# THE INTERACTION OF IgE WITH RAT BASOPHILIC LEUKEMIA CELLS

## II. Quantitative Aspects of the Binding Reaction

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We have demonstrated that rat basophilic leukemia cells (RBL-1 cells)[1] bind rat IgE (1). Those studies and others (2) showed that the IgE is bound to the cell surface with a specificity similar to that observed with normal rat mast cells.

In this paper we present a quantitative analysis of the binding. For some of the experiments larger amounts of IgE were necessary than could be obtained easily from reaginic serum. For this reason an IgE myeloma protein (3) was used after demonstrating that its binding properties were indistinguishable from those observed for the reaginic antibodies used previously.

## Materials and Methods

*IgE Preparations.* IgE was purified from rat anti-*Nippostrongylus brasiliensis* serum by the method described previously (4). A rat IgE myeloma protein was purified from serum of rats bearing tumor IR 162 (3) in an analogous fashion: 12 ml serum were dialyzed vs. 0.2 M borate-buffered saline pH 8.0 (BBS) and the protein precipitating between 38 and 48% saturated $(NH_4)_2SO_4$ at 4°C collected. This fraction was chromatographed on a 4.1 × 105 cm column of Sepharose 6B and the major component in the effluent concentrated by ultrafiltration (Amicon Corp., Lexington, Mass.) and dialyzed against a solution which was 33 mM in Tris base, 25 mM in phosphate, having a conductivity of 2.3 mmho and a pH of 8.0 at room temperature. The protein (~ 100 absorbancy units at 280 nm) was next chromatographed on a 2.6 × 7.2 cm column of "DE-52" diethylaminoethyl cellulose (Whatman Biochemicals Ltd., Maidstone, England). The excluded component was dialyzed against BBS and applied successively to two Sepharose 2B immunoadsorbents containing rabbit antibodies to the normal rat serum fraction precipitating at 50% saturated $(NH_4)_2SO_4$ and rabbit antibodies to normal rat proteins excluded from DE-52 respectively (4). Both antibody preparations had been absorbed with a Sepharose 2B immunoadsorbent containing Fab fragments of rat IgG. The purified IgE preparations were stored at 4°C in BBS and radiolabeled with [125]I (New England Nuclear, Boston, Mass.) by the chloramine-T method (4, 5) as required.

*RBL-1 Cells.* Cells were cultured in Falcon flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) as described previously (1) or in spinner cultures (footnote 2). In the latter case the medium

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[1] *Abbreviations used in this paper:* BBS, borate-buffered saline; PCA, passive cutaneous anaphylaxis; RBL-1 cells, rat basophilic leukemia cells.

[2] Buell, D. N., Metzger, H., and C. Isersky. 1974. Cell cycle and morphologic changes during growth and differentiation of a rat basophilic leukemia cell line. Manuscript submitted for publication.

contained 15% fetal calf serum. Cells were harvested by centrifugation (~70 g) and washed once in the experimental medium.

*Other Materials.* Phosphatidyl serine was purchcased from Calbiochem (San Diego, Calif.). Fetal calf serum, heat-inactivated, purchased from Grand Island Biological Co. (Grand Island, N. Y.) was used in culture and binding assay media.

Rat myeloma sera rich in IgA (IR 22, IR 253), IgM (IR 201, IR 202), and in $IgG_1$ (IR 12, IR 250) were obtained from Dr. Hervé Bazin (6), as was a goat anti-IgE (anti-IR-2) serum. The concentrations of the myeloma proteins were estimated from scans of cellulose acetate electrophoresis strips.

*Binding Assays.* The binding of [$^{125}$I]IgE to the RBL-1 cells was measured as described previously (1) i.e., by pelleting the sample of cells through fetal calf serum in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and counting the pellet. Those experiments at 37°C and 4°C were conducted in a warm room and cold room respectively. In many experiments the reaction was stopped by adding a 50-fold excess of unlabeled IgE at appropriate times. In control experiments a 100-fold excess of unlabeled IgE was added before the addition of [$^{125}$I]IgE. Unless stated otherwise, all incubations of IgE with the cells were performed in Eagle's minimum essential medium (7) with 10% fetal calf serum and containing 10 mM Hepes (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfuric acid) buffer, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and having a pH of 7.1.

In some instances a "modified" Tyrode's buffer was used. This consisted of 25 mM Tris base, 123 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, and 0.1% gelatin titrated to pH 7.6 with HCl.

To translate the counts bound/cell into molecules of [$^{125}$I]IgE bound/cell the measured specific activity of IgE (cpm/mg) and an assumed mol wt of $2 \times 10^5$ were used. To determine specific activities for the preparations, protein concentrations were calculated from the 280 nm absorbancy. The extinction coefficient was in turn determined by the refractive index increment method assuming a refractive index increment of 0.00188 (8).

*Other Methods.* Once it was established that the binding of IgE was by a simple reversible reaction appropriate calculations were made using the equation

$$k_i t = \frac{2.303}{\sqrt{-q}} \log \frac{1 - \left( \dfrac{K_D + A + B - \sqrt{-q}}{K_D + A + B + \sqrt{-q}} \right) X}{1 - X}$$

where $X$ = fraction of equilibrium, $K_D$ = dissociation constant, i.e. $1/K_A$, $-q = (K_D + A + B)^2 - 4AB$, $A$ and $B$ = initial concentrations of the reactants, $t$ = time and $k_1$ = the forward rate constant (9).

Time-sharing computer facilities were employed. Once an approximate $K_A$ was determined estimates of $k_1$ were refined as follows: the original $k_1$ was inserted into the equation along with $K_A$, and the percent site saturation at equilibrium calculated. The experimentally determined equilibrium value of occupied sites was then corrected upwards to give the true value of $R_o$. Using this value and the initial-rate data a more accurate $k_1$ could be calculated. In our most reliable experiments (e.g. Fig. 4; see Results for listing of criteria) these corrections were less than 5%. In other experiments reiterative corrections resulted in changes of 15-20%.

# Results

*Properties of Reaginic vs. Myeloma IgE.* Despite a 100- to 200-fold difference in concentration, the myeloma IgE (~ 10 mg/ml) behaved very much like the reaginic IgE (50-100 μg/ml (4, 10)) on purification. The IR 162 protein showed a similar precipitability pattern with $(NH_4)_2SO_4$ and its elution volume from Sepharose 6B was identical to that observed for reagins (4). On DEAE chromatography it was excluded at a somewhat lower conductivity (2.3 mmho) than the majority of reaginic antibodies (~ 6 mmho). The purified myeloma protein at 10 mg/ml showed a single line by immunoelectrophoresis against a potent rabbit antirat serum containing anti-light chain antibodies as well as

antibodies to multiple other serum proteins. The ^{125}I-labeled myeloma IgE was 94% precipitable by anti-IgE, whereas our purest preparations of labeled reaginic IgE were 70–80% precipitable (4). By radioimmunoassay using [125I]myeloma IgE the slopes of the inhibition curves of unlabeled IgE myeloma serum and reaginic serum were identical (10). The percentage of "active" [125I]IgE was determined by incubating the labeled protein with increasing number of cells as described previously (see Fig. 7 in Ref. 4). Approximately 50% of labeled reaginic IgE became bound whereas up to 70–80% of the [125I]myeloma IgE bound to the cells. When the unbound material was concentrated and incubated with another sample of cells only 5–10% of the radioactivity (1–2% of the total added originally) became cell bound. In the descriptions of experiments which follow, the IgE concentration in the incubation mixtures is given in terms of the active IgE, i.e. approximately (total IgE × 0.75) unless stated otherwise. The extinction coefficient of the myeloma IgE was 13.6 at 280 nm for a solution containing 1% protein as estimated from the refractive index increment. As a test of our technique the extinction coefficient for bovine serum albumin was simultaneously determined and was within 1% of the literature value (8).

The labeled myeloma IgE behaved almost identically to unlabeled myeloma IgE and to reaginic IgE in its binding properties. This was determined as follows. Increasing amounts of unlabeled IgE were mixed with a standard amount of [125I]myeloma IgE. The labeled IgE was itself in ~ fourfold excess to the number of receptors for IgE on the cells which were then added. As shown in Table I stoichiometric inhibition of binding was observed. In addition we have used the binding parameters determined for the myeloma IgE (below) to evaluate the binding for reaginic IgE.

Both the data in which labeled rabbit anti-light chain antibodies were used to assess reagin binding (Table IV, Ref. 1) and the results of [125I]reagin binding (Fig. 2, Ref. 1) have been analyzed. The data are consistent with the reaginic and myeloma IgE having similar binding characteristics for the RBL-1 cells. As was true of the reaginic IgE, binding of the myeloma IgE was not measurably inhibited by normal rat serum or by fetal calf serum. In the present study we also tested several rat myeloma sera having high concentrations of IgA, IgM, or IgG$_1$ (6).[3] Despite an estimated 1,000-fold excess of these proteins no inhibition of IgE binding was observed.

*Evaluation of Number of Binding Sites for IgE.* When increasing amounts of [125I]IgE were added to a given number of cells the number of cell-associated counts increased (open squares, Fig. 1). When a 100-fold excess of unlabeled IgE was mixed with the [125I]IgE before the addition of the cells very little radioactivity was present in the cell pellet at low doses of [125I]IgE ($\leq 3$ µg/ml). With higher doses of [125I]IgE somewhat more radioactivity was associated with the cell pellet even in the presence of the unlabeled IgE (open triangles, Fig. 1). Subtraction of the counts found with unlabeled IgE present from the counts found in the absence of unlabeled IgE yields the net specifically bound IgE (filled circles, Fig. 1). As can be seen from these data the slope of the least squares line is not significantly different from zero over the seven- to eightfold range of IgE added.

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[3] A more complete analysis of the binding of rat IgG proteins to the RBL-1 cells is underway.

TABLE I

*Inhibition of [$^{125}I$]IgE (IR 162) Binding by Unlabeled IgE*

| Inhibitor | Percent of total IgE | Percent inhibition of [$^{125}I$]IgE (IR 162) binding* |
|---|---|---|
| None | 0 | 0 |
| IgE (IR 162) | 62 | 58 |
| IgE (IR 162) | 83 | 80 |
| IgE (IR 162) | 94 | 93 |
| IgE (IR 162) | 98 | 97 |
| IgE (IR 162) | 99.5 | 98 |
| Reaginic rat serum | 75† | 67 |
| 10% Normal rat serum | <0.6§ | −7 |

* Measured at 90 min in an incubation mixture containing 0.98 μg [$^{125}I$]IgE (uncorrected) and 1.3 × 10⁶ cells. Temperature, 37°C.

‡ IgE was determined by a radioimmunoassay method described elsewhere (10). Its binding activity to RBL-1 cells was not reassessed but had been ~80% some months earlier (4).

§ Young rat serum similarly obtained had less than 100 ng/ml (10).

Initially, when different cultures were compared, considerable variability in the amount of IgE bound/cell was observed. A detailed analysis showed this phenomenon to be related to changes in the number of cell receptors during the cell cycle and culture growth.[4] Some typical results are given in Table II along with other data. For most of the studies presented here, cells from stationary phase cultures were used and the number of receptors was analyzed in each experiment.

*Kinetics of IgE-RBL-1 Cell Association.* [$^{125}I$]IgE was added to the RBL-1 cells and the rate of binding examined. At very early times the initial rates were constant. Some typical data are illustrated in Fig. 2. In this experiment the IgE concentration was held constant while the receptor concentrations differed by a factor of ∼ 2. The relative initial rates were linearly related to the receptor concentration; the ratio Δ rate/Δ cell concentration equaled 1.03 (Fig. 2) and 0.98 in another experiment. Similar results were obtained when the receptor concentration was held constant and the initial IgE concentration varied two- or fourfold. More complete binding curves from such an experiment are illustrated in Fig. 3.

These results suggested that the binding was by a simple bimolecular reaction: IgE + receptor → complex. The initial rate of such a reaction, $v_o$, is governed by the relationship:

$$v_o = k_1 (IgE_o) (R_o)$$

where $k_1$ equals the forward rate constant, $IgE_o$ the initial IgE concentration, and $R_o$ the initial receptor concentration. $IgE_o$ was measured directly and $R_o$ determined from the amount of IgE bound after prolonged incubation

[4] Isersky, C., Metzger, H., and D. Buell. 1974. Cell cycle-associated changes in the receptors for IgE during growth and differentiation of a rat basophilic leukemia cell line. Manuscript submitted for publication.
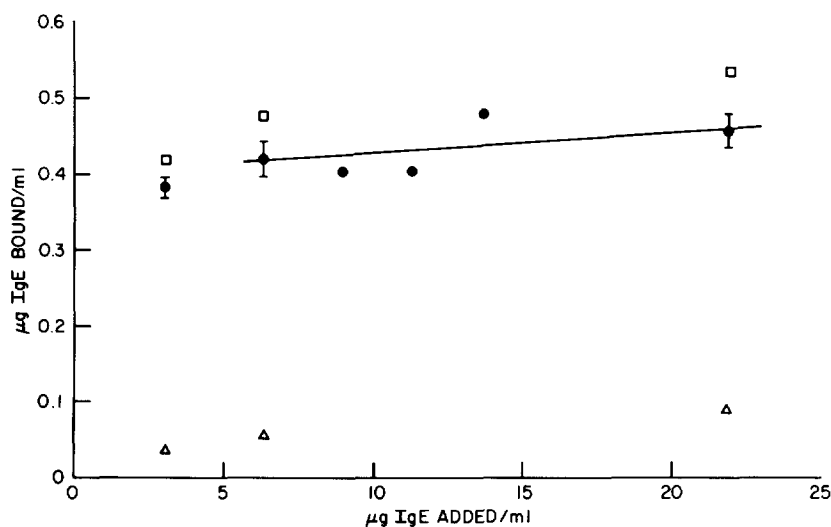
Fig. 1. Saturation of RBL-1 binding sites. $1.1 \times 10^6$ RBL-1 cells were incubated with varying concentrations of [$^{125}$I]IgE at 37°C in 1 ml. Duplicate samples had a 130-fold excess of unlabeled IgE added in addition. Bound [$^{125}$I]IgE was assayed at three time points between 1-2 h. Some concentrations were studied in triplicate and for these both the gross counts (no unlabeled IgE) (□), background counts (excess unlabeled IgE) (△), and net counts (gross minus background) (●), are shown. Where only single assays were performed only the net counts (●) are shown. The error bars indicate standard errors of the mean. The line of least mean squares (with appropriate weighting for the triplicate points) is shown.

(Methods). Several determinations of $k_1$ derived from initial rate studies are collected in Table II. It is apparent that there was reasonably good correspondence between the calculated values of $k_1$ despite considerable variations in cell source and the concentrations of receptors and IgE used. The more reliable experiments are those in which (a) very early points were collected, (b) the reaction was quenched at appropriate times with excess unlabeled IgE, (c) where the counts in control tubes incubated with excess unlabeled IgE at the start of the reaction were subtracted, and (d) under conditions where the experimentally determined receptor concentration closely approximated the calculated value (Methods). A representative experiment is illustrated in Fig. 4. It can be seen that the least squares line does not pass through the origin although the y intercept represents only 3.6% of the total IgE bound at equilibrium. In any case since this small unexplained "background" exhibited no time dependence it can have no effect on the estimation of $k_1$ which is calculated from the slope of the line. The average $k_1$ calculated from such experiments is $9.6 \times 10^4$ $M^{-1}$ $sec^{-1}$.

The rate of binding is sensitive to the incubation temperature. Fig. 5 presents results on the temperature dependence of $k_1$, in the form of an Arrhenius plot. Log $k_1$ is plotted vs. $1/T$ where $T$ is the temperature in degrees Kelvin. Since $2.3 \log k_1 = E_a/RT$, where $R$ is the gas constant and $E_a$ the activation energy, the latter can be calculated from the slope of the line and equalled 7.8 kcal/mol.

To study the sensitivity of the rate of binding to pH, samples of media were adjusted to various pH's between 6 and 8 at room temperature, [$^{125}$I]IgE was
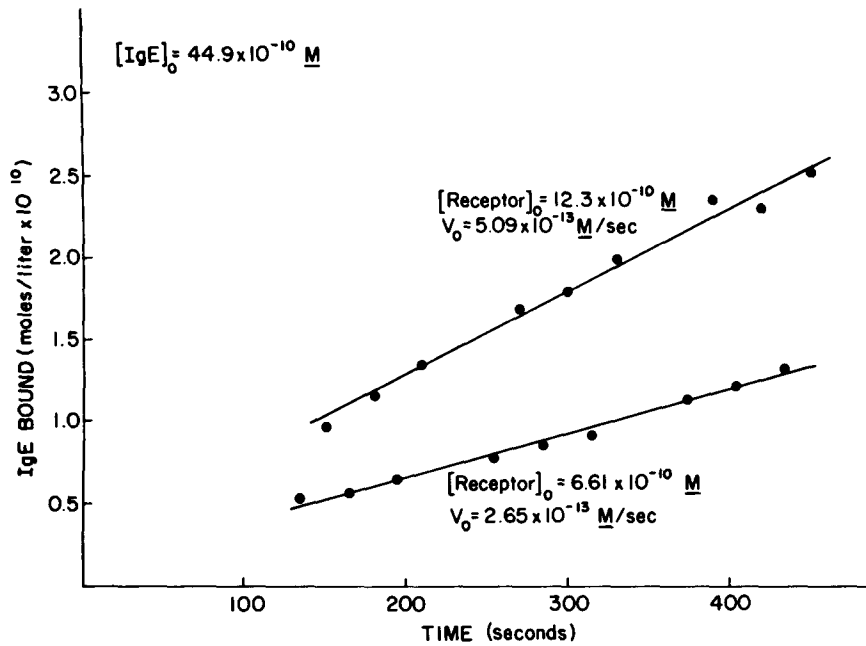
FIG. 2.   Dependence of initial rate of binding on receptor concentration. 1.15 $\mu$g/ml [$^{125}$I]IgE was incubated with either 1.2 $\times$ 10$^6$ (bottom) or 2.26 $\times$ 10$^6$ (top) RBL-1 cells/ml at 37°C. Total vol, 2.0 ml. Samples were periodically assayed for cell bound IgE. Lines of least mean squares for the data points are shown. The initial receptor concentration was estimated by determining the amount of IgE bound under these conditions at equilibrium and making a correction for incomplete saturation (Methods).
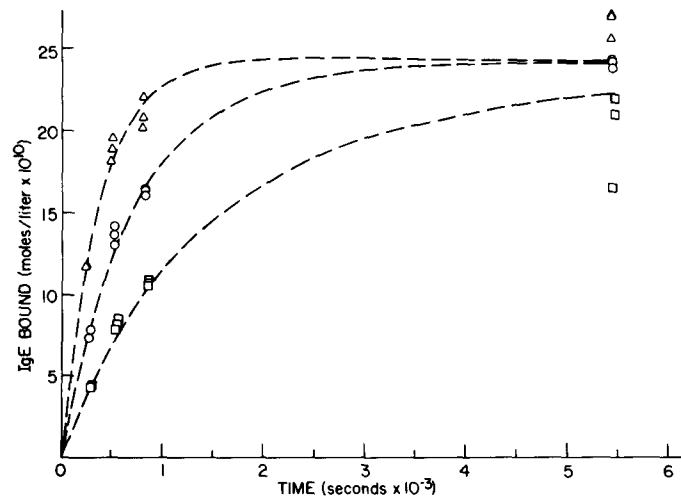


FIG. 3.   Dependence of initial rate of binding on IgE concentration. 0.99 $\times$ 10$^6$ RBL-1 cells were incubated at 37°C with 1.15 $\mu$g ($\square$), 2.33 $\mu$g ($\bigcirc$), or 4.48 $\mu$g ($\triangle$) of [$^{125}$I]IgE. Bound [$^{125}$I]IgE was determined periodically; total vol, 1 ml. The dashed lines indicate the theoretical curves for $K_A$ = 6 $\times$ 10$^9$ M$^{-1}$ and $k_1$ = 1.3 $\times$ 10$^5$ M$^{-1}$ sec$^{-1}$. In this experiment the samples were not quenched which may account for the $k_1$ being falsely high. Nevertheless, the results are consistent with the rate of binding being linearly related to the IgE concentration.

TABLE II

*IgE Binding Characteristics of RBL-1 Cells*

| Exp. | Type of culture* | Age of culture | Density at time of harvest | Via-bility | Receptors/cell ($\times 10^{-6}$) | Concentrations used for experiments | | $k_l$ |
|------|------|------|------|------|------|------|------|------|
| | | | | | | Recep-tors | IgE | |
| | | $h$ | *Cells/ml* ($\times 10^{-6}$) | | | $(M \times 10^9)$ | $(M \times 10^9)$ | $(M^{-1} sec^{-1}$ $\times 10^{-5})$ |
| 1 | F | 44 | 0.56 | 92 | 0.33 | 1.23 | 4.5 | 0.92 |
| | | | | | | 0.66 | 4.5 | 0.89 |
| 2 | F | 68 | 0.69 | 95 | 0.28 | 1.29 | 4.4 | 1.2 |
| | | | | | | 0.72 | 4.4 | 1.2 |
| 3 | S | 48 (0) | 1.3 | 99 | 0.85 | 1.9 | 11.1 | 0.88 |
| 4 | S | 96 (19) | 2.1 | 89 | 1.03 | 2.7 | 11.2 | 1.10 |
| 5 | S | 72 (24) | 0.70 | 94 | 1.12 | 2.7 | 11.2 | 0.69 |
| 6 | S | 101 (48)† | 1.6 | 97 | 1.46 | 2.5 | 5.6 | ~1.3§ |
| | | | | | | 2.5 | 11.4 | ~1.3 |
| | | | | | | 2.5 | 22 | ~1.3 |

* F, Falcon flasks; S, spinner cultures.

† Numbers in parenthesis refer to the approximate time in h that the cell density had remained constant at the time the cells were used.

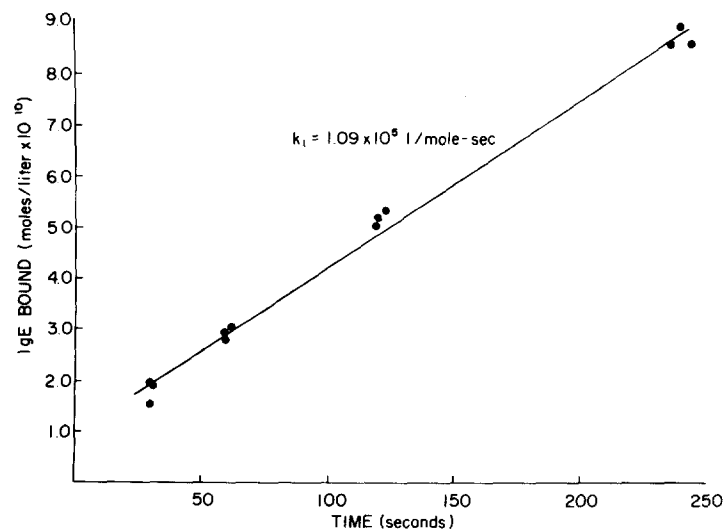§ No data from very early in the reaction. See Fig. 3.



FIG. 4.  Initial rate of IgE binding. 1.42 ml of a mixture containing 3.04 μg [$^{125}$I]IgE/ml and 1.56 × 10⁶ RBL-1 cells/ml was incubated at 37°C. Portions were added to a 50-fold excess of unlabeled IgE at the indicated times and shortly thereafter assayed. Control tubes contained a 100-fold excess of unlabeled IgE at the start of the reaction. The data shown are the net values.
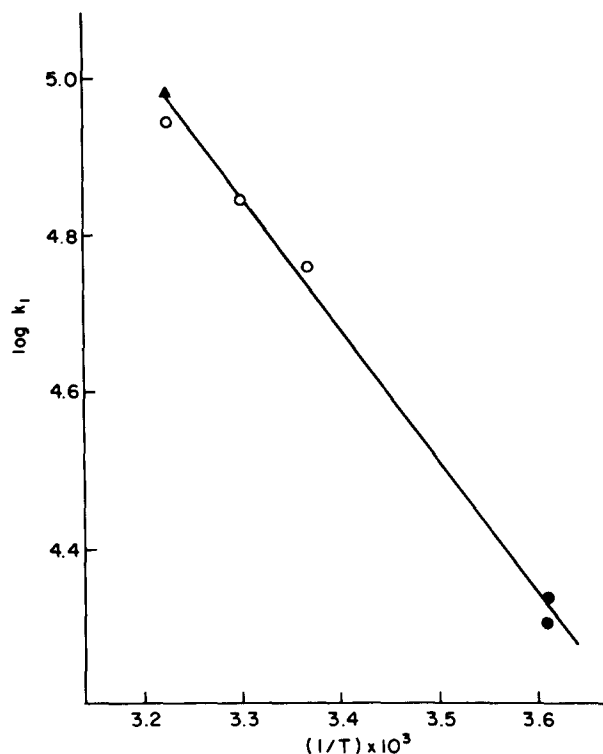
Fig. 5. Temperature dependence of the binding reaction. $k_1$ determinations from two experiments (O and ●) as well as the average of the most reliable data at 37°C (▲) are shown. The line of least mean squares is shown for log $k_1$ vs. the reciprocal of the absolute temperature.

added to 0.84 μg/ml and after equilibration at 37°C, RBL-1 cells were added to a concentration of 2.3 × 10⁶ cells/ml. Portions of each incubation mixture were removed periodically for analysis of cell-bound IgE and the values were expressed as a ratio to the mean for all samples at each time point. In this experiment the samples were not quenched and no corrections for noninhibitable binding (≤5%) were made. The results listed in Table III show that no systematic variation was observed.

During the course of these studies a variety of incubation media were tested (all within the pH range 7.1-7.6): modified Tyrode's buffer with 0.1% gelatin, Tris-A (with 0.03% human serum albumin), RPMI 1640 containing 4 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin with or without 10% fetal calf serum. The rate in Tyrode's buffer was reproducibly a few percent less but no consistent variations were seen otherwise.

The effect of divalent cations was studied with cells suspended in modified Tyrode's buffer containing 0.1% gelatin. The results are given in Table IV A. Higher concentrations of $Ca^{++}$ and $Mg^{++}$ depressed the rate of binding without a detectable affect on cell viability.

The addition of 4 mM EDTA alone had little effect. However, in the presence of 4 mM EDTA, increasing concentrations of $Ca^{++}$ or $Mg^{++}$ were associated with a pronounced dose-related decrease in the rate of binding. In 4 mM EDTA-4 mM

$Ca^{++}$ cell viability was reasonably maintained over the 1 h period of the experiment but dropped markedly (11–22%) in a similar experiment when cells were examined at 2 and 3 h. In 4 mM EDTA-8 mM $Ca^{++}$ viability dropped 30% in 2–3 h. For 4 mM EDTA-8 mM $Mg^{++}$ viability dropped 10% in 2–3 h.

Several other additives have been reported to affect the degree of sensitization of basophils and/or mast cells. Their effects on the rate of IgE binding to the RBL-1 cells are indicated in Table IV B. High concentrations of phosphatidyl serine caused some increase in the binding but heparin and $Al_2(SO_4)_3$ produced no change in the rate and equilibrium values of IgE binding.

*Kinetics of IgE-RBL-1 Cell Dissociation.* Cells were incubated with [125I]IgE and at an appropriate time $(t_o)$ a 100-fold excess of unlabeled IgE was added. The

TABLE III

*Relative Rates of Binding of IgE to RBL-1 Cells at Different pH's*

| Time of sampling | Saturation | pH: 6.0 | 6.4 | 6.8 | 7.2 | 7.6 | 8.0 |
|---|---|---|---|---|---|---|---|
| *min* | % | | | | | | |
| 5 | 20 | 0.86 | 0.94 | 1.06 | 0.96 | 1.03 | 1.15 |
| 17 | 42 | 0.94 | 0.95 | 1.08 | 0.99 | 0.98 | 1.06 |
| 30 | 55 | 0.96 | 0.96 | 1.13 | 0.98 | 0.97 | 0.99 |
| 90 | 80 | 1.01 | 0.97 | 1.12 | 0.88 | 1.00 | 1.01 |
| Average | | 0.94 | 0.96 | 1.10 | 0.95 | 1.00 | 1.05 |

TABLE IV A

*Effect of Various Additives on IgE Binding*

| Exp. | Additive | Concentration | Relative binding at: | | Viability |
|---|---|---|---|---|---|
| | | | 15 min | 61 min | |
| | | *mM* | % | % | % |
| 1 | None | — | 100* | 100* | 99 |
| | $Ca^{++}$ | 2 | 92 | 95 | 99 |
| | | 4 | 88 | 91 | 99 |
| | | 8 | 80 | 85 | 98 |
| | $Mg^{++}$ | 2 | 93 | 70 | ND |
| | | 4 | 89 | 64 | ND |
| | | 8 | 82 | 62 | 98 |
| | $Ca^{++}$ and $Mg^{++}$ | 2, 2 | 86 | 70 | ND |
| 2 | EDTA | 4 | 98 | 96 | 99 |
| | EDTA (4 mM) plus $Ca^{++}$ | 2 | 78 | 88 | 97 |
| | | 4 | 43 | 46 | 93 |
| | EDTA (4 mM) plus $Mg^{++}$ | 2 | 85 | 97 | 92† |
| | | 8 | 44 | 53 | 86† |

* Percent saturation of cell receptor sites was 44 and 89 at 15 and 61 min respectively.
† Average viability at 2–3 h.

incubation was continued and samples were analyzed periodically for cell-bound radioactivity. It was anticipated that any radioactive IgE which dissociated would not rebind because of the competition from the large excess of unlabeled IgE. In control tubes no unlabeled IgE was added. The results of such an experiment are presented in Fig. 6. It is apparent that the cell-bound [$^{125}$I]IgE in the control tubes (squares) remained constant over the ~ 7-h period of the experiment. However, those cells to which excess unlabeled IgE had been added showed a progressive loss in bound IgE. The decay curve was linear when the log of the residual cell-bound radioactivity was plotted versus time (Figs. 6, 7 A and B). The initial concentration of receptor-IgE complex could be varied sixfold (constant percent site saturation) without an observable change in the normalized rate of decay (Fig. 6). Similarly the rate of decay was the same when the percent saturation with [$^{125}$I]IgE at $t_o$ was varied eightfold (not shown).

These results are consistent with a simple first order decay process:

$$\text{complex} \rightarrow \text{IgE} + \text{receptor}.$$

The backward rate constant for such a reaction can be calculated from the relationship:

$$\text{rate} = k_{-1} [\text{complex}]$$

TABLE IV B

*Effect of Various Additives on IgE Binding\**

| Additive | Concentration | Relative binding at: | | Viability |
| --- | --- | --- | --- | --- |
| | | 20 min | 60 min | |
| | | % | % | % |
| None | — | 100§ | 100§ | 99 |
| Phosphatidyl serine | 12.5 | 105 | 112 | ND |
| (µg/ml) | 50 | 116 | 114 | ND |
| | 200 | 135 | 121 | 93, 98 |
| Heparin (µg/ml) | 3.3 | 101 | 99 | ND |
| | 10 | 110 | 97 | ND |
| | 30 | 102 | 101 | ND |
| Al$_2$(SO$_4$)$_3$ (M × 10$^7$) | 0.7 | 110 | 101 | ND |
| | 7.0 | 108 | 97 | ND |
| | 70 | 105 | 96 | ND |
| Human serum albumin | 0.3 | 93 | 99 | ND |
| (mg/ml) | 0‡ | 47 | 50 | 84, 91 |
| | 0.03‡ | 94 | 88 | 93 |
| | 0.3‡ | 98 | 99 | 94 |
| | 3.0‡ | 102 | 102 | ND |

\* Unless indicated otherwise incubation was in Tyrode's buffer with 1 mg/ml gelatin.
‡ Medium was Tris buffer without added protein.
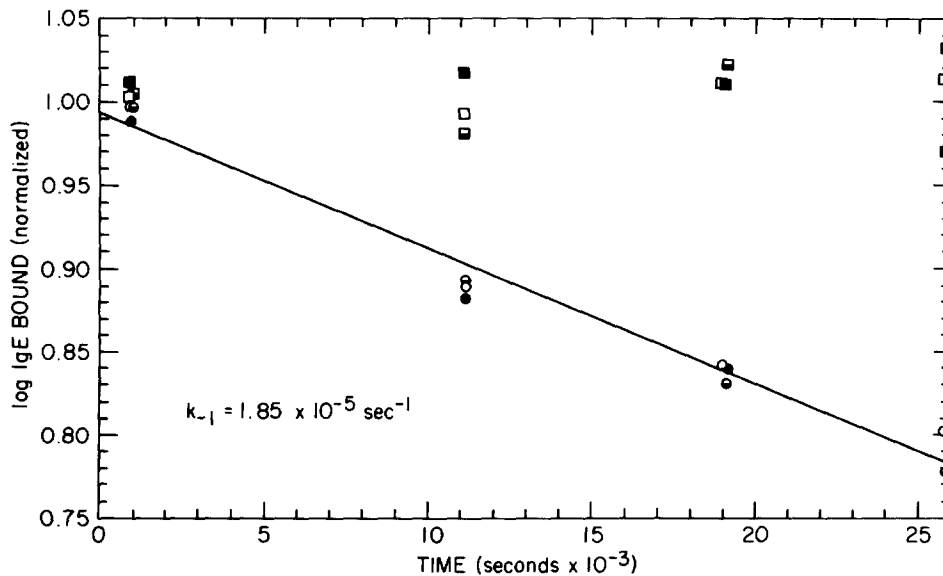§ Percent saturation of cell receptor sites was 50 and 87 at 20 and 60 min respectively.

FIG. 6.    Dependence of IgE dissociation rate on IgE-receptor complex concentration. 2.44 ×
10⁶ RBL-1 cells/ml were incubated with 2.47 μg/ml of [¹²⁵I]IgE for 35 min at 37°C. At $t_o$ a
112-fold excess of unlabeled IgE (circles) or buffer (squares) was added to each of three aliquots
some of which were then diluted. Final cell concentrations: 2.35 × 10⁶ (filled symbols), 9.62 ×
10⁵ (half-filled symbols), and 3.88 × 10⁵ (open symbols) cells/ml. Cell-bound [¹²⁵I]IgE was
determined at the times indicated. The data have been normalized to compensate for the
differences in the bound IgE concentration at $t_o$. The line of least mean squares for the
dissociation data is shown. Cell viability at the start and end of the experiment was 89 and
90% respectively.

or simply from the slope of the line (which equals $-k_{-1}/2.303$) when the data are
plotted as in Figs. 6 and 7. Since the reaction was made "irreversible" by the
addition of excess unlabeled IgE, this relationship is valid during the entire
reaction and not just initially. The average value of $k_{-1}$ from our more reliable
experiments (those in which 25-35 points were measured) was $1.6 \pm 0.4 \times 10^{-5}$
$\text{sec}^{-1}$. Cells harvested at 20-100 h and bearing $0.8$- $1.5 \times 10^6$ receptors per cell
gave equivalent values.

Fig. 7 represents the results of an experiment in which the temperature
dependence of dissociation was studied. The experiments at 37°C and 24°C (Fig.
7 A and B) again showed a constant amount of cell-bound radioactivity in the
absence of added unlabeled IgE while in the presence of the latter a progressive
loss was observed. At 4°C (Fig. 7 C) the results were more complicated. A
progressive loss in cell-bound [¹²⁵I]IgE even in the absence of added unlabeled
IgE was observed after about 3 h of incubation. During the first 3 h the dis-
sociation rate was consistent with a first order decay but after that time the
dissociation rate was difficult to assess in view of the changes in the control tubes.
The $k_{-1}$ derived from the 4°C data must be considered an approximate value
only.

The apparent loss of receptors observed in the control tubes at 4°C was also
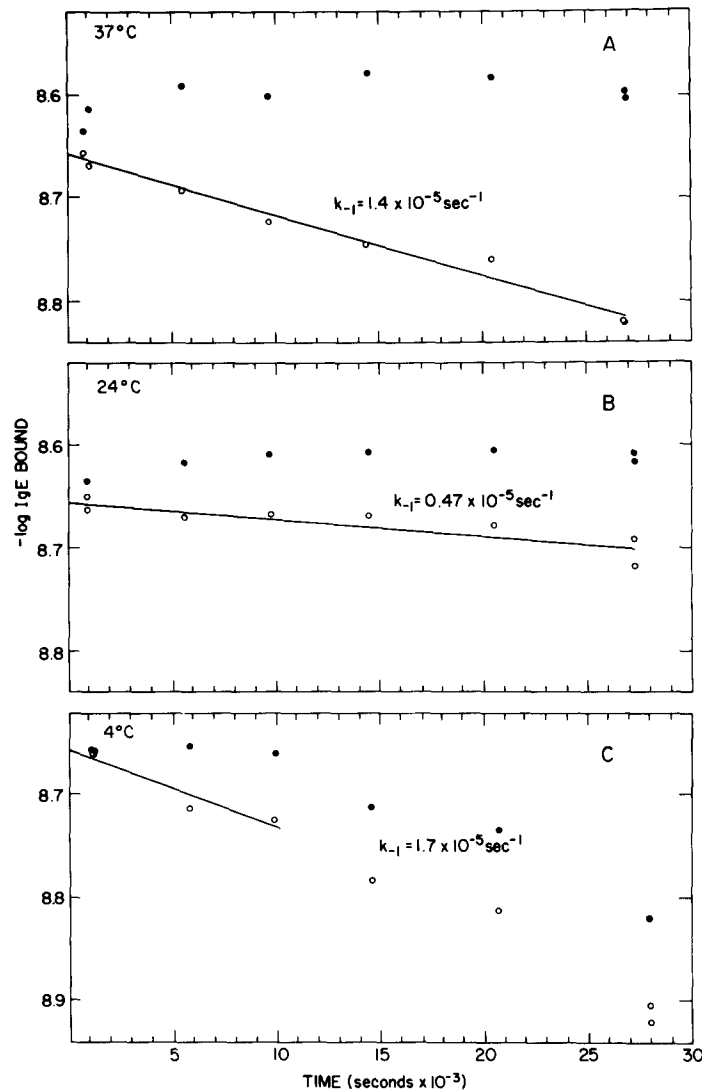observed at higher temperatures. Particularly in less complete media (e.g.

Fig. 7.   Temperature dependence of IgE-receptor dissociation. Three tubes each containing 1.6 × 10⁶ RBL-1 cells/ml and 2.25 μg/ml [¹²⁵I]IgE were preincubated for 35 min at 37°C. To duplicate samples of each of the three either a 114-fold excess of unlabeled IgE (open symbols) or buffer (filled symbols) was then added at $t_o$. The appropriate pairs were then incubated at 37°C (A), 24°C (B) or 4°C (C). Triplicate specimens were removed from each tube at the indicated times and assayed. Lines of least mean squares have been drawn through those data for which the appropriate control tubes showed a constant amount of bound IgE.

Tyrode's buffer) such a loss was quite regularly seen even at 37°C and as early as 4 h of incubation.[5] Even in the more complete media a small (~10% loss) loss of

[5] Since this manuscript was completed we have obtained direct evidence for shedding of particulate material containing IgE receptors from the cells under unfavorable growth conditions. D. Carson, A. Kulczycki, Jr., and H. Metzger. Interaction of IgE with rat basophilic leukemia cells. III. Release of intact receptors on cell-free particles. J. Immunol. In press.

receptor activity was seen at 8 h and sometimes earlier. We shall discuss the implications of these findings later. Here we wish primarily to indicate that we were therefore constrained to follow the dissociation for only a limited time—a time too short to permit us to observe more than 30-40% of the bound IgE dissociate.

The rate of dissociation is markedly enhanced at acid pH's. Fig. 8 illustrates an experiment in which $2.2 \times 10^6$ cells/ml were first incubated with 8.8 $\mu$g/ml IgE at 37°C for 1 h. The cells were pelleted, washed, and resuspended in Tyrode's buffer at $8 \times 10^6$ cells/ml. 50 microliters ($4 \times 10^5$ cells) were added to duplicate tubes at 0°C containing 2 ml of either a Na acetate buffer (50 mM acetate, 85 mM NaCl, 5 mM KCl, 10 mM EDTA, and 0.03% human serum albumin, pH's 3-5.6) or a Tris-phosphate buffer (50 mM Tris, 36 mM phosphate, 85 mM NaCl, 5 mM KCl, 0.03% human serum albumin and 10 mM EDTA, pH 6.4) or Eagle's medium with 10 mM Hepes buffer and 10% fetal calf serum (pH 7.2). After 12 min and 1 h, specimens were centrifuged and the pellet assessed for cell viability and the supernate for released [$^{125}$I]IgE. As can be seen in Fig. 8 (only the 1 h data are shown) substantial loss of bound IgE occurs at pH's below 4 with only modest losses in cell viability. Shorter times and a glycine-HCl buffer, pH 3.0, gave even more favorable results which are detailed in Table V A. Almost complete loss of cell-bound IgE occurs in 1 min without significant loss in cell viability. The data in Table V B document that the cellular receptors remained intact after such an
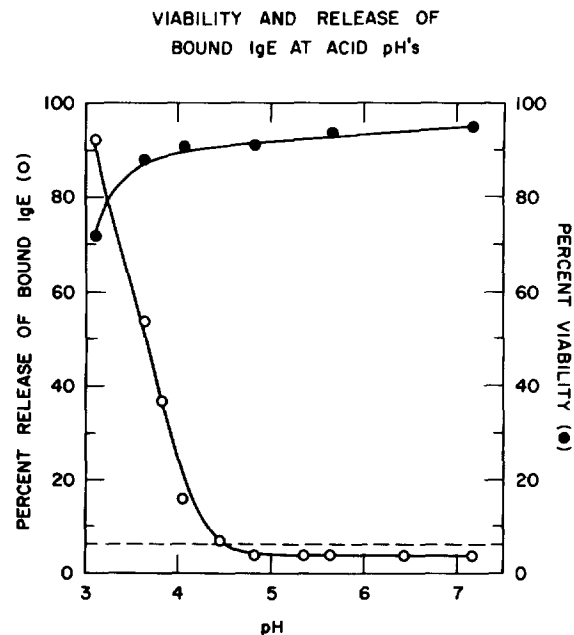


VIABILITY AND RELEASE OF
BOUND IgE AT ACID pH's

FIG. 8.   10 ml containing $2.18 \times 10^6$ RBL-1 cells/ml and 8.8 $\mu$g/ml [$^{125}$I]IgE were incubated at 37°C for 1 h. Cells were pelleted, washed, and resuspended in 2.5 cc of Tyrode's buffer. 50 $\mu$l of this suspension ($4.35 \times 10^5$ cells) were added to 2 ml of various buffers (see text) at 0°C for 1 h. After centrifugation, supernates were assayed for radioactivity and pellets for cell viability. Dashed line represents expected dissociation at 4°C in 1 h calculated from Fig. 7 C.

exposure to acid. Cells incubated at pH 3 for 1 min at 0°C were mixed with [$^{125}$I]IgE at neutral pH and 37°C in parallel with untreated cells. Neither the rate of binding nor the equilibrium value of bound IgE was substantially different for the treated and untreated cells.

## Discussion

*Qualitative Aspects of IgE-RBL-1 Cell Interaction.* In this paper and elsewhere we have established a number of characteristics of the interaction of rat IgE with the RBL-1 cells. (*a*) The reaction is specific: the sites are clearly saturable, rat and mouse IgE bind to the cells but, as shown either by direct binding studies or inhibition of rat IgE binding, a variety of other proteins (reduced and alkylated rat IgE, heated rat IgE, rat IgM, rat IgA, rat IgG$_1$, a mixture of normal rat serum proteins, human IgE, rabbit IgG, and porcine insulin) do not. (*b*) The IgE is bound to the surface of the cell: it is available to rabbit anti-light chain or anti-IgE binding, it can be visualized to be surface-bound by fluorescent antibody (2) or radioautographic techniques (1), its surface distribution can be shifted (2), and within a minute it can be quantitatively released under conditions where cell viability and receptor function are unimpaired (Table V A, B). (*c*) The binding is reversible; rapid dissociation without loss of receptor function can be demonstrated as just noted and slower but readily detectable dissociation can be observed under more physiological conditions (Figs. 6, 7).

*Mechanism and Quantitative Aspects of IgE-RBL-1 Cell Interaction.* The kinetic analyses reported here indicate that the binding of rat IgE to the RBL-1 cells can be most simply described by the reaction:

$$\text{IgE} + \text{receptor} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{receptor} - \text{IgE complex.}$$
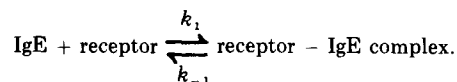
The concentration-dependence studies clearly showed that the association is first order with regard to both the IgE and receptor concentration, and precise

TABLE V A

*Dissociation of Bound [$^{125}$I]IgE at pH 3.0 at 0°C\**

| Time at acid pH | Viability | Dissociation of IgE |
|---|---|---|
| *min* | % | % |
| 0.25 | 97 | 69 |
| 0.50 | 99 | 75 |
| 1.00 | 97 | 86 |
| 4.00 | 89 | 90 |
| 16.00 | 57 | 89 |

\* All results are given in relative numbers compared to controls which were incubated in Eagle's medium at pH 7.1. Such cells had no detectable loss of bound IgE over the ~16 min of the experiment and were 96% viable.

TABLE V B

*Rate of Binding of [$^{125}I$]IgE to RBL-1 Cells Pretreated for 1 Min at pH 3.0 at 0°C\**

| Time | IgE bound (vs. control) |
|------|-------------------------|
| *min* | |
| 3.7 | 0.97 |
| 7.8 | 0.98 |
| 12.7 | 0.97 |
| 130‡ | 0.93 |

\* At the end of the pretreatment the cell mixture was neutralized, the cells washed, and suspended in Eagle's Medium at 37°. [$^{125}I$]IgE was then added.

‡ At the end of the experiment the acid treated cells were 85% viable compared to 93% for the untreated cells.

plots of the initial velocities failed to disclose any sigmoid features which would be indicative of positive cooperative phenomena. The value of $k_1$ is of the same magnitude as the values described for certain antibody: protein-antigen interactions (11). The dissociation reaction was studied by first filling the cell receptor sites with [$^{125}I$]IgE to a variable extent, and then adding 100-fold excess of unlabeled IgE. The concentration of the latter was sufficient to (a) saturate the unfilled sites and (b) prevent any released [$^{125}I$]IgE from rebinding. Thus, in all experiments dissociation was studied under conditions where the cell receptor sites for IgE were fully occupied. Under these conditions the relative rate of IgE release was independent of the receptor-IgE complex concentration consistent with a first-order decay process. Theoretically one could study the dissociation by simply diluting the system, but the absolute value of the $K_A$ combined with our finding that the cells fared poorly outside the concentration range of ~ $3 \times 10^5$ to $1-2 \times 10^6$ over prolonged periods, made such experiments impractical.

We wish to emphasize that the value of the dissociation rate constant, $k_{-1}$, which we determined here is a maximum value. This is so for the following reason. It is not impossible, indeed not unlikely, that there is a constant turnover of receptors—shedding of receptor molecules being balanced by synthesis of new receptors. If this occurred, our "control" specimens would still have shown a steady level of cell bound IgE (see Figs. 6, 7 A, 7 B) and the receptor shedding would be indistinguishable from receptor-IgE dissociation in the "experimental" samples. In the experiments described here we deliberately avoided using cells from the lag phase of culture growth—cells which show a relatively rapid net loss of receptor activity but which are otherwise healthy.[4] However, some receptor loss can be observed after prolonged periods even with cells from stationary cultures under the conditions used here; a loss accentuated by incubation in simple buffers or low temperatures (Fig. 7 C). Rather than construct elaborate (and never completely unambiguous) control experiments to gauge the possible contribution of receptor turnover to our results, we hope ultimately to reexamine the binding with cell-free receptor preparations.[5]

Since our estimate of $k_{-1}$ is a maximal value, $K_A$ calculated from $k_1/k_{-1}$ is a

minimal value. Its magnitude, $\sim 6 \times 10^9$ M$^{-1}$, is substantial and is consistent with the observation that the cells can be repeatedly washed without significant loss of cell bound IgE (1). All the estimated values of $k_1$ in this paper are based on an assumed mol wt of $2 \times 10^5$ for rat IgE. If the true value is somewhat different, e.g. 10% lower (3), the estimates of $k_1$ and $K_A$ would have to be lowered correspondingly.

We did not perform any equilibrium experiments to determine $K_A$ directly for the following reason. Given the estimated values of $K_A$, $k_1$ and $k_{-1}$, and the constraints of feasible cell concentrations, sample size for analysis, and errors in the cell bound IgE determinations, one can calculate that only a limited range of receptor site occupancy could be studied. Even under such conditions, prolonged incubations would be required to achieve equilibrium. We were convinced that such experiments would be unlikely to yield substantially more precise and accurate values, than those already determined.

There are no equivalent direct binding data on the interaction of normal human basophils with human IgE or of normal rat mast cells with rat IgE, the systems most frequently studied, to which we can easily compare our results. Values of $K_A$ have been estimated indirectly for the IgE-human basophil interaction. The value of $K_A$ for 12 of 13 preparations studied was reported as 0.1–1.3 $\times 10^9$ M$^{-1}$ and for one as 1.2 $\times 10^{10}$ M$^{-1}$ (12). Whether this represents a true variability or a difficulty in the indirect analysis of cell-bound IgE which was required for those experiments is uncertain. In the same work qualitative evidence for the dissociability of the IgE-receptor complex was presented. A substantial dissociation of IgE (as measured by reduction in grain counts by autoradiography) appears to have occurred in 40 min at 0°C. This is considerably faster than we would have anticipated from our own data but it is difficult to evaluate this discrepancy. There are no similar data for rat mast cells. We have previously remarked that the capacity of RBL-1 cells to absorb skin fixing passive cutaneous anaphylaxis (PCA) antibodies from reaginic sera (1) appeared to be roughly equivalent to the value reported for rat mast cells (13). Similarly the conditions ordinarily used to "sensitize" rat mast cells for histamine release are not inconsistent with our kinetic data if one makes several rather gratuitous assumptions. However, Carson et al. have recently determined that the IgE protein level can vary widely in serums having similar PCA titers[6] so that we now feel such comparisons of diverse data are meaningless. Direct experiments using similar techniques and IgE preparations are the only appropriate way to evaluate the two types of cells.

Similarly, with regard to the number of receptors for IgE no direct binding data for human basophils or rat mast cells are available for comparison. The estimates for human basophils (12) were developed with a highly sophisticated but elaborate and indirect procedure and for a variety of reasons we are uncertain about the accuracy of the values calculated (Avg. 4.2 $\times 10^4$, range 3–8.5 $\times 10^4$/cell). In any case the number of receptors/cell might be a much more variable characteristic of different cell types and, as we have documented here (Table II)

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[6] Carson, D., H. Metzger, and K. J. Bloch. 1974. Serum IgE levels during the potentiated reagin response to egg albumin in rats infected with *Nippostrongylus brasiliensis. J. Immunol.* In press.

and more completely elsewhere (footnote 4), can be highly variable even for the same cells.

*Influence of Extrinsic Factors on Binding.* We studied the effect of a variety of extrinsic factors on the IgE-RBL-1 cell interaction for two reasons: to check that small variations in the incubation conditions did not produce major changes in the binding and to be able to compare the findings on our system with published results on other systems. It should be noted, however, that in the studies on other systems the effect on IgE binding was perforce measured indirectly. That is, cells were incubated with reaginic sera at various pH's, temperatures, etc. under conditions where histamine release did not occur (sensitization). Subsequently the cells were incubated under conditions for optimal histamine release. The extent of the latter was assumed to reflect the extent of IgE binding during sensitization. The assumption is reasonable but it is difficult to rule out other effects conclusively. It should also be noted that we are studying the binding of purified IgE whereas reaginic sera were used by others. The presence of other serum proteins could have affected the results.

The Arrhenius plot of the temperature dependence of the association of IgE to the RBL-1 cells was linear, suggesting that no changes in the mechanism of binding were occurring over the temperature range studied. The calculated value of the activation energy was 7.8 kcal/mol. Estimates of this parameter were made by Levy and Osler (14) for the human reagin-basophil interaction. Their value of 9 kcal/mol (15) is similar to ours.[7] As noted in Results our estimate of $k_{-1}$ at 4°C is suspect so that the unusual temperature dependence of $k_1/k_{-1}$, i.e. $K_A$ (Table VI) may be spurious. Again, cell-free receptor preparations may resolve this issue.[5]

No significant effect of pH on $k_1$ and $k_{-1}$ was observed in the range 6–8 (Table III). Levy and Osler (14), using human basophils, and Bach et al. (16), using rat mast cells, reported marked optima at pH 7.4 and 6.8 respectively. It is uncertain whether the pH effects on sensitization observed by them were directly related to the reagin-cell interaction or to some other factors.

Our results at low pH's closely resemble the findings of Ishizaka and Ishizaka (17) on normal human basophils, and we wish to acknowledge that we were motivated to perform such studies on the basis of their findings which they kindly communicated to us.

With the RBL-1 cells, the simultaneous presence of 4 mM EDTA and 4 mM $Ca^{++}$ caused substantial inhibition of IgE binding which was associated with evidence for a progressive loss of cell viability. The effect of $Mg^{++}$, which itself leads to moderate diminution of binding, is similary enhanced by the presence of EDTA. These effects are quantitatively comparable to the results reported by Levy and Osler on human basophils (14). We did not observe the enhancing effect of $Ca^{++}$ which Bach et al.'s data on rat mast cells implied (16).

Several other additives reported as facilitating reagin binding to human

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[7] The ratio of the slopes in Fig. 2 (Ref. 15) are incorrectly given in the text. The figure shows that the ratios are ∼ 4.4:1.7:0.7 as stated in the original work (14) and from which the value of 9 kcal/mol was correctly calculated by Osler et al. (15).

TABLE VI

*Temperature Dependence of IgE-RBL-1 Cell-Binding Parameters\**

| Temp | $k_1$ | $k_{-1}$ | $K_A$ |
|---|---|---|---|
| °C | $M^{-1}sec^{-1}$ | $sec^{-1}$ | $M^{-1}$ |
| 37‡ | $8.8 \times 10^4$ | $1.4 \times 10^{-5}$ | $6 \times 10^9$ |
| 24 | $5.7 \times 10^4$ | $0.47 \times 10^{-5}$ | $12 \times 10^9$ |
| 4 | $2.1 \times 10^4$ | $(1.7 \times 10^{-5})$§ | $(1.2 \times 10^9)$§ |

\* All data in body of the Table are from a single experiment conducted on a single batch of cells.

‡ Corresponding average values for all our experiments are $9.6 \times 10^4$, $1.6 \times 10^{-5}$, $6 \times 10^9$.

§ As indicated in text the value of $k_{-1}$ and therefore $K_A$ at 4°C may be in error.

basophils, rat mast cells, or both (15, 16) had only trivial effects on the IgE-RBL-1 reaction (Table IV B). Only phosphatidyl serine had a reproducible though still fairly small (20-35%) effect on the IgE binding. However, the experiments which first demonstrated that phosphatidyl serine enhanced histamine release (18) were more consistent with an effect on the later stages of the reaction and not the IgE-binding stage. Indeed the enhancement was seen with non-IgE-mediated histamine release. We have not tested a variety of other phospholipids.

*Concluding Remarks.* Assuming that normal basophils, mast cells, and the RBL-1 cells have similar properties with regard to IgE binding, one can use the quantitative data reported here to begin to evaluate a variety of in vivo phenomena. The PCA reaction can be taken as an example. It is known that skin sites sensitized with reagins may remain reactive for weeks. Our value of $k_{-1}$ yields a half-life of dissociation ($t_{0.5} = ln\ 2/k_{-1}$) of $\sim$ 12 h. Thus in 1 wk the cell-bound IgE should have fallen to $\sim 6 \times 10^{-5}$ of the amount bound originally. Since this is clearly not the case (It has been estimated that the skin-bound IgE has a haf-life of $\sim$ 2 wk [19] one must invoke other considerations (e.g. limited diffusion of the IgE with rebinding to unoccupied sites) as explaining the prolonged sensitization. One may also anticipate the degree to which peripheral blood cells in a particular individual may be saturated with IgE given the level of serum IgE. Using their own values for $K_A$, Ishizaka et al. have discussed such calculations (12). The degree to which cells may be "sensitized" is of course a much more complicated problem which will depend upon the specific IgE antibody: total IgE protein ratio, the number of molecules which must react with antigen to cause cell triggering and so on.

The RBL-1 cells are permitting us to study rigorously the interaction of an immunoglobulin with a cell-bound receptor for the first time. While immediately relevant to the IgE-basophil (mast cell) interaction it may be that such studies will aid in the understanding of other Ig-receptor systems of interest.

## Summary

Previous studies indicated that cultured rat basophilic leukemia cells have surface receptors which bind IgE with high specificity. In this paper we describe some quantitative aspects of the phenomenon. The reaction mechanism appears to consist of a simple reversible binding reaction with $k_1 = 9.6 \times 10^4 \, M^{-1} \, sec^{-1}$ and $k_{-1} \leq 1.6 \times 10^{-5} \, sec^{-1}$ at 37°C. The calculated $K_A$ is therefore $\geq 6 \times 10^9 \, M^{-1}$. The activation energy of binding was found to be 7.8 kcal/mol. The number of binding sites/cell varied between $3 \times 10^5$ to over $1 \times 10^6$. The binding was insensitive to pH's between 6–8 but at pH 3.0 complete dissociation of bound IgE occurred in $\sim 1$ min at 0°C leaving the receptors for IgE intact. $Ca^{++}$ plus EDTA and $Mg^{++}$ plus EDTA produce a fairly marked reduction in binding capacity though these reagents alone produce much smaller effects.

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