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ANNUAL PROGRESS REPORT

A STUDY OF "IMMEDIATE" SENSITIZATION BY ADSORPTION OF ANTIGENS AND ANTIBODIES *IN VITRO*

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PART I

ANAPHYLACTIC AND HEMOLYTIC CROSS-REACTIONS OF ANTI-BOVINE γ-GLOBULIN

ANAPHYLACTIC AND HEMOLYTIC CROSS REACTIONS

OF ANTI BOVINE γ-GLOBULIN

I. INTRODUCTION

During the course of studies in this laboratory on the mechanism of fixation of antiovalbumin a question arose whether the fixation would be affected if another species of antibody were used. Bovine gamma-globulin and the corresponding rabbit antibody were prepared and attempts to sensitize normal cardiac tissues of the guinea pig with anti-bovine gamma-globulin resulted in the appearance of immediate anaphylaxis in the heart. This was characterized by the same sequence of events as had been observed in the usual cardiac anaphylaxis described by Feigen, et al. (1) The sequelae were an increase in heart rate, an increase in the amplitude of contraction, a decline in coronary flow, heart block, and the appearance of histamine in the perfusate. The experiments reported in this communication deal with the nature of the cross-reacting systems involved. Because it was suspected that one of the antigens might have been a species specific hapten, which could exhibit hemolysis in the usual immunological sense, the anti-bovine gamma-globulin was examined for the hemolytic properties on a variety of red cells, and this study concerns itself with the relationship between the anaphylactic effect of the antibody and its hemolytic action as observed in various species of cells.

II. PURPOSE OF EXPERIMENTS

The purpose of the present experiments was to find out whether the anaphylactic activity was resident in the particular antibody produced or whether the reaction was a manifestation of some inherent toxicity of the anti-BGG preparation. If anaphylaxis were due to the antibody, then the normal rabbit gamma-globulin should not produce the effect in question but it should arise only as the result of immunization and one might expect that a closely related antibody, such as anti-BSA, should be without effect in this regard. Another purpose of the experiment was to find out whether the anaphylactic activity was correlated with the hemolytic activity. For this reason we studied the effects of cross-absorption on three variables: 1) immediate anaphylaxis in tissues, 2) hemolysis, and 3) the [illegible] gel-diffusion precipitation reaction. In addition to the foregoing we also attempted to localize the cross-reacting antigens on guinea pig tissue by means of preliminary experiments with isotopically labelled and fluorescently labelled antibodies.

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III. PREPARATION OF ANTI-BGG

Anti-bovine gamma-globulin was prepared by injecting rabbits either with the commercially available product obtained from Armour and Company, which was an alcohol precipitate of pooled bovine plasma, or with an ammonium sulfate-precipitated material prepared in this laboratory by repeated precipitation in the presence of third saturated ammonium sulfate at pH 7.8. The starting material was obtained from a local meat-packing house as defibrinated blood. The antibody was prepared by giving rabbits 11 injections of 20 mg, followed by 7 injections of 10 mg at intervals of 1 day. The animals were rested for 9 days and then bled by cardiac puncture. The blood was permitted to clot, the serum was harvested, and the γ-globulin was obtained by re-precipitation in 1/3-saturated ammonium sulfate, as indicated above. After the final dialysis the protein content was determined by means of the Kjeldahl-Nessler micromethod used in this laboratory. (2) The antibody content of the gamma-globulin was estimated by means of the quantitative precipitin technique. Most of the preparations made during the course of these studies averaged 20% specifically precipitable antibody.

IV. FRACTIONATION

A. ANTIGEN

Both the bovine and the rabbit γ-globulin were separated into three components by being passed through DEAE-cellulose columns, according to the method described by Fahey (3). Glass columns 35 cm long with a cross-section of 1 cm were packed under 10 lbs psi with DEAE-cellulose which had first been treated with 0.2N NaOH and then washed twice with doubly distilled water and twice with 0.02 M phosphate buffer at pH 8.0. Fifty milligrams of purified gamma-globulin was placed on the column and eluted with a phosphate buffer of constantly rising ionic strength. The initial concentration was 0.02 M and this rose to 0.13 M at the end of the elution. Three-milliliter aliquots were collected on a fraction collector and the protein content was estimated with the aid of the DU Spectrophotometer at 278mμ. A typical result is presented in Figure 1. Following this determination, the tubes were pooled according to the three natural groupings as exhibited in Figure 1. Each group was separately pervaporated to approximately 10% of the starting volume and frozen at -20°C. As subsequent material was prepared it was mixed with the preceding samples until sufficient material had been accumulated for extensive testing. When a sufficient quantity of each of the three fractions was available, they were separately purified by re-precipitation in the presence of 1/3 ammonium sulfate. Each fraction was dialyzed until sulfate free and then stored in the deep freeze.

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B. ANTIBODY

The preparation of anti-bovine γ-globulin, normal gamma-globulin, and rabbit antibodies to other antigens was carried out in the same manner as described in the previous section.

V. CHARACTERIZATION

A. IMMUNOLOGICAL

1. Quantitative Precipitation (N:UV Ratio):

In order to facilitate and to accelerate the estimation of protein in the fractions, as well as in the total antibody prepration, a calibration curve relating the optical density at 278 mμ to the nitrogen content was established. In addition to the calibration curve a method was worked out whereby it was possible to determine the composition of two species of protein in a mixture. This was done using the principle developed by Lenni, Feigen, and Le Rosen (4) and by Feigen, Campbell, Sutherland, and Markus (5). A plot of the UV/N slopes against composition was linear. This permitted the composition to be determined from a knowledge of the nitrogen content and the density in the ultra violet

2. Ouchterlony Gel-Diffusion Method:

Gel-diffusion experiments were made according to the original description of Ouchterlony (6), utilizing wells, and also by a modification employing the filter-paper micromethod.

3. Anaphylaxis:

a. Schultz-Dale tests: Schultz-Dale tests were made on isolated ileal strips of the normal guinea pig according to methods previously published from this laboratory. (7), (8), (9). The guts were set up in 4-ml baths and the specific contractions were recorded either on a kymograph or on the CDC oscillograph. The reactions were quantitated by referring the height of the contraction to a calibration curve derived from the logistic transformation of von Krogh as described by Feigen (1).

b. Cardiac anaphylaxis: The experiments on cardiac anaphylaxis were performed with the aid of the perfused Lagendorff heart as described in the same publication (1). After the heart had been removed and attached

to the Anderson perfusion apparatus it was permitted to "stabilize" until records of amplitude, rate, and coronary flow were constant. The anti-bovine gamma globulin was introduced either through the side-arm or the standpipe and records of mechanical performance were taken on the kymograph or the oscillograph. Following the initial challenge the organ was rechallenged with antibody as long as activity remained. From time to time, the organ was tested with histamine to determine whether sensitivity to histamine remained. Perfusate samples were collected at 30-second intervals for the duration of the experiment. These were stored in the deep freeze until they were needed for the estimation of histamine either by bioassay or by the fluorometric method of Shore, Burkhalter, and Cohen as modified in this laboratory (10).

c. Passive cutaneous anaphylaxis: Samples of the several reagents were given intradermally in volumes of 0.05 ml to nembutalized guinea pigs. One hour following the administration of the test doses on the back of the animal 1.0 mg of Evan's Blue was injected intracardially. If no bluing occurred at the site of injection the animals were tested with a specific antigen. At the conclusion of the test, the guinea pigs were killed and the skin of the back reflected for more direct visualization of the reaction.

4. Localization:

a. Isotopic method: Normal guinea pig γ-globulin, radio iodinated by the method of MacFarlane (11), was injected intraperitoneally into normal guinea pigs. The animals were killed, the heart removed, and perfused with Chenoweth's solution for 20 minutes. The experiment was performed by stopping the organ at a reduced temperature, counting, and re-perfusing with various test substances. The results are presented in Table V.

b. Fluorescent antibody: Anti-BGG, normal rabbit γ-globulin, and rabbit anti-actomyosin were labelled with fluorescein by the method of Sentner (12). These preparations were tested by covering 2 micron thick sections of the frozen heart with solutions of these reagents. The preparations were examined under the microscope equipped with fluorescent attachment and selected photographs were taken.

B. PHYSICAL

1. Electrophoresis:

Several attempts were made to fractionate anti-BGG by starch gel-electrophoresis. This technique appeared to be unsuitable for the elution

of activity for our purpose and was consequently discarded.

2. Ultracentrifugation:

Samples of anti-BGG as well as of the DEAE-separated fractions were studied in the analytical ultracentrifuge at 41,000 and 59,000 rpm.

VI. HISTAMINE RELEASE

Histamine release was studied on the isolated heart and on the Schultz-Dale preparations by the two methods mentioned previously. The bioassay method appeared to be rapid and reliable but, from time to time, quantitative chemical estimations were made on the butanol extracts of the perfusates which had been adsorbed and eluted from cotton acid succinate columns. The eluates were treated with orthophthalaldehyde and the concentration of the histamine hydrochloride was determined with the aid of either the Aminco-Bowman spectrophotofluorometer or the Turner photofluorometer.

Since our studies involved the parallel estimation of anaphylactic and hemolytic activity the results of the absorption experiments are given in quantitative rather than qualitative terms in order to estimate the degree of absorption achieved by each maneuver.

VII. QUANTITATIVE IMMUNE HEMOLYSIS

Experiments on immune hemolysis were made according to the modification of Mayer's (13) method as used in this laboratory. The experiments consisted of determining density of hemoglobin released from sensitized cells owing to the action of varying amounts of complement. The results were reduced according to the logistic transformation of von Krogh (op cit) and are expressed as K values and per cent hemolysis.

VIII. RESULTS

A. WHOLE ANTI-BOVINE GAMMA-GLOBULIN

1. Dose Response Curve:

Hearts were perfused with anti-BGG or anti-Xp-BGG preparations in doses ranging from 0.1 to 27 mg per heart. The heart rate and the amplitude

were increased, heart block was noted at higher doses but there was no other consistency of reaction with the anti-BGG dosage used. The histamine released turned out to be a reliable function of dose, approaching an asymptote at about 10^{-8} moles/gm/min. The coronary flow was first increased and then decreased as the reaction progressed.

2. <u>Absorption</u>:

Absorption with homogenates of guinea pig heart and kidney failed to reduce the anaphylactic effect; however, the maneuver of absorbing with the red cells of several species of animal materially decreased the anaphylaxis of the anti-BGG preparations. In addition, it was found that these red cells could be sensitized with the preparation and would undergo hemolysis when complement was added.

3. <u>Hemolysis</u>:

Since the only maneuver reducing anaphylactic activity appeared to be by absorption with red blood cells it was suspected that some hapten present on the red blood cell envelope might be the antigenic culprit. This inference was not unreasonable in view of the distribution of Forssman antigen in the tissues of various species of animal. Although the Forssman antigen is not supposed to occur in cattle, it was thought best to rule it out by a specific experiment. Accordingly, the F-hapten was prepared by the method of Brunius (14) for use in competitive inhibition tests. The Forssman hapten, prepared from horse kidney, inhibited the classical sheep cell--anti sheep cell system but failed to inhibit the sheep cell anti-BGG reaction, confirming the suspected independence of the present cross-reaction from the heterophile system.

The presence of soluble cross-reacting impurities in the commercial preparation was eliminated by exhaustively extracting commercial bovine γ-globulin with hot alcohol in a Soxhlet extractor and also by preparing the antigen from beef serum by ammonium sulfate precipitation. The alcoholic extract did not block hemolytic activity and the ammonium sulfate fractionation produced antibodies having effects indistinguishable from those of the commercial bovine γ-globulin.

4. <u>Relation Between Anaphylaxis and Hemolysis</u>:

The reduction of anaphylactic activity following absorption with various types of cells was further studied on the guinea pig ileum since that technique could provide a quantitative measure of the effect of treatment, and could furnish a means for comparing anaphylactic and hemolytic potencies in parallel tests. The results of single, double, and triple absorptions are presented in Table I. Complete absorption of the

hemolytic action of the anti-BGG preparation by one cell type reduced the anaphylactic activity of the antibody by about 1/3. A second complete absorption by an additional type of red blood cell reduced the activity by another 35%. The third absorption reduced the activity to less than 5% of that originally present. It appears from our results that each type of cell independently removes 1/3 of the activity present in the preparation regardless of the sequence in which it is presented to the antibody. The mixed absorption experiments uncovered a surprising feature of the hemolytic action of anti-BGG: When the hemolytic action with respect to one species of cell had been removed by exhaustive absorption with that cell type, hemolysis was still observed when the residual anti-BGG was tested with other cells, suggesting that a number of independent hemolytic antibodies are formed as a result of immunization with BGG.

5. Absorption With Guinea Pig Gamma-Globulin:

Since it has long been known that the γ-globulins of many species will cross-react, we tested the idea that the anaphylactic cross-reaction observed in the present work might have resulted, at least in part, from the cross-specificity of bovine and guinea pig gamma-globulins. Figure 2 shows the results of quantitative precipitation tests in which BGG and GPGG were used as antigens. Table II shows that absorption of the antibody by guinea pig gamma-globulin removed two-thirds of the original anaphylactic activity, and that the additional treatment of the anti-BGG with any of the erythrocyte species removed the remainder.

6. Ouchterlony Patterns:

Gel-diffusion experiments continued to show strong reactions between BGG and anti-BGG even after the latter had been cleared of all anaphylactic and hemolytic action. Absorption with bovine gamma-globulin cleared the system of any cross-reacting antigens and also completely eliminated hemolytic and anaphylactic actions of the parent preparation.

7. Passive Cutaneous Anaphylaxis:

Intradermal injection of the anti-BGG preparation did not result in the immediate formation of the typical passive cutaneous anaphylaxis, suggesting that the cross-reacting antibody had no direct effects on the skin. The fact that these antibodies could sensitize the skin, however, was shown by the appearance of wheals following the subsequent intravascular administration of the corresponding antigen.

8. **Tentative Summary of Studies with Anti-BGG:**

The foregoing results have shown that the anaphylactic activity was due not to a single antigenic species but at least to two kinds of substance. This view was based on the evidence that no single absorption could entirely clear the antibody preparation of anaphylactic activity but that it required the combined absorption either with guinea pig γ-globulin and red cells or with the use of several red cell types. Thus, the anaphylactic activity could have been due to guinea pig γ-globulin or to any of the cross-reacting antigens on three different cell types. The possibility was not ruled out that the anaphylactic cross-reaction might have been due to soluble antigens (other gamma-globulins) adsorbed to the surfaces of the red cells in question.

B. ANTI-BGG FRACTIONS

The anti-BGG fractions produced by gradient elution on DEAE columns were tested for their effects on anaphylaxis, hemolysis, and quantitative precipitation.

1. **Anaphylaxis:**

All fractions tested on the heart gave positive results with respect to anaphylaxis. The gut was used in these experiments to obtain a better quantitation of the results. All fractions were tested at the same total protein concentration on the gut and, as may be seen from Table III, anti-BGG fraction III had the least anaphylactic activity of all preparations tested.

2. **Hemolysis:**

Comparison of the hemolytic data given in Table III shows that practically all of the hemolytic activity for cells other than those of the Human group A and the bovine type seems to reside in Fraction III. Only the unfractionated anti-BGG gave significant hemolysis for human A cells. About one-half of the hemolytic activity directed against beef cells was lost by the fractionation procedure in all cases. The anti-goat cell activity of Fraction III was higher than of the unfractionated anti-BGG but the anti-sheep activity had declined by about forty per cent. In contrast to this the anti-goat activity of Fractions I and II was very low and the anti-sheep activity of these fractions was significantly lower than that of Fraction III.

In summary we can say that while the greatest reductions of hemolytic activity occurred in Fractions I and II the anaphylactic activity was unchanged. On the other hand, Fraction III showed the least reduction in hemolytic activity but after absorption with red cells exhibited a fifty per cent drop in anaphylactic activity. It is evident from these results that some separation between the hemolytic activity and the anaphylactic activity had occurred through the fractionation procedure.

3. Quantitative Precipitation:

Separate curves were run to determine the per cent specific antibody in the various fractions to the whole bovine-gamma-globulin preparation and the results are shown in Table VI. Comparison of the curves demonstrating specific antibodies for bovine and guinea pig γ-globulins is illustrated in Figure 2. Ouchterlony tests showed that Fractions I and II of the antibody would react with all three fractions of the antigen; however, Fraction III of the antibody could not be made to give positive reactions with any of the antigens tested, probably because it was present in such low concentration. An additional feature of the Ouchterlony tests was the finding that Fraction I of the antigen gave only one line with the whole antibody whereas Fractions II and III each gave two lines. The common line appearing in all the fractions was a rather heavy one, while the second line appearing only in Fractions II and III was very thin and very prominently displayed. These findings further support the distinction between the anaphylactic and hemolytic activities of the fractions discussed in the preceding section.

C. LOCALIZATION

In order to determine whether one of the cross-reacting systems could be guinea pig γ-globulin, we tested the ability of radio-iodinated normal guinea pig γ-globulin to adhere to normal cardiac tissue. Physical adherence was established, but whether or not this implies sensitization is open to question. However, the fact that normal guinea pig γ-globulin can adhere to normal guinea pig heart suggested that possibly one of the cross-reacting antigens, as demonstrated by the quantitative precipitin curve, responsible for anaphylaxis was guinea pig γ-globulin.

Studies with the fluorescent γ-globulins showed a diffuse distribution of fluorescein-labelled anti-BGG which was indistinguishable from that of the normal rabbit γ-globulin. This was in high contrast to the exquisite localization obtained with rabbit antibody to guinea pig heart actomyosin, which also produced anaphylaxis.

D. ULTRACENTRIFUGATION

The sedimentation patterns of the ammonium sulfate-precipitated anti-BGG, as well as those of Fractions II and III, were studied by ultracentrifugation. The whole preparation presented a uniform pattern of a 6.6 S gamma-globulin molecule. The anti-BGG and the anti-XpBGG contained, respectively, 5 and 2 per cent specifically precipitable γ-globulin. These experiments were run at 59,000 rpm. Subsequent studies in this laboratory showed the presence of a fast moving component in Fraction III, which presumably was a [illegible] globulin of a molecular weight much larger than the 6.6 S. Fraction II showed only a very slight proportion of this kind of gamma-globulin. It is the expectation at the present time that the macro-globulin is the antibody primarily responsible for the hemolytic activity. Direct proof will have to await studies made with separation cells in which the macroglobulin can be isolated and tested for hemolytic and anaphylactic activity.

E. MISCELLANEOUS

1. Other Cross-Reacting Systems

a. Red cells: Anti-BGG did not produce hemolysis in type [illegible] or B human cells nor did it seem to affect guinea pig cells although it produced anaphylaxis in guinea pig tissues.

b. The specificity of anti-BGG is well shown by the fact that anti-BSA was entirely devoid of both hemolytic and anaphylactic [illegible].

2. Species Specificity of Anti-BGG

Immunization of chickens and [illegible] with the commercial preparations of bovine γ-globulin produced precipitating antibodies to BGG but the [illegible] anti-BGG had no anaphylactic effect on guinea pig tissue. [illegible] was produced both in the serum as well as in the [illegible] preparations precipitated the specific antigen but [illegible] antibody produced an anaphylactic effect of the type [illegible] rabbit preparation.

It appears, therefore, that some specificity resides in rabbit [illegible] bodies for guinea pig tissues and also that the anaphylactic antibody might be screened out by the vascular system during extravasation [illegible] the peritoneum.

3. Other Antibodies Producing Anaphylaxis:

The relationship between the anaphylactic and the hemolytic activity was further studied in other antibody systems known to produce anaphylaxis in guinea pigs. With exception of the anti-sheep antibody, which was chosen because of the particularly strong hemolytic effect that anti-BGG exhibited on sheep cells, all other antibodies were made against guinea pig tissues. The following antibodies were studied: anti-kidney, anti-gamma-globulin, anti-serum albumin, and anti-actomyosin. The gamma-globulin fractions of each of these anti-sera, with the exception of the anti-sheep preparation, was obtained from third-saturated ammonium sulfate precipitation.

Anti-sheep cell serum produced no detectable anaphylaxis and no cross-reaction in hemolytic tests. It was highly specific for sheep cells. All of the anti-guinea pig preparations were absorbed with a variety of red cells and until no further hemolysis was obtained and they were then tested on the isolated guinea pig gut. The strongest reactions of the unabsorbed sera were demonstrated by anti-guinea pig serum albumin. The extraction of hemolytic antibodies with the various cells was successfully accomplished in the case of anti-guinea pig kidney, anti-guinea pig albumin, and anti-actomyosin. In the case of anti-guinea pig gamma-globulin it was difficult to remove completely the cross-reactions with human type A cells and with beef cells. In the case of anti-guinea pig kidney all absorptions reduced the anaphylactic activity to a level of 25 to 50 per cent but, as mentioned before, type A and beef cells failed to eliminate the hemolytic activity. The results exhibited in Table VII, then, can be taken as confirming the independence of other cross-reactions of anti-BGG.

A survey of species heterogeneity of gamma-globulins was made by means of the Ouchterlony double diffusion technique. The following γ-globulins cross-reacted with anti-BGG: cow, guinea pig, cat, goat, rat, human, and mouse; but not that of duck or chicken.

SUMMARY AND CONCLUSIONS

Evidence has accumulated to suggest that the hemolytic and anaphylactic activities of anti-BGG may be entirely unrelated phenomena and that they may be due to the production of at least two kinds of antibody to bovine γ-globulin.

The reaction arises specifically from the immunization of rabbits with bovine γ-globulin and, apparently, is limited to that protein as immunization with BSA does not produce the hemolytic or anaphylactic manifestations discussed in this report. The hemolytic and anaphylactic properties of the

antiserum probably arise from different antigenic moieties of the BGG or, possibly, because of the formation of several kinds of functional antibody to BGG. The following evidence is consistent with these views: 1) Anti-sheep rbc antibody does not produce anaphylaxis in guinea pig tissues although sheep cells are regularily lysed by anti-BGG. 2) Antibodies directed against guinea pig serum proteins, which produce anaphylaxis in the heart and gut, do not react hemolytically with red cells although these erythrocytes can be used to absorb the anaphylactic action of anti-BGG. 3) Whereas both the hemolytic and the anaphylactic actions of anti-BGG can be completely removed by BGG, they are not removed by tissue homogenates of heart or gut, by red cells, nor by the individual absorption of the guinea pig gamma globulin.

Part of the anaphylactic cross-reaction is undoubtedly due to the presence of guinea pig gamma-globulin on the tissue, and the adherence of this protein to the heart is verified by the persistence of the labelled normal guinea pig gamma-globulin on the organ. Anti-actomyosin can produce anaphylactic effects on the guinea pig heart but it has never been shown in this laboratory to react with guinea pig gamma globulin or with BGG.

Ultracentrifugal evidence suggest that two kinds of antibody are formed against BGG--regular 6.6 S kind, comprising the bulk of fractions I and II, and a macroglobulin present with the 6.6 S pattern of Fraction III. Modern evidence points to the latter as possessing most of the hemolytic activity (15). Since we have shown that the hemolytic activity is virtually independent of the anaphylactic effect, the anaphylactic power of antibody Fraction III is probably not ascribable to the macroglobulin but must be due to some other property of that fraction.

It is revealing that the anti-BGG Fraction I which has the greatest anaphylactic power reveals only one antigenic component in Fraction I of the antigen, but shows two lines in Fractions II and III. Since it has not been established that the precipitating and the anaphylactic actions are due to the same antibody individual it is impossible to identify BGG Fraction I as the antigen responsible for the anaphylactic effect of the preparation. It is, of course, not clear why the present cross-reaction should occur. It is evidently not due to an impurity. Since one of the cross reacting antigens is a γ-globulin a reasonable explanation may be advanced that this molecule is particularily susceptible to alteration during the purification step. It has been shown by a variety of authors (16), (17), (18), that even autologous γ-globulins can produce hypersensitivity if these proteins are subjected to such relatively mild procedures as freezing and thawing, or to exposure to a high pH.

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TABLE I

HEMOLYTIC AND ANAPHYLACTIC ACTIVITY OF ABSORBED ANTI-BOVINE GAMMA GLOBULIN

HEMOLYSIS			GUT ANAPHYLAXIS		
Absorbed with	Times absorbed for Zero hemolysis	First K value ml of 1/50 Comp for 50% hemolysis	Times tested	Mean equivalent Histamine Conc.	% of Unabsorbed Antibody
0	0	13.0	13	6.5×10^{-6}M ± 2.6	100
Human A rbc	3	13.0	1	4.5×10^{-6}M	70
Beef rbc	4	3.4	1	4.0×10^{-6}M	62
Goat rbc	3	4.1	1	6.0×10^{-6}M	92
Sheep rbc	7	0.4	1	4.0×10^{-6}M	62
Human A rbc Beef rbc	3 2		4	2.1×10^{-6}M *$0.8\text{-}4.0 \times 10^{-6}$	32
Goat rbc Beef rbc	3 2		2	2.4×10^{-6}M	37
Human A rbc Beef rbc Sheep rbc	3 2 3		2	3.5×10^{-7}M *$0\text{-}7 \times 10^{-7}$	4.6

TABLE II

HEMOLYTIC AND ANAPHYLACTIC ACTIVITY OF ABSORBED ANTI-BOVINE GAMMA GLOBULIN

HEMOLYSIS		ANAPHYLAXIS		
Absorbed with	Times Absorbed for zero hemolysis	Times Tested	Histamine Equivalent	% of Unabsorbed Anti-BGG
Unabsorbed	0	13	$6.5 \times 10^{-6}M \pm 2.6$	100
Guinea Pig γ-globulin at optimal prop.	1	3	$2.3 \times 10^{-6}M$ *2.0-2.6	35
+ Human grp A rbc	2	2	$1.2 \times 10^{-7}M$ *0-2.4	1.8
+ Beef rbc	3	2	$1.5 \times 10^{-7}M$ *0-3.0	2.3
+ Goat rbc	2	2	$7.0 \times 10^{-7}M$ *6-8	10.7
+ Sheep rbc	2	2	$7.0 \times 10^{-8}M$ *0-1.4	1.1

*Range

TABLE III

ANAPHYLACTIC AND HEMOLYTIC ACTIVITY OF
DEAE-CELLULOSE SEPARATED FRACTIONS OF ANTI-BOVINE GAMMA-GLOBULINS

ANTI-BODY	GUT ANAPHYLAXIS			HEMOLYSIS			
	No. of Tests	Mean Equivalent Histamine Conc.	% of Whole Antibody	No. of Tests	Red Cell Type	K	% Lysis
Unfractionated	14	6.5×10^{-6} M	100	1	Human group A	13	23
Anti-BGG		$\pm$ 2.6		1	Beef	3.4	60
(0.750 mg/ml)				1	Goat	4.1	49
				4	Sheep	0.4	100
Fraction I of	6	9.0×10^{-7} M	83	2	Human group A	∞	0
Anti-BGG		$\pm$ 4.6		1	Beef	8.1	31
(0.125 mg/ml)				2	Goat	14.3	18
				2	Sheep	5.4	38
Fraction II of	7	10.3×10^{-7} M	95	2	Human group A	∞	0
Anti-BGG		$\pm$ 4.2		1	Beef	8.2	21
(0.125 mg/ml)				2	Goat	22.0	10
				2	Sheep	5.4	30
Fraction III of	6	4.5×10^{-7} M	42	2	Human group A	∞	0
Anti-BGG		$\pm$ 3.6		1	Beef	11.8	21
(0.125 mg/ml)				2	Goat	3.8	54
				2	Sheep	3.0	62

TABLE IV

INHIBITORY ACTIVITY OF PURE FORSSMAN HAPTEN

Forssman Hapten	Anti-Bovine γ-globulin		Anti-Sheep rbc	
mg added	Per Cent Hemolysis	K*	Per Cent Hemolysis	K*
0	98	1.[illegible]	100	0.6
25	86	1.0	17	8.6
50	92	1.1	22	6.3
75 mg	100	.58	20	8.5
100 mg	87	1.1	15	11.5
125 mg	95	1.6	16	10.0

* mls of 1/50 complement for 50% hemolysis

TABLE V

TEST	RADIOACTIVITY (cpm)
Initial Prechallenge	4899
After Anti-BGG challenge	2814
After Guinea Pig γ-globulin	1345
After Anti-Guinea Pig γ-globulin	563

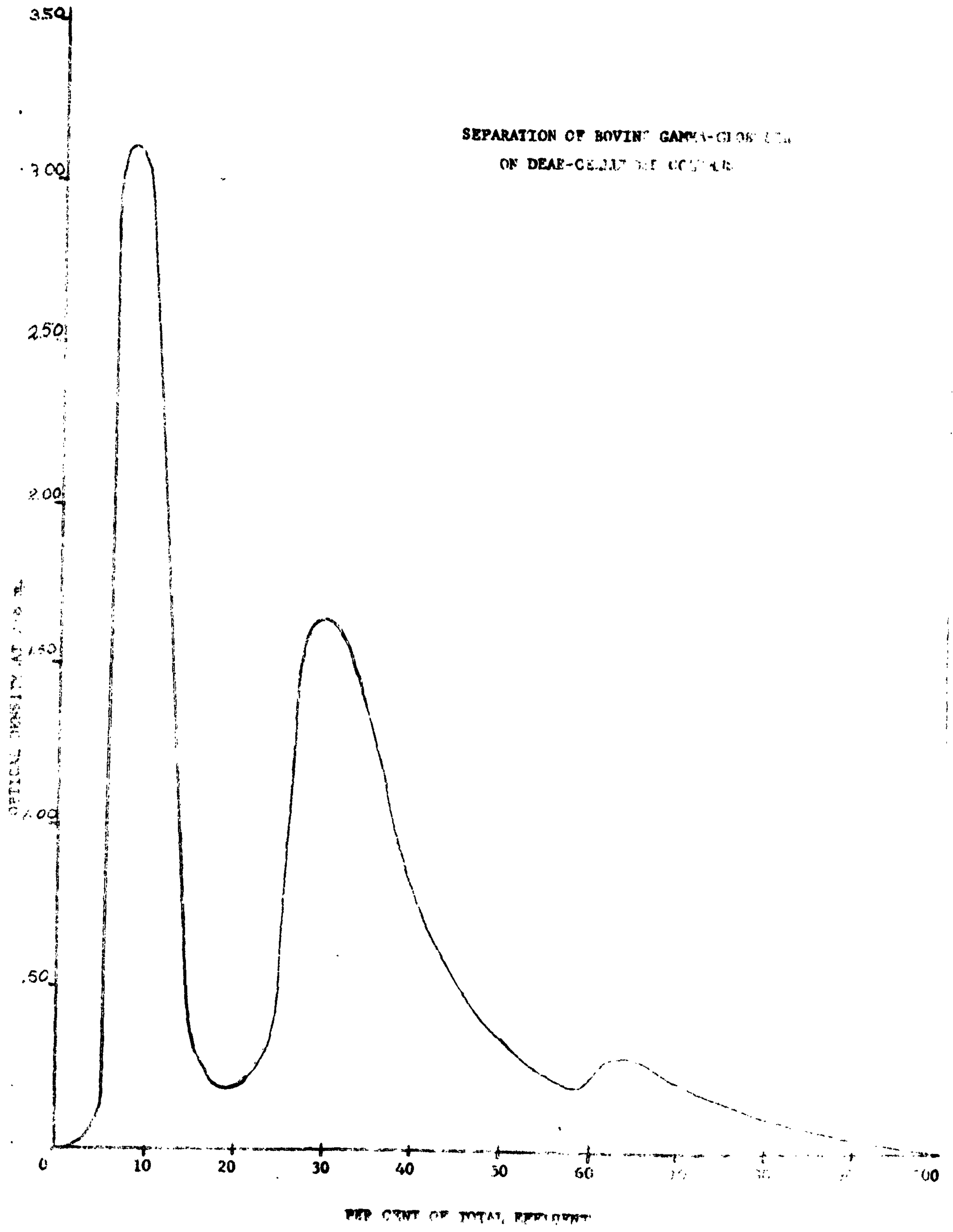
TABLE VI

ANTI-BGG PREPARATION	PER CENT SPECIFIC ANTIBODY
whole preparation	20
Fraction I	31
Fraction II	19
Fraction III	20

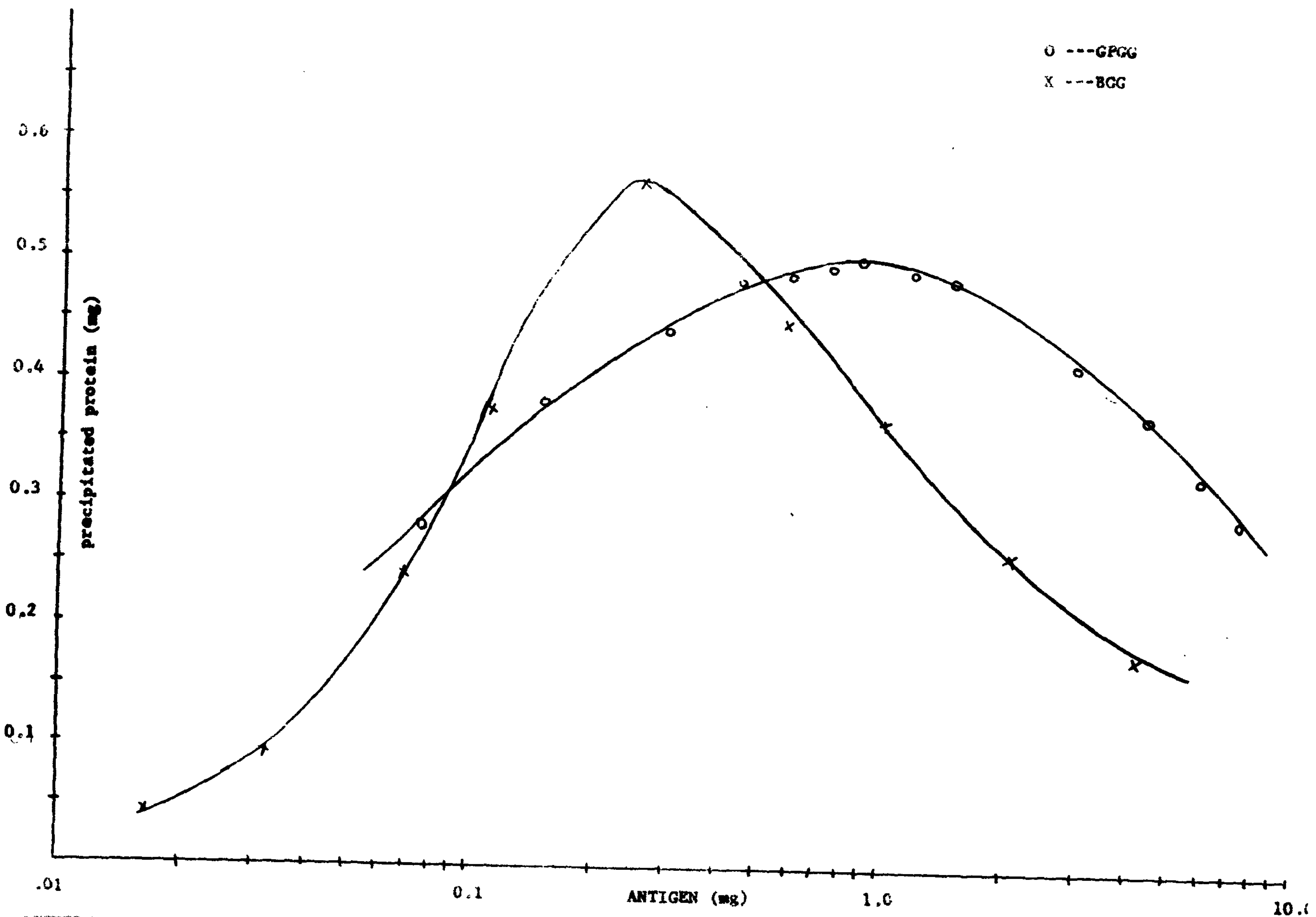
TABLE VII

ANAPHYLACTIC AND HEMOLYTIC
ACTIVITY OF OTHER ANTIBODY PREPARATIONS

		ANAPHYLACTIC ACTIVITY	HEMOLYTIC ACTIVITY
ANTIBODY	ABSORBED WITH	EQUIVALENT HISTAMINE CONCENTRATION	PER CENT HEMOLYSIS
Anti-sheep	0	0	---
	Human A rbc	0	0
	Beef rbc	0	0
	Goat rbc	0	
	Sheep rbc	0	100
Anti-Guinea Pig Kidney	0	4×10^{-6}	---
	Human A rbc	1.7×10^{-6}	0
	Beef rbc	1.0×10^{-6}	0
	Goat rbc	3.2×10^{-6}	---
	Sheep rbc	1.6×10^{-6}	100
Anti-Guinea Pig γ-globulin	0	2×10^{-5}	---
	Human A rbc	1×10^{-5}	[illegible]
	Beef rbc	8×10^{-6}	19
	Goat rbc	4.3×10^{-6}	---
	Sheep rbc	1.5×10^{-5}	100
Anti-Guinea Pig Albumin	0	1.3×10^{-5}	---
	Human A rbc	---	[illegible]
	Beef rbc	8×10^{-6}	[illegible]
	Goat rbc	---	---
	Sheep rbc	2.4×10^{-6}	100
Anti-Actomyosin	0	2×10^{-5}	---
	Human A rbc	6.4×10^{-6}	[illegible]
	Beef rbc	---	[illegible]
	Goat rbc	2×10^{-6}	---
	Sheep rbc	---	100



SEPARATION OF BOVIN
ON DEAE-C
3.50
3.00
2.50
2.00
1.50
1.00
.50
0
10
20
30
40
50
60



PRECIPITIN CURVES OF ANTI-BGG vs BGG and GPGG
O ---GPGG
X ---BGG
precipitated protein (mg)
0.6
0.5
0.4
0.3
0.2
0.1
.01
0.1
1.0
10.0
ANTIGEN (mg)

PART II

STUDIES ON THE PERIPHERAL NEUROMUSCULAR ACTION OF TETANUS TOXIN

STUDIES ON THE PERIPHERAL NEUROMUSCULAR ACTION OF TETANUS TOXIN

I. INTRODUCTION

Although the mechanism of the paralytic action of purified tetanus toxin appears to have been clarified by the work of Brooks, Curtis, and Eccles (1,2) and their collaborators as being due to the blocking of inhibitory impulses, and the receptor unit identified by van Heyningen (3-6), there still remain significant questions concerning the mechanism of its peripheral effect. It seemed to us that in order to settle this problem, tetanus toxin would have to be tested at the most peripheral site of the neuromuscular apparatus--the motor end-plate.

During the past year sufficient evidence has accumulated as the result of our joint studies to indicate that a principle or moiety present in "purified" tetanus toxin has a direct effect on the peripheral structures. This effect is recognized by the increase in the frequency of random discharge of miniature end-plate potentials, as recorded intracellularly by micro-electrodes inserted into the intercostal musculature of the mouse, an animal exquisitely susceptible to the paralytic effects of toxin. There has been considerable discussion over the past 30 years concerning the genuineness of the peripheral effect. This had arisen, in part, because of the want of direct means of testing and, in part, because experiments had been made with relatively impure materials. The recent findings of van Heyningen concerning the high degree of specificity of the paralytic moiety of tetanus toxin for ganglioside offered a means of assessing whether the paralytic and peripheral actions of tetanus toxin were separate manifestations of the toxicity of one protein molecule or whether they were due to different species of molecules present in the "purified toxin".

Evidence will be presented in this communication to establish the existence of a peripheral neuromuscular effect and to support the position that the paralytic and the peripheral actions of tetanus toxin are not correlated.

II. METHOD

A. PHYSIOLOGICAL

Male Swiss-Webster mice, averaging 20 grams in weight, were used as the source of intercostal muscle preparations in these experiments. Each

animal was killed by a blow to the base of the skull; the entire thoracic cage was quickly presented and the entire spinal column, together with the adhering musculature, was cut away in a container of well gassed (95% O_2--5% CO_2) solution described by Liley (7). One-half of the thorax was then mounted in a lucite clamp and placed in a muscle bath (8) while the second half was set aside in oxygenated medium for subsequent use. Intracellular potentials were measured by means of micropipettes filled with 3M KCl; these electrodes, which had a tip resistance of 1.1-11.0 megohms, were connected by an Ag-AgCl bridge to the input of a cathode follower circuit (8) and were displayed on a Tektronix 502 oscilloscope, and photographed with a Grass oscilloscopic camera. Potentials were recorded from at least 12 cells before and after the administration of a test dose of toxin. A period of 15 seconds was allowed to elapse from the time that a cell was impaled until recording was begun; after that, records of cellular activity were taken for at least 10 seconds. Cells having resting potentials of less than 60 mV were not measured. Potentials were considered acceptable only if the frequency remained stable and the amplitude averaged at least 250 µV. The amplitude was found to vary with the quality of the seal around the tip, its proximity to the myoneural junction, and with the cell size. The frequency appeared to be little influenced by these variables. Accordingly, frequency rather than amplitude was considered the significant parameter in these experiments.

B. CHEMICAL

The purified tetanus toxin (TD 464-D), supplied to one of us (W. E. van Heyningen) by the Wellcome Research Laboratory, was a lyophilized powder. This preparation was approximately 40% protein, of which 75% was active toxin, and had 2 x 10^7 LD_{50} mouse units/mg. "Top substance", the supernatant remaining after the paralytic activity of the toxin was reduced by complexing it with ganglioside and separating it by ultracentrifugation, was prepared at Oxford from a less concentrated starting material, batch TD 594-B. The paralytic activity was reduced to 1.25% by six successive adsorptions with protagon and the almost pure protein (a9g) had 5 x 10^4 LD_{50} mouse units/mg of toxicity.

Both the toxin and the impurity were made up by dissolving the dry powder in 0.15 M phosphate buffer (pH 7.0) containing 0.1% gelatin. Stock solutions containing 10^6 LD_{50} toxin units per milliliter were subdiluted so as to provide the proper concentration for testing and aliquots of 3-5 ml were delivered into 5 ml serum bottles, stoppered, and stored in the deep freeze until needed. Preliminary experimentation showed that these preparations would be stable for at least 7 days. Results obtained with materials stored for longer periods often were erratic and, for this reason, in the subsequent portions of the study, we arbitrarily discarded any samples which remained in the deep-freeze for over 4 days.

III. RESULTS

A. STANDARDIZATION

1. Consistency of Measurements:

In the preceding section it was stated that the frequency of miniature end-plate potentials appeared to be the more reliable variable for the assessment of the activity of toxin. This decision was based on data which were obtained from control studies on 61 mice and which include observations made, in most cases, on both hemithoracic sections. The average frequency observed for 100 hemithoraces at 35.1 ± 0.1° C was 5.35 ± 0.29 sec^{-1}, and the average amplitude was 402 ± 13μV. The question next arose whether the variation between the hemithoraces of one mouse was significantly less than that existing among the mice taken at random. Sufficient data were at hand in the 61 mice which permitted a statistical analysis to be made of the consistency between the first and the second hemithorax. In 40 duplicate tests the mean frequency of the first hemithorax was 5.27 sec^{-1}, and that of the second hemithorax was 4.73 sec^{-1}. The slope of the regression line, b, was 0.29 and its standard error was 0.14. The ratio of the slope to its standard error, t, was 2.18 which was significant at the 5% confidence level. The correlation, r, between the two halves was 0.33. A similar analysis of the amplitude data, on 36 duplicate cases, gave the following statistical information: The mean amplitudes were 410 and 443 μV for the first and second halves, respectively; the regression coefficient of the first half or the second half was 0.39 ± 0.18, and the t value of 2.20 was significant at the 5% level. The correlation was 0.35.

Since both tests showed such poor internal correlations, it was not advantageous to use one half as a control for the second, and therefore all subsequent experiments were made with a single preparation from a given animal, using the ratio between test and control as a means of expressing the effect of toxin.

2. Effects of Selected Drugs and Cations:

a. Cations: The possibility that the increased frequency of MEPPS observed after the administration of the diluted toxin might have come about because of the small amount of potassium present in the gelatin and from the ionic calcium in the toxin preparation itself was ruled out by direct experiment. The nutrient solution contained Ca and K in concentrations of 2.0 and 5.0 mM/L, respectively. The effect of quadrupling

the Ca concentration was found, in 3 experiments, to double the natural MEPP frequency. The frequency of discharges was increased 4-15-fold when the potassium concentration of the medium was increased 2.5 times. If the toxin had been pure $CaCl_2$, the final [Ca] in the bath would have been 1.98 mM/L when toxin was added in its most concentrated form, i.e. 1 ml of 10^6 LD_{50} to a 67 ml bath; additionally, this dose would have reduced the [K] from 5.0 to 4.96 mM/L. It is clear that the effects described in this communication could not have resulted from the cations in question.

b. Drugs: Strychnine given in doses ranging from 8×10^{-5} to 3×10^{-2} mg/ml of bath fluid appeared to increase the MEPP frequency irregularly, but the increments were within the same range as those observed with toxin. No significant results were observed when 5-hydroxytryptamine was tested. Curare, at a final concentration of 3.7×10^{-4} mg/ml of bath fluid stopped the appearance of miniature end-plate potentials both in the control preparations as well as in those in which the frequency had been increased by toxin.

B. DOSE-RESPONSE CURVES

The relationship between the concentration of the active agent and the response was determined at 35° C for both the total toxin preparation and the supernatant fraction over approximately the same range of concentrations. The results presented in Table I give the average control and post-toxin frequencies observed on 4-7 hemithorax preparations at each dosage-level. Control records were taken after an equilibration period of at least 30 minutes and post-toxin measurements were made 10 minutes after the addition of a 1 ml test dose to the bath. Control and test records were taken from muscle cells supplied by the same nerve twig and, insofar as was possible, the same cells were sampled before and after the addition of toxin.

The results plotted in Figures 1 and 2 are the ratios of the mean test/control frequencies. An inspection of Figure 1 shows at once that the principal distinction between the two preparations lies not in the median effective (gram) dose, but in the heterogeneity of the sigmoid curve displayed by the parent preparation. It is evident that one of the consequences of adsorption with protagon was to increase the uniformity of the preparation by reducing the number of molecular species capable of producing the peripheral effect. The separation of the central paralytic effect from the peripheral effect by treatment with protagon is quite evident in Figure 2, which shows the mean peripheral activity to lie at about 10^{-1} LD_{50}/ml in the case of the whole toxin and at 1.5×10^{-3} LD_{50} for that of the supernatant.

The degree of purification of the MEPP factor achieved by treating the crude preparation with protagon can be estimated by comparing the $MEPP_{50}/LD_{50}$ ratios per mg of protein for the two preparations. Taking the mid points of the MEPP curves as effective reference values, and correcting the toxicity and the MEPP data for activity per milligram of protein, we obtain the results exhibited below:

	LD_{50}/mg protein	$MEPP_{50}$/mg protein	$MEPP_{50}/LD_{50}$
Toxin	5×10^7	0.5 to 1.1×10^9	10-22
Top Substance	5×10^4	3.1×10^7	620

From this summary it is immediately evident that the MEPP activity is concentrated in the supernatant owing to the removal of the paralytic effect by protagon.

C. EFFECT OF TEMPERATURE

One of the classical features of the paralytic action of tetanus toxin is the dependence of its toxicity upon the body temperature. This feature was dramatically illustrated in the recent work of Rowson (9) who showed that frogs, which are normally unaffected by tetanus toxin, become sharply susceptible to it when the ambient temperature is raised above the critical point of 20° C. The studies described in this section were made to find out whether the peripheral effect had a temperature dependence of the same character and magnitude as that exhibited in the paralytic action.

The experiments were performed essentially as described under "Methods" with the exception that a larger muscle bath (114 ml) was used to insure temperature stability. The frequency of MEPPS was studied at 5 temperatures ranging from 27.55 to 39.50° C before and after the administration of a dose of tetanus toxin which provided a final concentration in the bath of 0.064 mg/ml. Six to eight preparations were tested at each point in temperature and the average values for the control and test frequencies are given in Table II.

The uniformity of the response with respect to temperature was tested by plotting the logarithm of the average frequencies against the corresponding absolute temperatures. Figure 3 shows that this function is linear. The slope, hence the activation energy, of the toxin line is greater than that

of the control function and both least squares lines converge upon a common point at 27.55° C. A statistical analysis performed on the data showed the difference between the slopes to be significant at the 0.001% confidence level.

The activation energy was calculated by means of the Arrhenius equation,

$$\log \frac{K_2}{K_1} = \frac{E_a(1/T_1 - 1/T_2)}{2.303R}$$

in which E_a is the energy of activation in calories, R is the gas constant = 1.987 cal. degree^{-1} mole^{-1}, and K is the frequency of MEPP in sec^{-1}.

Since the functions are linear and the slopes are given by

$$\frac{-E_a}{2.3R},$$

the activation energy may be calculated from any two coordinates taken from the regression line. Taking the values of K from the line at $1/T = 32 \times 10^{-4}$ and 33×10^{-4}, we get an activation energy of 39.9 Kcal and 47.3 Kcal for the control and the toxin categories, respectively.

D. NEUTRALIZATION WITH ANTITOXIN

The effect of antitoxin on the MEPP response to toxin was studied by treating 3 LD_{50} units of the latter with enough dialyzed antitoxin to provide a just-limiting neutralization. The comparison between neutralized and un-neutralized toxin, based on 4 preparations in each case, showed the effect to be completely neutralized at 37°C. The results were as follows: Toxin (3 LD_{50}/115 ml) alone: + 26.8%; Toxin-antitoxin:+3.7%or, essentially, nil.

IV. DISCUSSION

Experiments presented in this communication have established that a peripheral neuromuscular effect can be demonstrated by the increased frequency in the random appearance of miniature end-plate potentials.

The peripheral effect can be separated from the paralytic action of toxin by treating the parent preparation with protagon, or pureganglio-side, which reduces the lethal toxicity out of proportion to the reduction of the peripheral effect.

Although the resulting preparation is less heterogenous with respect to peripheral activity than the starting material, it is impossible, at the present time, to say whether the two activities observed here are due to a single species of molecule--having both actions--or whether they are due to two or more different types of molecule. If the effects are due to separate kinds of molecules, as suggested by the appearance of 2 antigenic lines in the immunodiffusion tests made on the parent preparation, it is relevant to note that both species are heat labile and antigenic, and it is very likely, though not certain, that the species having the MEPP action is a protein.

As far as we are aware, the present data concerning the activation energy of the MEPP frequency are the most complete presently available over the temperature range used, and therefore it is not surprising that they differ in magnitude from those given by other authors. Whereas Q_{10} values ranging from 3 to 4.8 have been observed in the frog (10)(11) and 2.1-3.5 in mammals (7)(12), our data give a figure of about 5-8 depending on the temperature picked (Table II). The fact that an additional 7 Kcal is required for the action of toxin suggests that this process is enzymatic and involves the breaking of a bond of moderate strength.

It is implicit in the experimental arrangement used in the present study that the peripheral activity observed comes about because of the accelerated release of transmitter from structures proximal to the end-plate (11). If this early "cholinergic" effect of the toxin had been used as an argument by the proponents of the "peripheral" school, notably Harvey (12), to support their view of the mechanism of action of tetanus toxin, then our results have contributed a solution to this controversy by demonstrating that the central and peripheral effects are separable by chemical treatment.

V. CONCLUSIONS

1. A reliable quantitative method for the study of the peripheral activity, based on the frequency of miniature end-plate potentials, is described.

2. Treatment of the crude material with protagon complexes the paralytic activity, which can be separated by ultracentrifugation, and concentrates the peripheral activity in the supernate. The ratio of the $MEPP_{50}/LD_{50}$ is 10-22 and 620 in the case of the toxin and the supernatant, respectively.

3. The activation energy of MEPP frequency is about 40 Kcal in the absence of toxin and 47 Kcal in its presence.

4. The peripheral effect of toxin can be neutralized by pretreating it with antitoxin.

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TABLE I

DOSE-RESPONSE SUMMARY

TETANUS TOXIN

Bath Concn LD_{50}/ml	Bath Concn mg/ml	Control Freq.	± S.E.	Test Freq.	± S.E.	Change	n
2.188×10^{-4}	1.094×10^{-11}	5.34	± 0.41	5.11	± 0.49	- 4.3%	7
2.188×10^{-3}	1.094×10^{-10}	4.17	± 0.58	4.12	± 0.66	- 1.2%	6
2.188×10^{-2}	1.094×10^{-9}	3.83	± 0.34	4.40	± 0.53	+14.9%	4
2.188×10^{-1}	1.094×10^{-8}	5.80	± 0.90	7.52	± 2.02	+29.7%	5
2.188×10^{0}	1.094×10^{-7}	5.80	± 1.07	7.90	± 2.02	+36.2%	4

SUPERNATE

Bath Concn LD_{50}/ml	Bath Concn mg/ml	Control Freq.	± S.E.	Test Freq.	± S.E.	Change	n
5.472×10^{-5}	1.094×10^{-9}	5.53	± 1.33	5.46	± 1.26	- 1.3%	6
2.736×10^{-4}	5.47×10^{-9}	4.31	± 1.24	4.30	± 0.86	- 0.2%	4
4.10×10^{-4}	8.205×10^{-9}	4.91	± 1.17	6.22	± 1.75	+24.6%	5
5.472×10^{-4}	1.094×10^{-8}	4.28	± 1.15	6.20	± 1.54	+44.9%	7
5.472×10^{-3}	1.094×10^{-7}	6.91	± 2.53	9.61	± 3.33	+39.1%	4

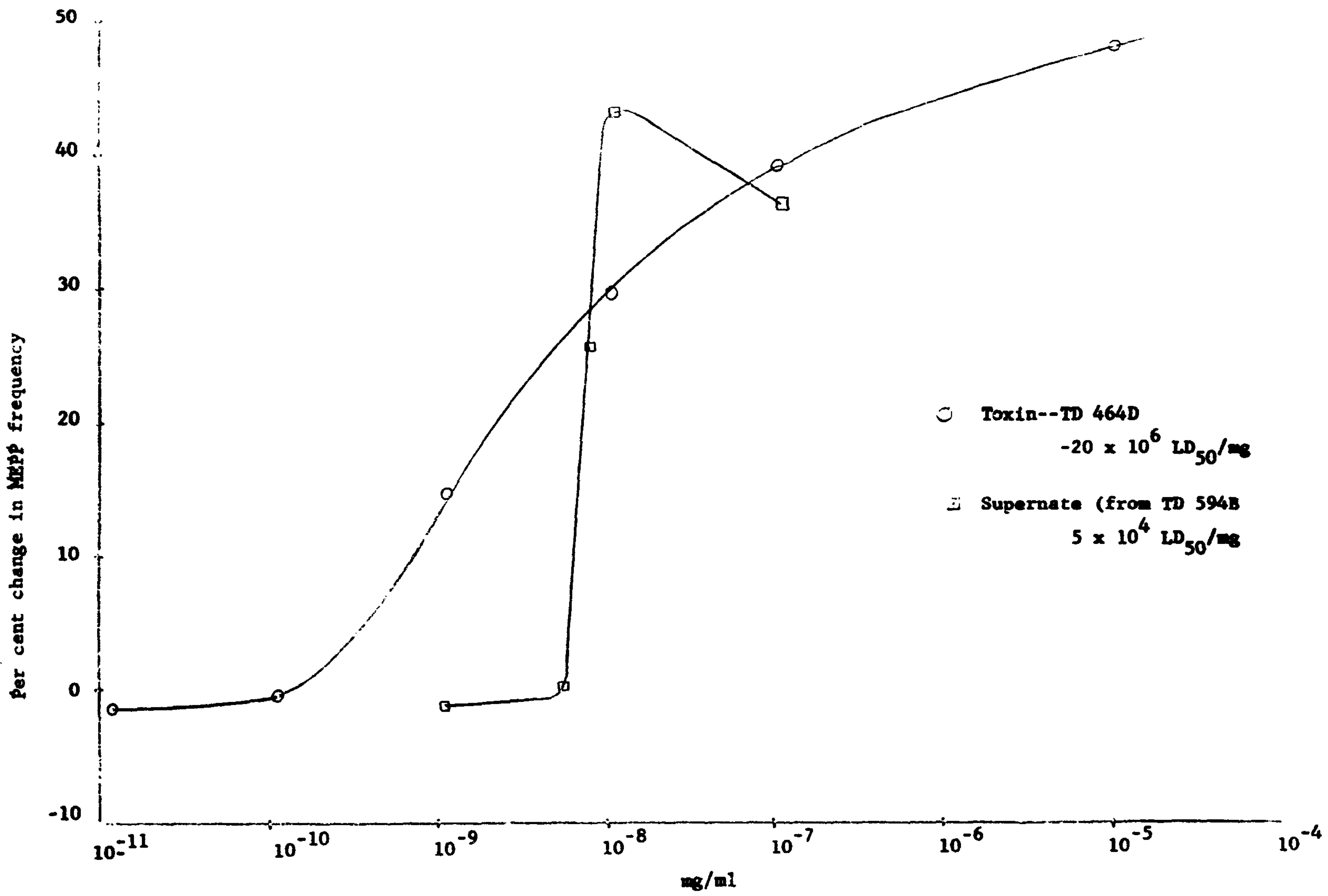
TABLE II

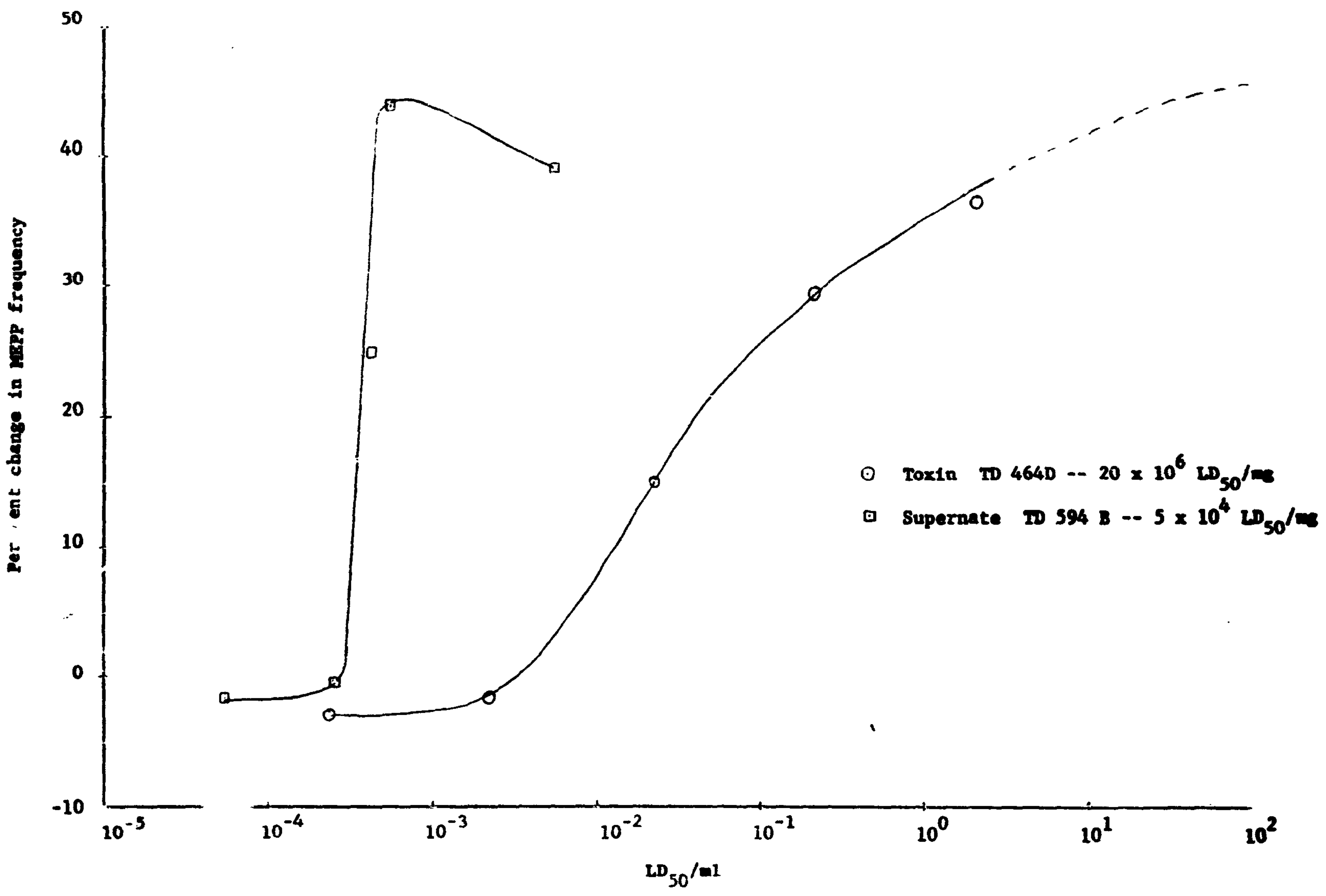
EFFECT OF TEMPERATURE AND TETANUS TOXIN

ON FREQUENCY OF MEPPS

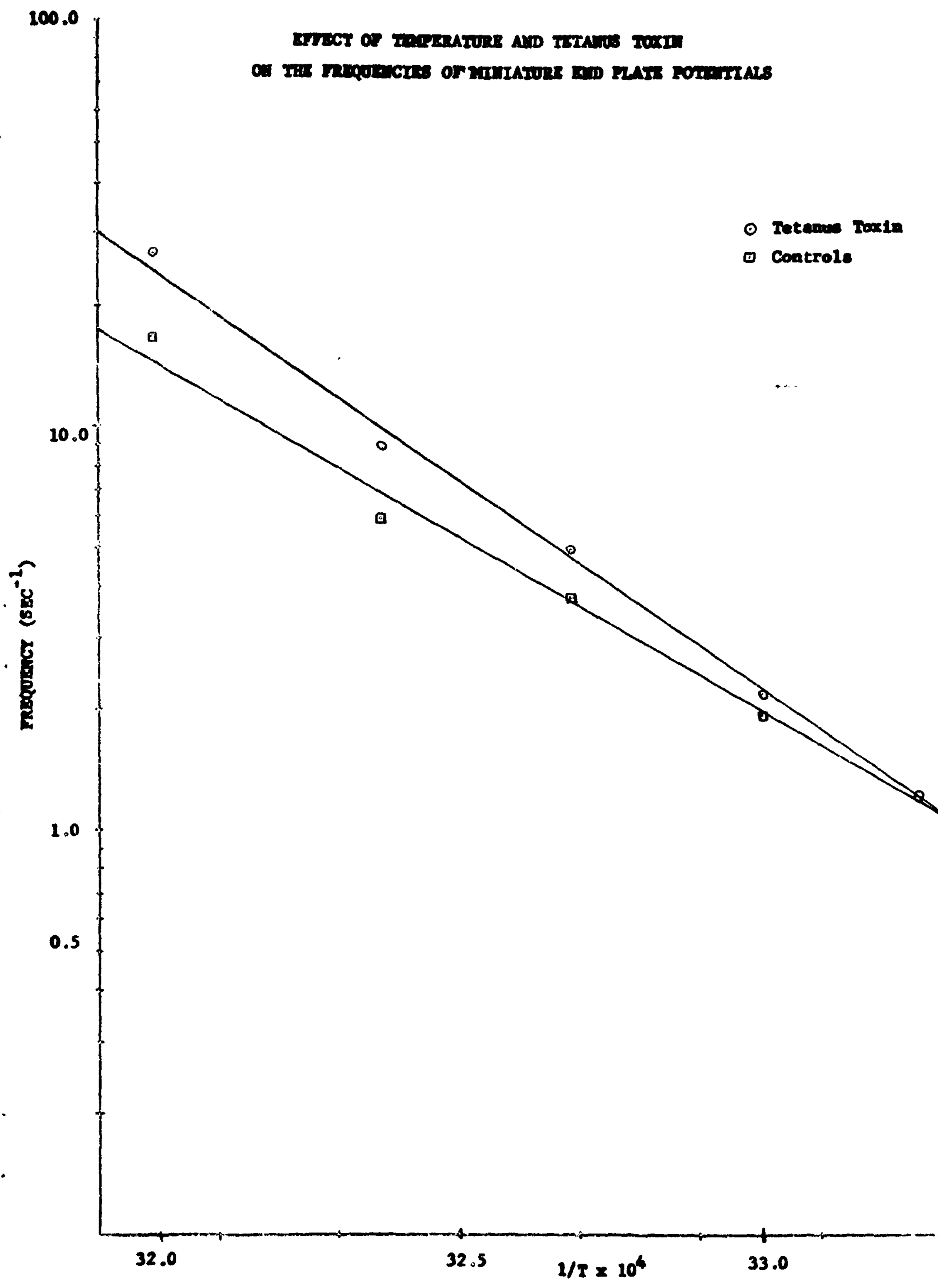
Temp °C	Control Freq.	Test Freq.*	n
27.55	1.21 ± 0.14	1.20 ± 0.10	6
29.90	1.92 ± 0.14	2.13 ± 0.11	7
32.85	3.79 ± 0.36	4.88 ± 0.41	7
38.80	5.95 ± 0.47	8.93 ± 1.37	8
39.50	16.8 ± 2.0	26.9 ± 3.2	7

* In the presence of 0.064 mg/ml of Tetanus Toxin.


COMPARISON OF TOXIN AND SUPERNATE BY WEIGHT
Per cent change in MEPP frequency
50
40
30
20
10
0
-10
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
mg/ml
Toxin--TD 464D
-20×10^{6} LD_{50}/mg
Supernate (from TD 594B
5×10^{4} LD_{50}/mg



COMPARISON OF TOXIN WITH SUPERNATE BY TOXICITY
Per cent change in MEPP frequency
50
40
30
20
10
0
-10
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
LD_{50}/ml
Toxin TD 464D -- 20×10^{6} LD_{50}/mg
Supernate TD 594 B -- 5×10^{4} LD_{50}/mg


EFFECT OF TEMPERATURE AND TETANUS TOXIN
ON THE FREQUENCIES OF MINIATURE END PLATE POTENTIALS
Tetanus Toxin
Controls
100.0
10.0
1.0
0.5
FREQUENCY (SEC-1)
32.0
32.5
33.0
1/T x 10^4

PART III

MISCELLANEOUS PRELIMINARY STUDIES

MISCELLANEOUS PRELIMINARY STUDIES

I. STUDIES OF IN VITRO SENSITIZATION WITH THERMALLY ALTERED ANTIBODIES

In order to compare the mode of action of soluble (or insoluble) antibody-antigen complexes with "fixed" antibody it was necessary to have a single antigen-antibody system which could have the following functional properties: (1) a precipitating system, (2) a non-precipitating system, (3) a complement-fixing system, and (4) a system which would not fix complement (C'). Weil has shown that heated antiserum would no longer precipitate but would passively sensitize guinea pigs, and that by reducing the pH of this antiserum to below 4.0 and then neutralizing back to 7.0, the antibody would no longer fix C'.

A. HEAT TREATMENT OF RABBIT ANTISERUM AND GUINEA PIG SERUM

Maurer (1960) has shown that with increasing temperature a diluted rabbit antiserum will become less and less precipitable. The present work shows that when undiluted serum is used the effect of temperature is increased, and that the effect of heat is not so great on guinea pig serum as on rabbit serum. At 70°C for 12-15 minutes rabbit serum forms a gel which will not disperse in a 1% NaCl solution, while guinea pig serum at 70°C for 25 minutes will form a gel which can easily be broken with a salt solution.

B. HEAT TREATMENT OF FRACTIONATED RABBIT ANTIOVALBUMIN

1. Rabbit antiovalbumin serum loses its precipitating capacity at 70°C for 12-15 minutes. On the other hand, when fractionated antibody is heated to 70°C for 30 minutes, it will give an equivalence zone much broader than that of the unheated control, and it is apparent that the amount of precipitate has increased considerably by this maneuver.

2. When Rabbit γ-globulin (antiovalbumin) is heated with guinea pig serum at 70°C for 30 minutes, the system will not precipitate antigen. The product can passively sensitize tissues as well, if not better, than an equal concentration of fractionated antibody.

3. Treatment of rabbit γ-globulin (antiovalbumin) with rabbit serum instead of guinea pig serum at 70°C for 12 minutes produces a non-precipitating system which is, perhaps, less effective in passive sensitization than the original antibody.

C. HEAT TREATMENT OF A COMPLEX OF GUINEA PIG γ-GLOBULIN AND OVALBUMIN (65°C-30 minutes)

The soluble product of this reaction will passively sensitize guinea pig gut so that it will respond to challenge with either rabbit guinea pig γ-globulin or rabbit antiovalbumin. Normal guinea pig gut will respond to rabbit anti-guinea pig γ-globulin.

D. HEAT TREATMENT OF CHICKEN ANTI-SERUM RABBIT TO γ-GLOBULIN SERUM WITH NORMAL GUINEA PIG γ-GLOBULIN

A complex is formed that will passively sensitize guinea pig gut so it will respond to a challenge of rabbit γ-globulin. The response is weak.

E. PAPER ELECTROPHORESIS OF HEATED NORMAL GUINEA PIG SERUM (or normal guinea pig γ-globulin)

1. The albumin band is greatly reduced.

2. γ-globulins of both sera eventually disappear.

3. A new, non-migrating, band is established in the presence of Veronal buffer at pH 8.6 which has the mobility of a β-globulin. Since this above complex can sensitize guinea pig gut as well as, if not better than, an equal concentration of antiovalbumin, the sensitizing capacity must reside in the non-migrating band. It is hoped that this complex can be separated by suitable fractionation procedures.

II. STUDIES ON SEA URCHIN TOXIN

Intoxication with venoms of marine forms has a long history but the documentation is usually sparse, and many of the reports available have been highly overcast with the folk-lore of littoral inhabitants. In recent years, owing to the practical problems arising from injuries to commercial fisherman, professional and amateur divers, and military personnel there has been a renewed effort to investigate the chemical nature and mechanism of action of some of these materials with the view of providing rational therapeutic maneuvers.

The sea urchin, *Tripneustes gratilla*, contains a powerful venom in the pedicellariae which is capable of killing mice and, from certain undocumented reports, of being implicated in the death of abalone divers. This material is a protein and has been purified by fractionation with ammonium sulfate.

The present studies were conducted to characterize the toxicological properties of this substance.

A. MATERIALS

The pedicellaria toxin was received from Mr. Charles Alender of the Zoology Department of the University of Hawaii as a lyophilized powder which contained about 70% protein. According to Mr. Alender this protein killed mice, produced a fatal drop in blood pressure of the rabbit, and lysed fish and rabbit erythrocytes. Work was undertaken in this laboratory to extend and amplify his observations with a view to determining the action of toxin on isolated organ and tissue systems.

B. RESULTS

1. Lethal Toxicity:

Dose-mortality studies were made on groups of Swiss-Webster mice, averaging 20 g in weight, by injecting them intravenously with various concentrations of toxin, ranging from 2.5 to 15μ, made up in 0.10 ml volumes in 1% NaCl solution. Death occurred immediately or within 10 minutes, depending on the dose used. From the results exhibited in Table I it may be estimated by graphical interpolation that the LD_{50} was 4.3γ/mouse or 0.15γ of the pure protein per gram of animal.

2. Skin Tests:

The intradermal injection of 0.05 ml portions of various concentrations of sea urchin toxin on the backs of young rabbits produced a transient wheal within 15-30 minutes of the administration of the test dose. The minimal effective dose was 1.7×10^{-3} mg. Doses as high as 1.7×10^{-2} mg produced a reaction in a shorter time, but the magnitude of the effect was not increased.

3. Rabbit Blood Pressure:

The intravenous administration of 3-6γ of toxin produced an appreciable reduction in the blood pressure of the anesthetized rabbit. Usually this could be reversed by adrenaline, but the precipitate--and fatal--drop produced by the injection of 22γ could not be so reversed. Samples of blood, withdrawn immediately post-mortem, showed that extensive hemolysis had occurred.

4. Isolated Guinea Pig Heart:

The pedicellaria toxin causes an initial increase in rate and amplitude, a decline in coronary flow and, in large doses (1.8×10^{-3} mg/g heart), an irreversible heart block. As seen from Table II the minimal effective dose is 9.1×10^{-4} mg/g heart.

5. Isolated Gut:

a. Guinea pig: The sea urchin toxin produces slow, sustained contractions of isolated segments of guinea pig gut which are characterized by having a long (60-90") latent period. The average minimal effective dose is 2.4×10^{-3} mg/100 mg of gut. Relaxation occurs after a rest period of 5-10 minutes during which the gut is washed repeatedly with Tyrode's solution. The action of the toxin is not blocked by either the separate or combined administration of atropine or pyribenzamine in doses which completely eliminate the action of acetylcholine and histamine.

b. Rat gut: Very large doses of toxin failed to produce contraction in rat gut.

6. Tests for Stability and Release of Diffusible Substances:

The effect on the guinea pig gut is eliminated by boiling the toxin in acid (pH 2) or alkali (pH 12) for 60 minutes. The toxin evidently does not cause guinea pig gut to release a specific diffusible substance capable of causing a second gut to contract.

These studies were carried out both at 20° and 37°C. Guinea pig guts were incubated in sea urchin toxin (6×10^{-4} mg/ml) and samples were withdrawn at intervals of 1, 5, 10, 15, 20, 25, 30, 45, and 60 minutes. The samples were dialyzed against an equal volume of saline and the dialyzates were tested on a second gut. Control samples were obtained by incubating the gut against Tyrode's and withdrawing samples at 1, 5, 30, 45, and 60 minutes. Neither the toxin-treated nor control samples were active on test strips.

Since the preparation was toxic to the guinea pig gut but not to rat intestine the possibility remained that histamine was released in the reaction but that it could not be detected because of the possible destruction of this agent by tissue enzymes. Accordingly, the perfusates were chemically analyzed for histamine according to the method described in Part I. Analyses for histamine proved to be negative.

7. Hemolysis:

The effect of two concentrations--2.4×10^{-2} and 2.4×10^{-1} mg/ml--of sea urchin toxin was studied on the time of appearance of lysis in the washed red cells of various species of animals. The results given in

Table III show that the fastest lysis was produced, in ascending order of velocity, by rabbit, human type O, and human type A cells, the times ranging from 15-45 minutes at room temperature. At the lower concentration of toxin lysis in guinea pig cells occurred in 10 hours, and at 15 hours in sheep and beef cells. Raising the [toxin] had no effect on the rate of lysis on the cells of group II. The same maneuver in the case of group I dramatically accelerated the rate of lysis in the guinea pig (20 minutes) but it had a much less pronounced effect on sheep and beef cells.

C SUMMARY

The toxin obtained from pedicellaria of Tripneustes gratilla is a relatively unstable protein which produces wheals; a reduction in blood pressure owing, principally, to its effects on the heart; gut contraction, and hemolysis. Since no diffusible material is produced by its action on isolated tissues and since its effect is not blocked by atropine or by pyribenzamine, the effect must be directly on the peripheral effectors. The fact that the toxin lyses red cells suggests that it may be a lecithinase. Although it is probable that all of the tissue reactions observed here could have been produced by a lecithinase, the presence of a separate substance having these pharmacological actions is not ruled out.

TABLE I

LETHAL TOXICITY OF PEDICELLARIA TOXIN

Dose (per mouse)	Total n	Died 4/10/63	Died 4/11/63	Total Dead	% Mortality
2.5γ	5	1	0	1/5	20
4.0γ	5	1	1	2/5	40
5.0γ	11	10	0	10/11	[illegible]
10.0γ	5	5	0	5/5	[illegible]
25.0γ	7	7	0	7/7	[illegible]

TABLE II

SEA URCHIN TOXIN PERFUSION STUDIES ON
ISOLATED GUINEA PIG HEART

EXPERIMENT	SEA URCHIN TOXIN DOSE mg/gm heart	EFFECT ON HEART			HISTAMINE RELEASED
		AMPLITUDE	RATE	BLOCK	
I	5.39×10^{-4}	---	---	0	0
	1.08×10^{-3}	---	+	0	0
	2.16×10^{-3}	---	+++	+++	0
II	4.58×10^{-4}	---	---	0	0
	9.14×10^{-4}	---	+	0	0
	1.83×10^{-3}	---	+++	+++	0

Minimum effective dose = 9.14×10^{-4} mg /gm heart

TABLE III

EFFECT OF PEDICELLARIA TOXIN ON THE SPEED OF HEMOLYSIS

Group I

Red Cell Type	2.4×10^{-1} mg/ml Results	Time	2.4×10^{-2} mg/ml Results	Time
Sheep	Lysis	10 hrs.	No lysis	15 hrs.
Beef	Lysis	10 hrs.	No lysis	15 hrs.
Guinea Pig	Lysis	30 min.	Lysis	10 hrs.

Group II

Red Cell Type	2.4×10^{-1} mg/ml Results	Time	2.4×10^{-2} mg/ml Results	Time
Rabbit	Lysis	15 min.	Lysis	15 min.
Human O	Lysis	30 min.	Lysis	30 min.
Human A	Lysis	30 min.	Lysis	45 min.

PART IV

EFFECT OF ANTIBODY CONCENTRATION ON THE VELOCITY OF SENSITIZATION

EFFECT OF ANTIBODY CONCENTRATION ON THE VELOCITY OF SENSITIZATION

The effect of antibody concentration on the velocity of sensitization of normal guinea pig guts to antiovalbumin was studied by determining the amount of histamine which could be released by antigen after various incubation periods in the presence of 0.0025, 0.005, 0.01, 0.02, and 0.08 mg/ml of specific antibody in the bulk phase. The tissues were washed for 1 minute before being challenged, and the histamine was estimated on an aliquot of the challenge fluid by the usual chemical method described in Part I.

Protocols for all the concentrations studied are given at the end of this section. The data were linearized by plotting the natural logarithm of the activity remaining (1 - y) against time for each concentration. As is evident from Figure 1 the lines could not be extrapolated to the origin at zero time but tended to coincide at a value somewhat greater than that. The first-order velocity constants displayed in Table I were obtained by dividing 0.693 by the appropriate time interval and were plotted against antibody concentration to produce Figure 2.

TABLE I

EFFECT OF [ANTIOVALBUMIN] ON THE VELOCITY CONSTANT

[Antibody] (mg/ml)	k(min^{-1})
0.0025	0.00976
0.0050	0.01444
0.0100	0.02390
0.0200	0.03746
0.0800	0.04620

Figue 2 shows that the velocity of sensitization rises rapidly with antibody concentration ranging between 0.0025 and 0.01 mg/ml but that it then decelerates between 0.001 and 0.02 mg/ml. Since this

function seemed to approach a limiting velocity with antibody concentration it appeared useful, if not theoretically quite appropriate, to be able to describe the general effect of antibody concentration on the reaction rate by means of the Michaelis-Menten equation.

In its reciprocal form the Michaelis-Menten equation,

$$\frac{1}{V} = \frac{1}{S} \cdot \frac{K_s}{V_{max}} + \frac{1}{V_{max}}$$

predicts that a plot of $\frac{1}{V}$ against $\frac{1}{S}$ should be linear with a slope of K_s/V_{max} and an intercept of $1/V_{max}$.

The physical meaning of the Michaelis constant, K_s, is that of the substrate concentration required to produce one-half the limiting velocity. Putting k (Table I) for V and the antibody concentration for S we see that the plot exhibited in Figure 3 fulfills the conditions of linearity. The value of V_{max}, calculated from the reciprocal of the intercept, is 0.0526 and the slope, K_s/V_{max}, is equal to 0.2175, whence K_s = 0.0115 mg/ml.

THEORETICAL DISCUSSION

The present results may be used to reopen the question of the role of antibody in the anaphylactic reaction and, possibly, to point out a method for arriving at a solution. Whereas the number of cells reacting to a given concentration of antibody can be calculated from the quantal release of histamine per cell, one of the problems which must be solved is that of the number of antibody molecules required per cell for the reaction to take place. The answer to this is complicated by the fact that we are dealing with tissues--not with individual cells--so that evidence from physical

studies of absorption are meaningless, for that process appears to take place with a velocity not susceptible of measurement by the methods at our disposal. The answer is further complicated by the justifiable inference made from our previous work that sensitization may be only velocity-limited, not concentration-limited, implying that a bare minimum of molecules might produce complete sensitization if the time is made sufficiently long. In previous studies of the effects of concentration on histamine release after one hour we have seen that the mid-point of the reaction curves tended to move in the direction of lower concentration as the temperature was increased, but that the magnitude of the final reaction was constant for all temperatures. This finding suggested to us that the observed values represented the net kinetic outcome of the effects of temperature and antibody concentration on the reaction velocity.

The participation of antibody in the anaphylactic process is understood only to the extent that the reaction of cell-fixed antibody with antigen probably activates a short-lived enzyme which releases histamine from the cell. The present results cannot be taken to suggest, merely because of their conformance to the Michaelis-Menten plot, that antibody acts as substrate: what can be said with certainty is that it does not behave, kinetically, as a typical enzyme.

The fact that increasing temperature shifts the reaction isotherms in the direction of lower concentration and permits us to calculate an activation energy from the variation of the reciprocals of the mid-concentrations at each temperature, implies that the rate constants vary regularly with temperature. Therefore, at a constant point in time, the same degree of reaction can obviously be achieved by a variety of concentrations and specified temperatures. The ratio of V/V_{max} can be shown to vary sigmoidally with the negative logarithm of the substrate concentration and it is evident that the mid-point of the curve is equal to $\log 1/K_m$.

If the distribution of our data at 60 minutes occurred because of a distribution of velocities and since the kinetics were shown to be first-order, we can calculate the velocity constants from the histamine release figures from the expression

$$k = \frac{\ln(1 - y)}{t}$$

in which $y = H/H_{max}$, $t = 60$ minutes, and $H_{max} = 18 \times 10^{-8}$ moles. These calculations are presented in Table III. The effect of changing variables from $\ln(1-H/H_{max})$ to $\ln H/H_{max}$ is merely to rotate the

curves around the value $H/H_{max} = 1/2$. Thus the behavior of v vs log s can be read off the graph for log H/H_{max} vs log s , and if the v vs log s curves have a common limit, the log H vs log s curves also should have a common limit.

Evidently the activation energy of 12 Kcal calculated from the reciprocals of the mid-point concentrations is the activation energy of the Michaelis constant. Information on the variation of the Michaelis constant with temperature is rather sparse because V_{max} of the reaction can vary independently of K_m .

In classical collision theory the reduction of K_m with temperature suggests that the proportion of molecules above the activation energy is increased by temperature. If histamine release is quantal then a given level of response obviously implies a constant number of wounded cells. On the assumption that a vulnerable cell requires the same average number of molecules for its activation we can provide a reasonable physical description of the isosteric activation energy. In order to wound a cell the reacting molecules have to be in an excited state. The proportion of molecules in a given population, having a minimum energy, is given by the simple form of the Maxwell-Boltzmann distribution equation,

$$\frac{n_1}{n_o} = e^{-E/RT}$$

in which E is the activation energy, R is the gas constant 1.987 cal/degree-mole, T is the absolute temperature, n_o is the total number of molecules, and n_1 is the number of molecules having the energy level, E . If a certain number of molecules is required to affect a given number of cells it is obvious that this value can be achieved by manipulating either the concentration or the temperature. Both variables have been shown to influence the velocity of the reaction, and the effect of increasing the temperature is to reduce the bulk phase

concentration necessary to provide a constant number of active molecules. In the present case the total number of molecules, n_o, required to furnish a constant value of n_1 declines as the temperature is raised. Since this is tantamount to saying that the value $\frac{n_1}{n_o}$ increases with temperature it is evident that the variation of log $1/n_o$ with $1/T$ is sufficient to evaluate the activation energy.

If the number of moles of histamine per reacting cell is known we can then establish the number of cells involved at any reaction level; and if n_o varies in such a way as to furnish a constant value for n_1 we can estimate the average number of molecules necessary to wound a cell. Assuming an activation energy of 12,000 (Table II) the constant value of n_1 needed for the 1/2 reaction is 1.76×10^5 molecules. However, if the activation energy were 18 Kcal as calculated from the results displayed in a recent communication from this laboratory,(Table III) we should expect to find only 7 molecules/ml above the activation energy to react with 3000 cells or, as the reaction was carried out in a 30-ml bath, 210 molecules to sensitize 15,000 cells.

Since simple collision theory provides an impossible value for the reaction ratio it is evident that the reaction mechanism involves factors of far greater complexity than the activation of γ-globulin molecules.

A more general expression of the Michaelis-Menten equation can be constructed from the theory of absolute reaction rates, in which the temperature dependence is taken into account.

According to its classical formulation the Michaelis-Menten equation expresses the velocity of the reaction at a given substrate concentration,

V_S , by the relationship

$$v_s = \frac{V\ (S)}{K_m + (S)}$$

in which K_m is the Michaelis constant and V the maximum velocity. An ammended form which would fit the data would be:

$$v(S,T) = \frac{V \cdot (S) e^{-U_1/kT}}{K_m e^{-U_2/kT} + (S)}$$

in which U_1 and U_2 are the activation energies for v and K_m , respectively, since both vary with temperature. Since K_m is usually a small number compared with (S), the influence of $K_m e^{-U_2/kT}$ on the slope of v(S,T) with respect to (S) will be small at large values of (S) , and values of k taken along a line of constant (S) can be evaluated in the usual manner for U_1 . This can then be identified as the activation energy of the overall reaction. Once U_1 has been specified numerically, the above equation for v(S,T) will describe the velocity of the reaction at all temperatures and concentrations.

The variation of V with temperature is specified by a Boltzmann distribution in accordance with the laws governing any reaction velocity: $k = Ae^{-U/kT}$, since

$$v = Ae^{-E/RT} \qquad (1)$$

(v = observed velocity, E = activation energy

and

$$A = \kappa'(kT/h)e^{\Delta S/R} \qquad (2)$$

(ΔS = entropy of activation, k = Boltzmann constant, κ' = transmission coefficient = 1, h = Planck's constant.

Thus the amended complete equation for v becomes

$$v = (kT/h)e^{\Delta S/R}e^{-E/RT} \qquad (3)$$

v_{max} will always vary with temperature, since v_{max} is defined as $v_{max} = k_3(E_o)$, where k_3 is an ordinary rate constant, which thus has a temperature dependence of the form given by (3). We can write this dependence as $v_{max} = (kT/h)e^{\Delta S_3/R}e^{-E_3/RT}(E_o)$ and thus explicitly demonstrate the variation with temperature. This means that if the temperature is raised, there will be more molecules with the correct energy available at all times, and <u>hence at all times the reaction will be able to go faster, even when the substrate concentration is maximal</u>. In other words: given two reactions, both taking place under conditions of maximal substrate concentration but at different temperatures, the velocity of the reaction in both cases will be the V_{max} appropriate to that temperature. However, at the higher temperature, more molecules of the necessary energy will be available at any given time and hence the reaction will be able to proceed at a faster rate than the reaction at the lower temperature.

We conclude that V_{max} will change with temperature, although it will remain true that at a specified temperature, V_{max} will be the maximum value which the velocity may attain.

Considering next the variation of K_m with temperature, we note that K_m can be defined as $K_m = \frac{k_2 + k_3}{k_1}$, where the k's can be identified from the scheme $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$. Since all of the k's are reaction constants, they will have a temperature dependence of the form given by (3) above. Substituting these values into the definition of Km,

we have

$$K_m = \frac{\kappa_2 (kT/h) e^{\Delta S_2/R} e^{-E_2/RT} + \kappa_3 (kT/h) e^{\Delta S_3/R} e^{-E_3/RT}}{\kappa_1 (kT/h) e^{\Delta S_1/R} e^{-E_1/RT}} \quad (4)$$

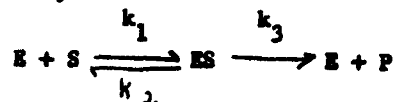
$$= \frac{\kappa_2}{\kappa_1} e^{(\Delta S_2 - \Delta S_1)/R} e^{-(E_2 - E_1)/RT} + \frac{\kappa_3}{\kappa_1} e^{(\Delta S_3 - \Delta S_1)/R} e^{-\frac{E_3 - E_1}{kT}} \quad (5)$$

where κ_1 , κ_2 , κ_3 are the appropriate transmission coefficients, and can be taken effectively as unity.

It appears that K_m will almost always vary monotonically with temperature since it contains factors of the form $e^{-(E_3 - E_1)/RT}$.

One interpretation of the increase of K_m with temperature is that since the upper limit on the maximum velocity has been increased (by raising the temperature) the concentration necessary to achieve this larger half-velocity must like wise be increased. This did not turn out to obtain in our experiments, and we might explain the phenomenon by saying that V_{max} may have greater temperature dependence than v itself.

A similar uncertainty about the variation of K_m with temperature was treated by Neurath, in traditional terms. Applied to the present case, if the reaction is enzymatic, it probably follows the usual pathway of intermediate complex formation and, since the enzyme is present in excess, the kinetics are determined by the substrate concentration and is a measure of the decomposition of $ES \rightarrow E + P$.
In the Michaelis reaction,



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

All rate constants are temperature dependent and the Michaelis constant is determined by the ratio k_3/k_1. There may be a significant dissociation of $ES \rightarrow E + S$ at a rate k_2 when the temperature drops so that K_m will be relatively great. As the temperature is raised k_2 may become negligible with respect to k_1 and the value of K_m will fall.

To apply the results of these analysis to the present data the following considerations must be borne in mind.

The sensitization reaction has a kinetic basis and eventually it comes to a constant value. The interaction between the cell receptor and the antibody molecule occurs very rapidly, as sensitization can proceed to completion if given sufficient time and temperature, after only a minute's exposure to antibody. The reversibility decreases with time, which allows us to suggest a plausible model to explain the activation energy.

1. Attachment is made by Fraction III and depends on S-S linkages arranged in a given spatial configuration. Enzymes present in cells in target tissues have the ability to break these bonds in such a way that the attachment between cells and γ-globulin becomes strong.

2. The amount of the reaction is a measure of the number of critical bonds formed per unit time.

3. The reaction follows the standard pathway of complex formation and the kinetics is determined by k_3/k_1.

4. As temperature increases, the dissociation of ES becomes negligible with the result that the value of K_m drops.

RATE STUDIES ADSORPTION HISTAMINE RELEASE

[Ab] = 0.08 mg Ab/ml Prep "53"

Sample No.	Incubation in Ab solution	Wash period (min)	Experimental Histamine released (moles/g wet weight)	t Total time from exposure to Ab (min)	1/t (min^{-1})	Normalized* values Hist. equivalents	log Hist. equivalent
99	15	1	1.72×10^{-9}	16	.0625	2.05×10^{-9}	-8.6882
100	20	1	3.38×10^{-9}	21	.0476	3.71×10^{-9}	-8.4306
101	25	1	4.73×10^{-9}	26	.0384	5.06×10^{-9}	-8.2958
102	30	1	5.76×10^{-9}	31	.0325	6.09×10^{-9}	-8.2154
104	40	1	7.50×10^{-9}	41	.0243	7.83×10^{-9}	-8.1062
105	45	1	8.20×10^{-9}	46	.0217	8.53×10^{-9}	-8.0691
106	60	1	9.80×10^{-9}	61	.0163	10.13×10^{-9}	-7.9957

*Normalized as follows:

An average value, at 46', = 8.53×10^{-9} for this [Ab]
Experimental value = 8.20×10^{-9}

Difference, + $.33 \times 10^{-9}$, added to experimental values

RATE STUDIES ADSORPTION HISTAMINE RELEASE

$[Ab] = 0.04$ mg Ab/ml Prep "53"

Sample No.	Incubation in Ab solution	Wash period (min)	Experimental Histamine released (moles/g wet weight)	t Total time from exposure to Ab (min)	1/t (min^{-1})	Normalized* values Hist. equivalents	log Hist. equivalent
115	15	1	0.77×10^{-9}	16	.0625	0.5×10^{-9}	-9.3010
116	20	1	1.28×10^{-9}	21	.0476	1.0×10^{-9}	-9.0000
117	25	1	1.95×10^{-9}	26	.0384	1.67×10^{-9}	-8.7773
118	30	1	3.17×10^{-9}	31	.0325	2.89×10^{-9}	-8.5391
119	40	1	5.26×10^{-9}	41	.0243	4.98×10^{-9}	-8.3028
	45		6.4×10^{-9} by curve	46	.0217	6.12×10^{-9}	-8.2140
120	50	1	6.58×10^{-9}	51	.0196	7.15×10^{-9}	-8.1457
121	60	1	8.26×10^{-9}	61	.0163	8.64×10^{-9}	-8.0635
122	75	1	9.17×10^{-9}	76	.0131	10.12×10^{-9}	-7.9957

*Normalized as follows:

An average value, at 46', $= 6.12 \times 10^{-9}$

Experimental value $= 6.40$

Difference $-.18 \times 10^{-9}$

RATE STUDIES ABSORPTION HISTAMINE RELEASE

[Ab] = 0.02 mg Ab/ml Prep "53"

Sample No.	Incubation in Ab solution	Wash period (min)	Experimental Histamine released (moles/g wet weight)	t Total time from exposure to Ab (min)	1/t (min^{-1})	Normalized* values Hist. equivalents	log Hist. equivalent
139	25	1	2.47×10^{-9}	26	.0384	2.85×10^{-9}	-8.5452
140	35	1	5.28×10^{-9}	36	.0277	5.56×10^{-9}	-8.2549
141	45	1	7.31×10^{-9}	46	.0217	7.69×10^{-9}	-8.1141
142	55	1	8.76×10^{-9}	56	.0178	9.14×10^{-9}	-8.0391
143	65	1	10.5×10^{-9}	66	.0151	10.88×10^{-9}	-7.9626
144	75	1	11.7×10^{-9}	76	.0131	12.08×10^{-9}	-7.9172
145	85	1	11.4×10^{-9}	86	.0116		
146	95	1	10.4×10^{-9}	96	.0104		

*Normalized as follows:

An average value at 46' = 7.69×10^{-9}
Experimental value = 7.31

Difference + $.38 \times 10^{-9}$ added to experimental curve

RATE STUDIES ADSORPTION HISTAMINE RELEASE

[Ab] = 0.01 mg Ab/ml Prep "53"

Sample No.	Incubation in Ab solution	Wash period (min)	Experimental Histamine released (moles/g wet weight)	t Total time from exposure to Ab (min)	1/t (min^{-1})	Normalized* values Hist. equivalents	log Hist. equivalent
179	20	1	$1.15 x 10^{-9}$	21	.0476	$0.94 x 10^{-9}$	-9.0269
180	30	1	$3.83 x 10^{-9}$	31	.0325	$3.62 x 10^{-9}$	-8.4413
181	40	1	$5.65 x 10^{-9}$	41	.0243	$5.44 x 10^{-9}$	-8.2644
			$6.35 x 10^{-9}$ (by curve)	46	.0217	$6.14 x 10^{-9}$	-8.2118
182	50	1	$6.93 x 10^{-9}$	51	.0196	$6.72 x 10^{-9}$	-8.1726
183	60	1	$8.78 x 10^{-9}$	61	.0163	$8.57 x 10^{-9}$	-8.0665
184	75	1	$10.2 x 10^{-9}$	76	.0131	$9.99 x 10^{-9}$	-8.0004
185	90	1	$11.5 x 10^{-9}$	91	.0109	$11.29 x 10^{-9}$	-7.9469
186	120	1	$12.2 x 10^{-9}$	121	.0082	$11.99 x 10^{-9}$	-7.9208

*Normalized as follows:

An average value at 46' = $6.14 x 10^{-9}$
Experimental value = 6.35
Difference $-.21 x 10^{-9}$ subtracted from experimental values

RATE STUDIES ADSORPTION HISTAMINE RELEASE

[Ab] = 0.005 mg Ab/ml Prep "53"

Sample No.	Incubation in Ab solution	Wash period (min)	Experimental Histamine released (moles/g wet weight)	t Total time from exposure to Ab (min)	1/t (min^{-1})	Normalized* values Hist. equivalents	log Hist. equivalent
187	25	1	1.54×10^{-9}	26	.0384	1.36×10^{-9}	-8.8665
188	30	1	1.95×10^{-9}	31	.0325	1.77×10^{-9}	-8.7520
189	40	1	3.35×10^{-9}	41	.0243	3.17×10^{-9}	-8.4989
	45	(by curve)	3.70×10^{-9}	46	.0217	3.52×10^{-9}	-8.4535
190	50	1	4.85×10^{-9}	51	.0196	4.67×10^{-9}	-8.3307
191	60	1	6.44×10^{-9}	61	.0163	6.26×10^{-9}	-8.2034
192	75	1	8.53×10^{-9}	76	.0131	8.25×10^{-9}	-8.0835
193	90	1	10.9×10^{-9}	91	.0109	10.72×10^{-9}	-7.9706
194	120	1	11.95×10^{-9}	121	.0082	11.77×10^{-9}	-7.9281

*Normalized as follows:

An average value at 46' = 3.52×10^{-9}

Experimental value = 3.70

Difference - .18 subtracted from experimental values.

RATE STUDIES ADSORPTION HISTAMINE RELEASE

[Ab] = 0.0025 mg Ab/ml Prep "53"

Sample No.	Incubation in Ab solution	Wash period (min)	Experimental Histamine released (moles/g wet weight)	t Total time from exposure to Ab (min)	1/t (min^{-1})	Normalized* values Hist. equivalents	log Hist. equivalent
195	30	1	$1.23x10^{-9}$	31	.0325	$0.40x10^{-9}$	-9.3979
196	40	1	$3.13x10^{-9}$	41	.0243	$2.3\ x10^{-9}$	-8.6383
	45	(by curve)	$3.25x10^{-9}$	46	.0217	$2.42x10^{-9}$	-8.6162
197	50	1	$3.81x10^{-9}$	51	.0196	$2.98x10^{-9}$	-8.5258
198	60	1	$5.65x10^{-9}$	61	.0163	$4.82x10^{-9}$	-8.3170
199	75	1	$8.34x10^{-9}$	76	.0131	$7.51x10^{-9}$	-8.1244
200	90	1	$11.7x\ 10^{-9}$	91	.0109	$10.87x10^{-9}$	-7.9666
201	105	1	$13.4x\ 10^{-9}$	106	.0094	$12.57x10^{-9}$	-7.8996
202	120	1	$15.0x\ 10^{-9}$	121	.0082	$14.17x10^{-9}$	-7.8477

*Normalized as follows:

An average value at 46' = $2.42x10^{-9}$

Experimental value = 3.25

Difference -0.83×10^{-9} subtracted from experimental values.

TABLE II

Temp.	[Ab]	H.E. Released	y	1-y	-ln(1-y)	k	1/k	1/C	No. Cells lysed at 60'
20°	.0091	9.57×10^{-8}	.05	.947	0.054	0.0009	1111.1	101.9	360
	.0183	1.9×10^{-8}	.105	.895	0.111	0.0019	526.3	54.6	700
	.0274	4.9×10^{-8}	.272	.728	0.317	0.0053	188.7	36.5	1,800
	.0365	7.72×10^{-8}	.429	.571	0.56	0.0093	107.5	27.4	2,860
	.0456	9.96×10^{-8}	.553	.447	0.805	0.0134	74.6	21.9	3,680
	.0548	1.23×10^{-7}	.683	.317	1.14885	0.0192	52.08	18.2	4,560
27°	.00456	1.9×10^{-8}	.106	.894	0.11205	0.0019	526.3	219.3	700
	.0091	2.5×10^{-8}	.139	.861	0.15	0.0025	400.0	109.9	920
	.018	5.2×10^{-8}	.289	.711	0.341	0.00568	176.0	55.6	1,920
	.031	10.8×10^{-8}	.60	.4	0.916	0.0153	6[illegible].4	32.26	4,000
	.044	14.2×10^{-8}	.789	.211	1.556	0.0259	38.6	22.7	5,260
37°	.000282	5.1×10^{-8}	.028	.972	0.0284	0.00047	2128	3546.0	186
	.00056	7.7×10^{-9}	.043	.957	0.044	0.00073	1370	1786.0	280
	.00112	1.55×10^{-8}	.086	.914	0.09	0.0015	666.7	892.9	580
	.00224	2.6×10^{-8}	.144	.856	0.1[illegible]	0.0026	384.6	446.4	960
	.00334	3.7×10^{-8}	.206	.794	0.244	0.0037	270.3	299.4	1,380
	.00456	4.45×10^{-8}	.247	.753	0.284	0.0047	212.8	219.3	1,640
	.018	11×10^{-8}	.611	.389	0.944	0.0157	63.7	55.6	4,080
	.036	16.2×10^{-8}	.900	.100	2.303	0.0384	26.04	27.8	6,000

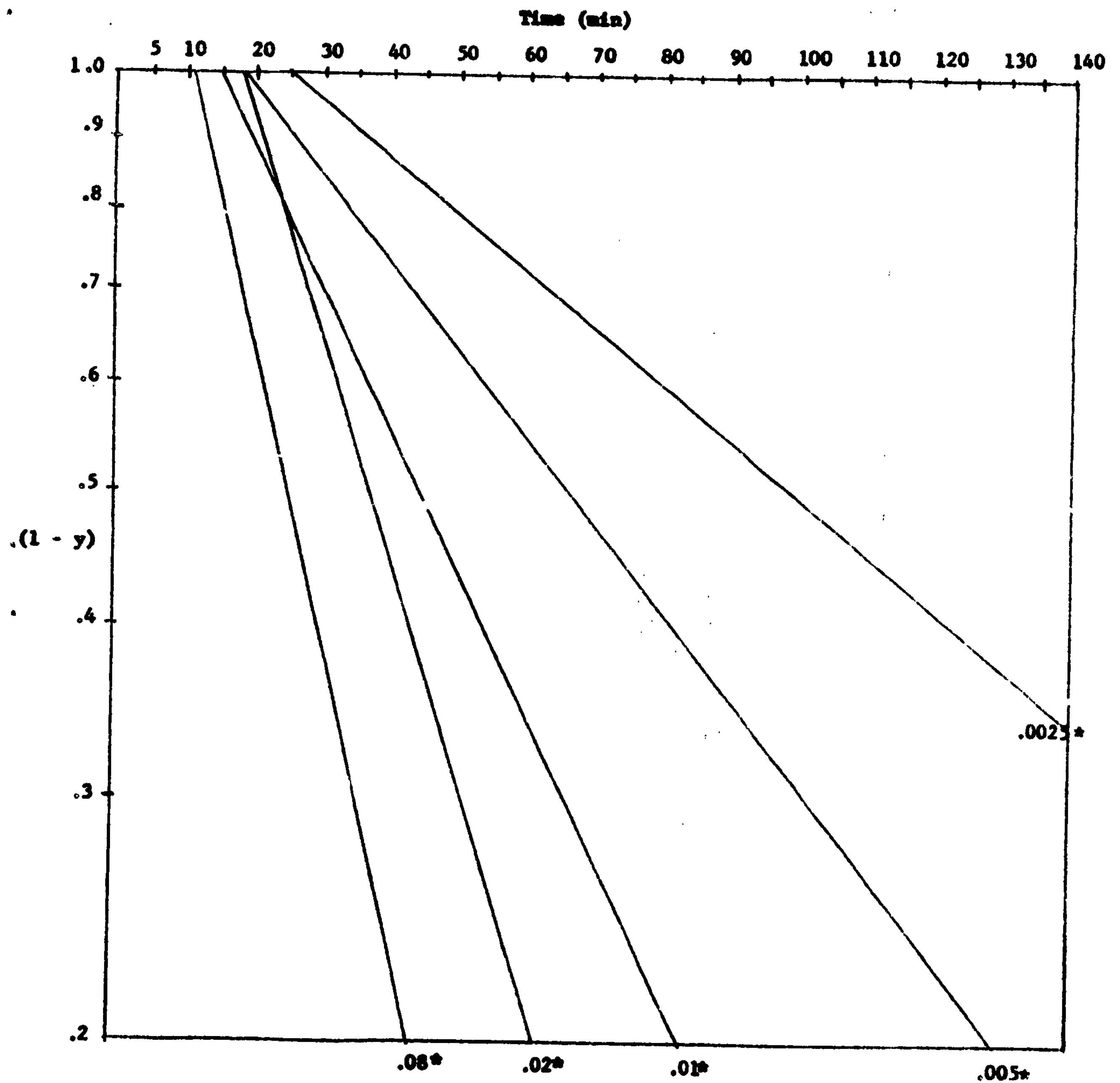
TABLE III

*Specific Ab/tissue
**Bulk-phase

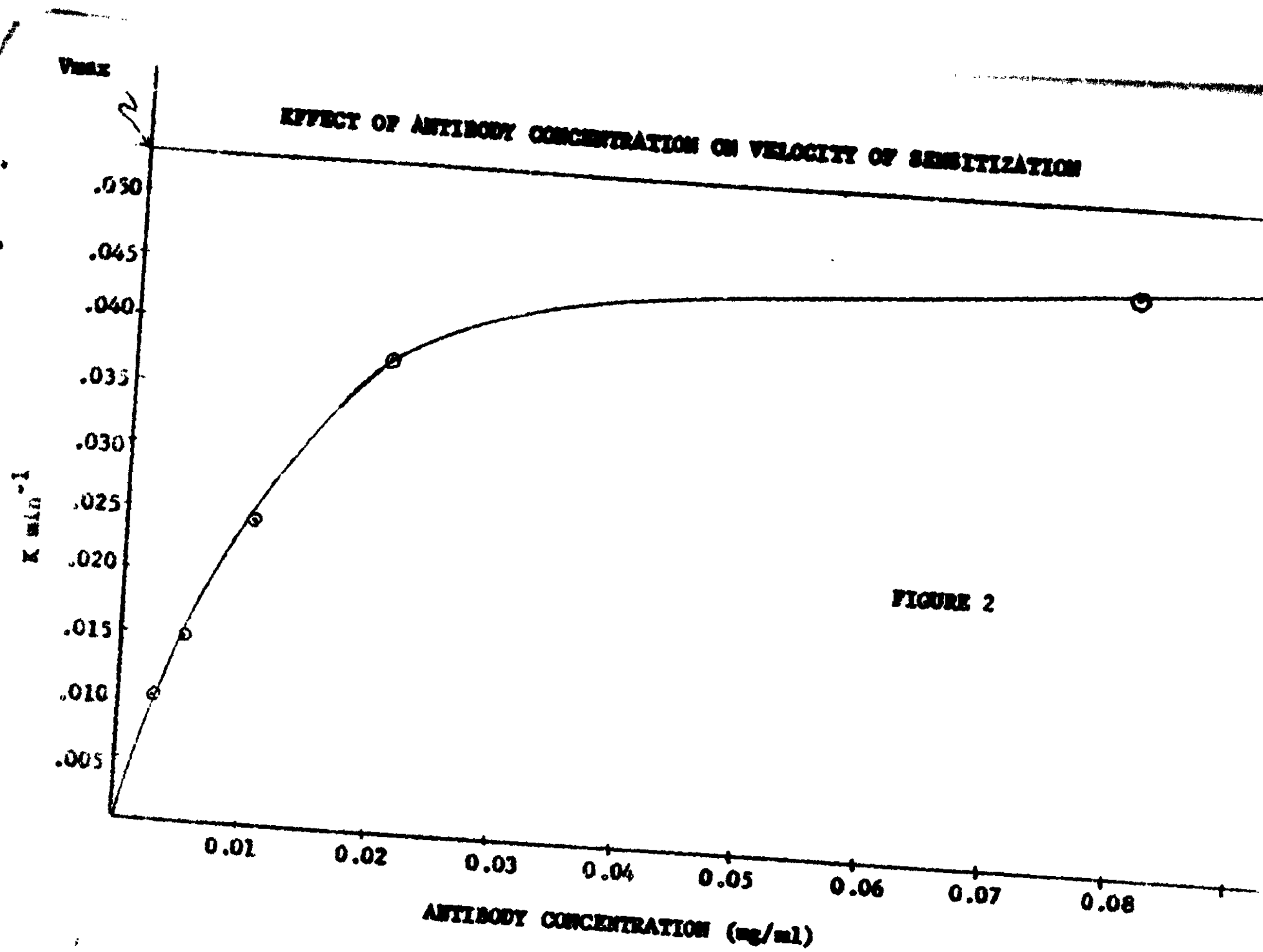
TEMP	C*	H. E.	y	1-y	-ln(1-y)	k	1/k	1/C**	C	1/spec. Ab Tissues	No. Cells Lysed
37°	.0386	3.84×10^{-8}	.96	.04	3.219	0.0537	18.6	4.39	0.228	25.9	28,800
	.0317	3.73×10^{-8}	.93	.07	2.66	0.0443	22.6	5.49	0.182	31.5	27,900
	.0162	3.56×10^{-8}	.89	.11	2.207	0.0368	27.2	11.00	0.091	61.7	26,700
	.0072	2.66×10^{-8}	.665	.335	1.094	0.0182	54.9	21.74	0.046	138.9	19,950
	.0019	2.32×10^{-8}	.58	.42	0.868	0.0145	69.0	55.0	0.018	526.3	17,400
	.0010	2.16×10^{-8}	.54	.46	0.777	0.013	76.9	111.1	0.009	1000.0	16,200
27°	.0371	3.40×10^{-8}	.85	.15	1.9	0.0317	31.5	3.66	0.273	26.95	25,500
	.0354	2.48×10^{-8}	.62	.38	0.968	0.0161	62.1	4.39	0.228	28.2	18,600
	.0321	2.64×10^{-8}	.66	.34	1.08	0.0180	55.5	5.49	0.182	31.1	19,800
	.0215	2.00×10^{-8}	.50	.50	0.693	0.0116	86.2	7.3	0.137	46.5	15,000
	.0153	1.83×10^{-8}	.46	.54	0.616	0.0103	97.1	11.0	0.091	65.4	13,800
	.0081	1.34×10^{-8}	.335	.665	.408	0.0068	147.0	21.74	0.046	123.5	10,050
20°	.0482	2.37×10^{-8}	.59	.41	.891	0.0149	67.1	3.66	0.273	20.75	17,700
	.0391	2.70×10^{-8}	.675	.325	1.124	0.0187	53.5	4.39	0.228	25.6	20,250
	.0331	1.87×10^{-8}	.468	.532	.63	0.0105	95.2	5.49	0.182	30.2	14,040
	.0236	1.58×10^{-8}	.395	.605	.5	0.0083	120.4	7.3	0.137	42.4	11,850
	.0216	1.32×10^{-8}	.33	.67	.4	0.0064	150.0	11.0	0.091	46.3	9,900
	.0103	1.08×10^{-8}	.27	.73	.315	0.00525	190.5	21.74	0.046	97.1	8,100

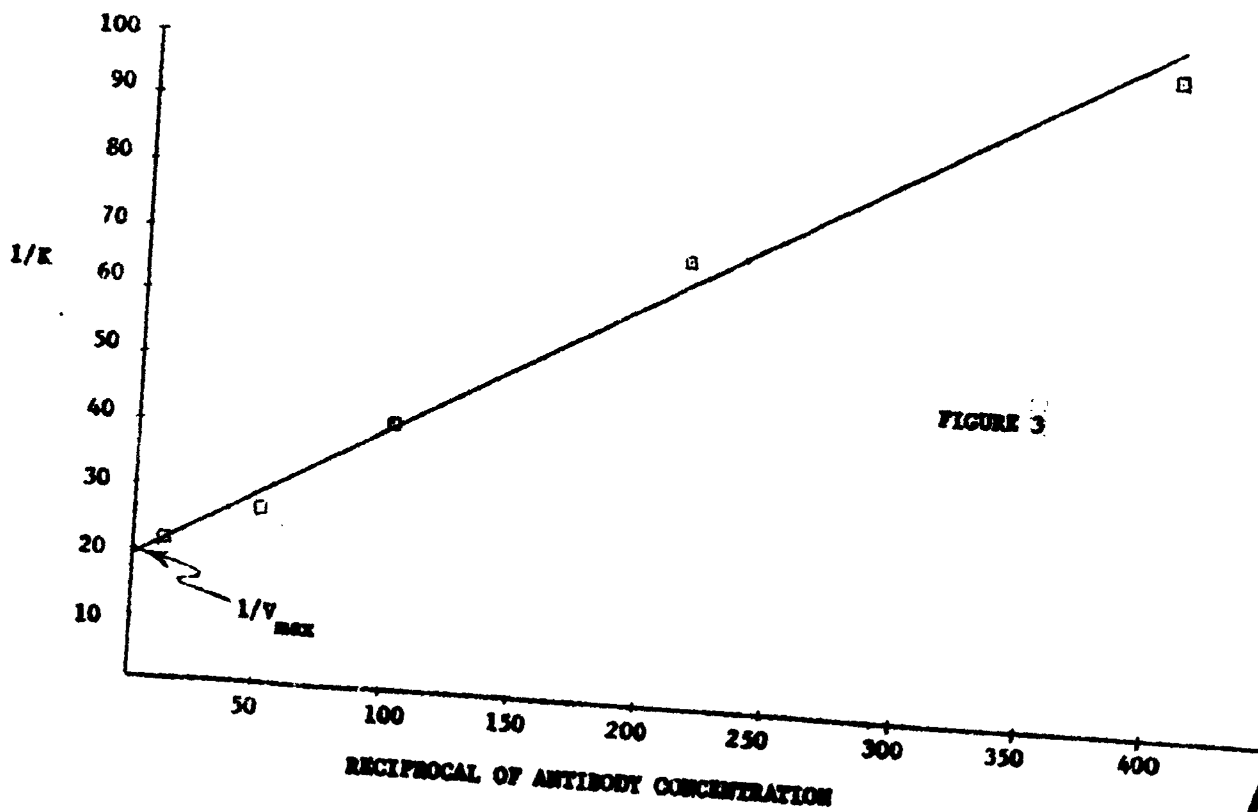
FIGURE 1

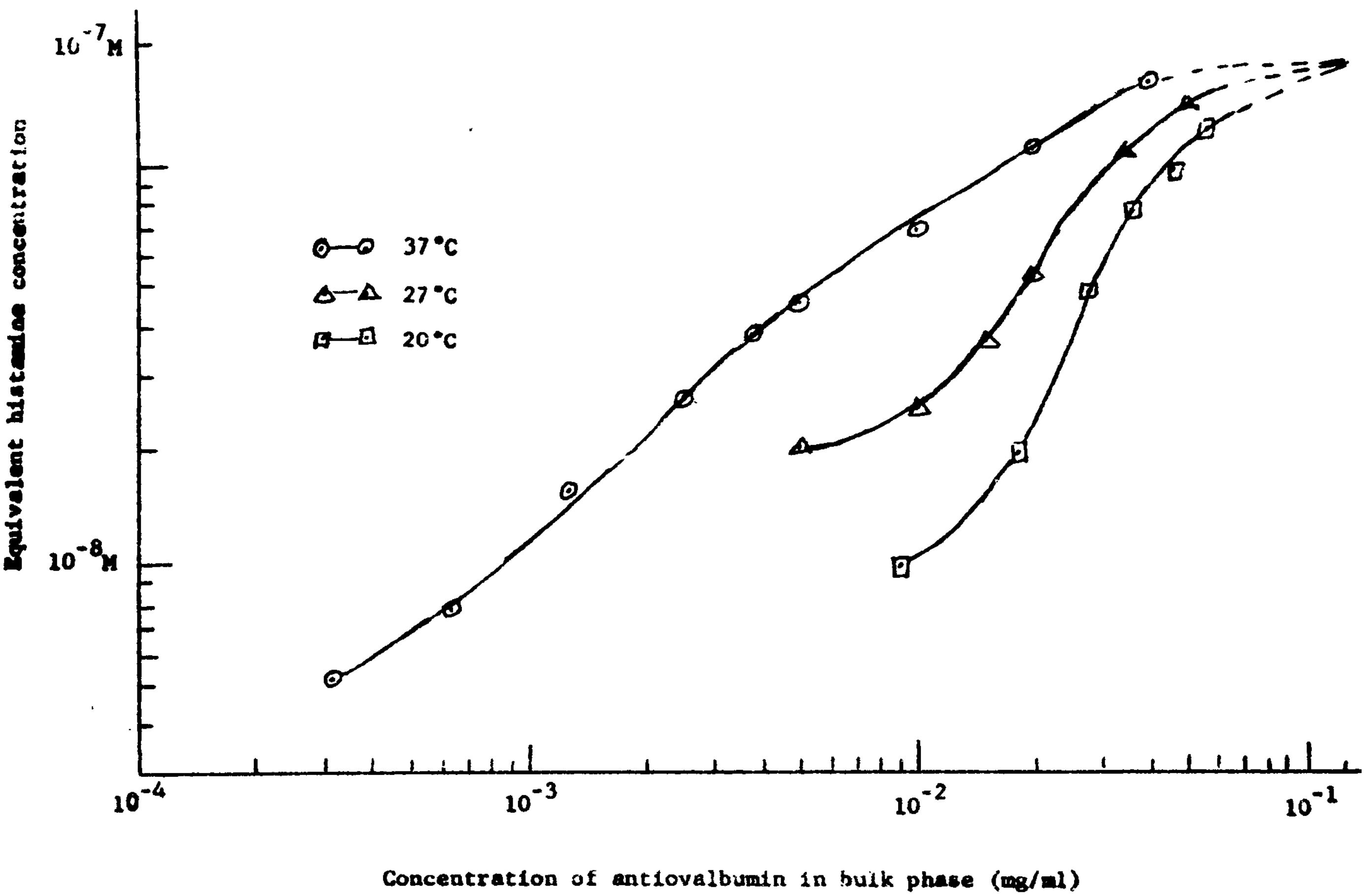
TIME COURSE OF SENSITIZATION AS A FUNCTION OF ANTIBODY CONCENTRATION



* Milligrams of antiovalbumin per milliliter


Vmax
EFFECT OF ANTIBODY CONCENTRATION ON VELOCITY OF SENSITIZATION
.050
.045
.040
.035
.030
.025
.020
.015
.010
.005
K min-1
FIGURE 2
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
ANTIBODY CONCENTRATION (mg/ml)


100
90
80
70
60
50
40
30
20
10
1/K
FIGURE 3
1/Vmax
50
100
150
200
250
300
350
400
RECIPROCAL OF ANTIBODY CONCENTRATION



In vitro sensitization:
Effect of antibody concentration and incubation temperature on reaction to antigen
Equivalent histamine concentration
10^{-7}M
10^{-8}M
37°C
27°C
20°C
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Concentration of antiovalbumin in bulk phase (mg/ml)