody is native and that its binding is not affected by the reaction with Bolton–Hunter reagent.

The assay reported here incorporates the approach of Kulczycki and Metzger (3) with certain significant modifications. The use of an 8% solution of II as a cell cushion and of microsediment tubes in the isolation of cell-bound radioactivity provided a more facile and reproducible assay. Considerable savings of time and animals were made possible by the use of mixed peritoneal cells. A protocol with optimal ionic strength, pH, and time of incubation conditions for the interaction of myeloma IgE with rat mast cells has been elaborated in this paper. Although high concentrations (10 m*M*) of calcium and magnesium appeared to inhibit the binding of IgE to mast cells, the inclusion of low amounts of Ca^{2+} (0.1–1 m*M*) may be desirable to maintain membrane and cell integrity. Adequate shaking during the incubation was important for reproducible results.

Compounds that lyse mast cells, making them incapable of penetrating the sucrose–polymer (II) cushion in the microsediment tube (Fig. 1), show up as apparent inhibitors of IgE binding. Therefore, active compounds should be examined for their ability to release cytoplasmic enzymes. In these inhibition studies, concentrations of test compound and ^{125}I-IgE of 100 μM and 3 n*M*, respectively, were generally employed. The cytolytic activity of some agents may be circumvented by decreasing the concentrations of compounds and antibody (keeping the ratio constant) and by increasing the specific activity of IgE by reacting less protein (see *Experimental*) with Bolton–Hunter reagent.

The insensitivity of the assay system to low concentrations of dimethyl sulfoxide has facilitated the testing of lipophilic compounds. A concentration of solvent that not only does not affect the binding parameters of IgE to its receptor but also does not affect the interaction of the drug with the cells must be used. Dimethyl sulfoxide was found to be more suitable than dimethylformamide, acetone, or ethanol, both as a solvent for dissolving compounds and for its lack of interaction with the biological system (data not shown). To avoid solvent–drug artifacts, however, dimethyl sulfoxide should be used at a final concentration below 0.2% (v/v).

This assay to quantitate cell-bound ligand appeared more convenient than the micromethod suggested by Davis *et al.* (15), which employed a two-tube assembly with a quartz-wool filter to trap the cells. Whereas the method described here had a one-step sedimentation procedure for separating cell-bound counts, the method described by Davis *et al.* required three washing steps.

Pentapeptide III, Asp-Ser-Asp-Pro-Arg, was reported by Hamburger (5) to inhibit the Prausnitz–Küstner (P.–K.) reaction. This finding implied that the pentapeptide could block the binding of IgE to its mast cell receptors. Direct binding studies (16) of pentapeptide III, as well as the competition experiments reported here, failed to indicate strong ($K_a < 5 \times 10^5\ M^{-1}$) or specific binding. This finding was supported by the report (17) that the pentapeptide did not affect the binding of human IgE to human basophils, nor did it block antigen-induced histamine release.

Two recent communications (18, 19), however, supported Hamburger's (5) original results. One article stated that the pentapeptide could inhibit antigen-induced degranulation of rat mast cells in several models, as assessed by the estimation of the number of degranulated mast cells in a mixed peritoneal cell sample (18). These researchers suggested that the pentapeptide could interfere with both IgE–mast cell interactions and IgE–antigen combination. The other article reported the inhibition of wheal and flare reactions by the pentapeptide when used against certain antigens in the human Prausnitz–Küstner and baboon passive cutaneous anaphylaxis systems (19). The experiments reported here, however, failed to show any evidence of a specific interaction between pentapeptide III and the IgE receptors on rat mast cells.

This assay should facilitate the discovery and characterization of modulators of the binding of IgE to its mast cell receptor.

REFERENCES

(1) K. F. Austen, *Fed. Proc.*, **33,** 2256 (1974).

(2) S. Newman, G. Rossi, and H. Metzger, *Proc. Natl. Acad. Sci. USA*, **74,** 869 (1977).

(3) A. Kulczycki, Jr., and H. Metzger, *J. Exp. Med.*, **140,** 1676 (1974).

(4) D. Conrad, H. Bazin, A. Sehon, and A. Froese, *J. Immunol.*, **144,** 1688 (1975).

(5) R. N. Hamburger, *Science*, **189,** 389 (1975).

(6) T. Ishizaka, T. Adachi, T.-H. Chang, and K. Ishizaka, *J. Immunol.* **118,** 211 (1977).

(7) H. Bazin, P. Querinjean, A. Beckers, J. F. Heremans, and F. Dessy, *Immunology*, **26,** 713 (1974).

(8) U. K. Laemmli, *Nature*, **227,** 680 (1970).

(9) A. E. Bolton and W. M. Hunter, *Biochem. J.*, **133,** 529 (1973).

(10) C. J. Coulson, R. E. Ford, S. Marshall, J. L. Walker, K. R. Wooldridge, K. Bowden, and T. J. Coombs, *Nature*, **265,** 545 (1977).

(11) J. A. Beavo, N. L. Rogers, O. B. Crofford, J. G. Hardman, E. W. Sutherland, and E. V. Newman, *Mol. Pharmacol.*, **6,** 597 (1970).

(12) P. B. Stewart, J. P. Devlin, and K. R. Freter, *Fed. Proc.*, **33,** 762 (1974).

(13) I. Tauber, M. Kaliner, D. J. Stechschulte, and K. F. Austen, *J. Immunol.*, **111,** 27 (1973).

(14) N. Levy and J. Dawson, *ibid.*, **116,** 1526 (1976).

(15) E. Davis, D. Tsay, M. Schlamowitz, and E. Walborg, Jr., *Anal. Biochem.*, **80,** 416 (1977).

(16) S. Coutts, D. Reid, and I. Weinryb, *Fed. Proc.*, **36,** 743 (1977).

(17) H. Bennich, U. Ragnarsson, S. Johansson, K. Ishizaka, T. Ishizaka, D. Levy, and L. Lichtenstein, *Int. Arch. Allergy Appl. Immunol.*, **53,** 459 (1977).

(18) N. Vardinon, Z. Spirer, M. Fridkin, J. Schwartz, and S. Ben-Efraim, *Acta Allergol.*, **32,** 291 (1977).

(19) D. Stanworth, M. Kings, P. Roy, and D. Moran, *Int. Arch. Allergy Appl. Immunol.*, **56,** 409 (1978).

ACKNOWLEDGMENT

The authors thank Dr. H. Bazin (Louvain) for furnishing a sample of IgE-containing ascitic fluid. They also acknowledge helpful comments from Dr. Metzger and Dr. Bazin during preliminary work.