level of inhibition is concentration dependent. The inhibitor is destroyed by heating and is apparently not dialyzable, which may be indicative of a macromolecular nature.

1. Vohra, P., Kratzer, F. H., Poultry Sci., 1964, v43, 1164.
2. Anderson, J. O., Warnick, R. E., ibid., 1964, v43, 1091.
3. Bakshi, Y. K., Couch, J. R., Creger, C. R., ibid., 1964, v43, 1302.
4. Couch, J. R., Bakshi, Y. K., Prescott, J. M., Creger, C. R., Fed. Proc., 1965, v24, 687.
5. Borchers, R., Ackerson, C. W., J. Nutrition, 1950, v41, 339.
6. Dijkstra, N. D., Landbouwkundig Tijdschrift, 1962, v74, 822.
7. Anson, M. L., J. Gen. Phys., 1938, v22, 79.
8. Northrop, J. H., Kunitz, M., Herriott, R. M., Crystalline Enzymes, Columbia Univ. Press, New York, 1948, Chap. VI, p125.

Received June 27, 1966. P.S.E.B.M., 1966, v123.

Termination of Immunologic Tolerance in Mice by Antigen-Antibody Complexes.* (31462)

F. E. HEMPHILL,† D. SEGRE, AND W. L. MYERS
College of Veterinary Medicine, University of Illinois, Urbana

The theory of antibody formation of Eisen and Karush(1) proposes an extracellular control of tolerance and immunity exerted by circulating antibodies. According to Eisen and Karush, the ratio of the concentrations of antigen and antibody (natural or immune) determines whether the outcome of the exposure of an animal to an antigen will be immunity or tolerance. Thus, it should be possible to terminate immunologic tolerance by manipulations intended to shift the ratio of antigen to antibody from one giving rise to complexes of 2 molecules of antigen with one of antibody (Ag_2Ab) assumed by the model to result in tolerance, to one favoring the formation of complexes of one molecule of antigen with one of antibody (AgAb) which, according to the model, will induce immunity. Since the Ag_2Ab complexes are assumed to be unable to gain entrance into the immunologically competent cells, a specific prediction of this model is that tolerant animals should contain cells immunologically competent to respond to the tolerated antigen. Because other theories of antibody formation explain tolerance by the absence or the repression of immunologically competent cells genetically capable of synthesizing antibody specific to the tolerated antigen, the demonstration of the existence of inducible cells in lymphoid tissues of a tolerant animal, and their induction by bimolecular complexes of antigen and antibody, would provide strong support for an extracellular control of tolerance and immunity of the type postulated by Eisen and Karush. Successful attempts to terminate tolerance by the means outlined are described here.

Materials and methods. Fifty-four NIH strain white mice‡ received 3 subcutaneous injections of 20 mg of crystallized human serum albumin (HSA)§ each on the day of birth and at 2 and 3 weeks of age. A fourth injection of 20 mg HSA was given intraperitoneally at 6 weeks of age to insure maintenance of the tolerant state. At 6 weeks of age the mice also received 20 μg of radioiodinated human serum albumin (I^{131} HSA).‖

* These studies were aided by Contract Nonr-1834 (37) (NR-103-509) with Office of Naval Research, Dept. of the Navy, and by contract AT (11-1)-1628 with U. S. Atomic Energy Commission.

† Captain, U. S. Air Force, assigned to Univ. of Illinois under sponsorship of Air Force Inst. of Technology. Present address: U. S. Army Biological Center, Fort Detrick, Maryland.

‡ Adult mice were obtained from Belair Acres Laboratory Animals, Danville, Ind., and bred at the authors' laboratory.

§ Pentex, Inc., Kankakee, Ill.

‖ Abbott Laboratories, North Chicago, Ill. I^{131} HSA had a specific activity of approximately 10 μc/mg and was used within 48 hours of receipt.

Throughout the experiment, the mice had access to drinking water containing sodium iodide, to prevent excessive uptake of I^{131} by the thyroid. The mice were challenged at 5 weeks of age with 100 μg of soluble HSA given intravenously. The state of tolerance was ascertained by the absence of antibody in blood obtained from the periorbital sinus, as determined by the hemagglutination (HA) test of Boyden (2) as modified by Stavitsky (3), and by measurement of the rate of I^{131} HSA eliminated from the mice as determined by whole-body counting. HA titers were expressed as the number of the endpoint tube in a series of doubling serum dilutions starting with a dilution of 1:20. At 7 weeks, the tolerant mice were divided into 5 groups, 4 of which were splenectomized and subjected to the treatments described below. The fifth group was untreated at this time and served as an indicator of the spontaneous termination of tolerance.

Antigen and antibody were mixed by adding 1.7 volumes of rabbit anti-HSA¶ to 1 volume of a 0.1% solution of HSA in Hanks' balanced salt solution (HBSS). Each 0.27 ml of the mixture contained 100 μg of HSA and 260 μg of rabbit anti-HSA. These quantities of antigen and antibody were calculated to be equimolar on the basis of molecular weights of 60,000 for HSA and 160,000 for rabbit immunoglobulin G antibody. A mixture with an antigen: antibody molecular ratio of 10:1 was prepared similarly, but with the rabbit anti-HSA previously diluted 1:10 in HBSS. The mixtures were stored overnight at 5C.

Single-cell suspensions were made separately from the spleens of the mice in Groups I to IV. The spleen cells were washed 3 times in HBSS. The packed cells from mice of Group I were then resuspended in 0.27 ml of the mixture made with equimolar solutions of antigen and antibody. Cells from mice of Group II were resuspended in the same quantity of the mixture made at an antigen: antibody molecular ratio of 10:1. Cells from mice of Groups III and IV were resuspended in preparations in which antibody, and both antigen and antibody respectively, had been replaced by equal volumes of HBSS.

The resuspended spleen cells were incubated for 2 hours at 37C. Vital and total cell counts were made with a hemocytometer, with and without addition of trypan blue, both prior to and after incubation of the cell suspensions.

The splenic cells were then injected intraperitoneally in the same mouse from which they had been removed. One week later, the mice were again assayed for antibody by HA test and by measurement of the rate of I^{131} HSA elimination following intraperitoneal injection of 20 μg I^{131} HSA. At 9 weeks of age, the mice were challenged by foot pad injection of 100 μg of HSA in incomplete Freund's adjuvant.** Normal, non-tolerant mice (Group VI), as well as the tolerant, but previously untreated, mice of Group V, were similarly challenged. At weeks 10 and 11, all mice were tested by HA and, at week 10, by measurement of the rate of antigen elimination to assess their immunologic state. The experiment was terminated when the mice were 11 weeks old. The number of mice per group, the treatments of the various groups and the sequence of treatments are summarized in Table I.

Results. Induced immunologic tolerance was terminated in 55% of the mice in the principal Groups I and II, whose splenic cells had been exposed *in vitro* to antigen-antibody complexes in molar ratios of 1:1 and 10:1 respectively. In contrast approximately 90% of the control animals (groups III and IV) whose cells were exposed *in vitro* to HSA only or were not exposed to antigen or antibody remained tolerant. Only one mouse in each of the control groups III and IV showed an antibody response following a challenge injection of HSA in incomplete Freund's adjuvant at 9 weeks of age. Group V was composed of 5 tolerant mice, 2 of which spontaneously broke tolerance during the 10th week of the experiment. Group VI, consisting of normal non-tolerant controls, received only

¶ Lot No. 1104632, obtained from Sylvana Co., Millburn, N. J. The serum contained 1.5 mg of antibody/ml as determined by quantitative precipitation with HSA.

** Difco Laboratories, Detroit, Mich.

the challenge injection of 100 μg of HSA in incomplete Freund's adjuvant. All of the mice in this group gave an immune response (Table II).

The mean tube titers of the principal groups (I and II) were greater than the mean tube titers of control groups III, IV and V; however, they were not as high as the mean tube titer of the normal non-tolerant mice receiving the same immunizing dose of antigen (Table II).

The results were statistically analyzed using the Chi-square test. Table III summarizes the statistical comparison between the various groups of mice.

No significant difference was observed between Groups I and II since the percent responders in both groups were the same. Similarly, no significant difference was observed between the antigen only or HBSS controls (Groups III and IV). When the percent of responders in Groups I and II were pooled and compared with the percent of responders of pooled Groups III and IV, there was a significant difference ($P = 0.014$) between the two pooled groups. There was also

TABLE I. Treatment of Principal and Control Groups of Mice.

Age	Treatment	Groups I	II	III	IV	V	VI
1 day	20 mg HSA subcutaneously	+	+	+	+	+	—
2 wk	*Idem*	+	+	+	+	+	—
3 "	"	+	+	+	+	+	—
5 "	100 μg HSA intravenously	+	+	+	+	+	—
6 "	HA test	+	+	+	+	+	—
6 "	20 μg I^{131} HSA + 20 mg cold HSA intraperitoneally	+	+	+	+	+	—
6-7 "	Measurement of rate of HSA elimination	+	+	+	+	+	—
7 "	Splenectomy	+	+	+	+	—	—
	Exposure of spleen cells *in vitro* to:						
	1. AgAb (1:1 molar ratio)	+	—	—	—	—	—
	2. AgAb (10:1 molar ratio)	—	+	—	—	—	—
	3. Ag only (100 μg)	—	—	+	—	—	—
	4. HBSS*	—	—	—	+	—	—
8 "	HA test	+	+	+	+	+	—
8-9 "	Measurement of rate of HSA elimination	+	+	+	+	+	—
9 "	100 μg HSA in incomplete Freund's adjuvant in 3 foot pads	+	+	+	+	+	+
10 "	HA test	+	+	+	+	+	+
10-11 "	Measurement of rate of HSA elimination	+	+	+	+	+	+
11 "	HA test	+	+	+	+	+	+
	No. of mice	11	9	9	8	5	12

+ = treatment received. — = treatment described was not done.

* Hanks' Balanced Salt Solution.

TABLE II. Immune Response of Principal and Control Mice.

Group	No. of responders/No. in group	% Responders	Range of HA tube titers	Mean tube titer (±S.D.*) of: Responders	Group
I					
AgAb complex (1:1 ratio)	6/11	55	2–7	5.16 ± 1.70	2.81 ± 2.86
II					
AgAb complex (10:1 ratio)	5/9	55	3–11	5.20 ± 2.99	2.88 ± 3.42
III					
Ag only	1/9	11	2	2	.22
IV					
HBSS†	1/8	12	5	5	.62
V					
Observation	2/5	40	1–3	2.00 ± 1.00	.80 ± 1.36
VI					
Normal nontolerant	12/12	100	1–6	3.66 ± 1.26	3.66 ± 1.26

* S.D. = Standard deviation.

† Hanks' Balanced Salt Solution.

a significant difference ($P<0.05$) when either Group I or Group II was compared with Group III. Although 40% of the mice in Group V spontaneously responded, the Chi-square test showed no significant difference between this group and the controls of Groups III and IV. No significant differences were found between principals and controls so far as mean HA titers are concerned.

Discussion. The immune or tolerant state of the mice was assessed by both HA testing and measurement of the rate of antigen elimination. In general, there was good agreement between the results of the two procedures. The half time (T ½) of I^{131} HSA in mice with HA antibodies was approximately 4.5 hours. The T ½ of I^{131} HSA in mice considered to be tolerant because their sera were negative by HA was 20 hours or longer. However, occasionally mice positive by the HA test eliminated the antigen in a non-immune manner (T ½$\geqq$20 hr), and mice negative by the HA test eliminated the antigen at an accelerated rate (T ½$\leqq$6 hr). Some inconsistencies in the rates of antigen elimination were also found upon examination of the results obtained at successive challenges of the same individual mouse. For example, a mouse in Group III eliminated HSA with a T ½ of 22.5 hours at 6 weeks of age, one week after the first challenge. The T ½ of HSA in the same mouse was 6 hours one week after splenectomy (8 weeks of age), and 20 hours one week after challenge with HSA in incomplete Freund's adjuvant (10 weeks of age).

The reasons for the occasional lack of correlation between the results of the HA test and the rate of HSA elimination are not clear, although the failure of certain antibodies to cause accelerated elimination of HSA in mice has been reported(4). In view of the inconsistencies in rates of HSA elimination observed in individual tolerant mice on repeated testing, however, it was decided to rely on the results of the HA test in the few cases in which there was no agreement between HA and rate of HSA elimination.

It was important to ascertain the effect of the *in vitro* treatments to which the spleen cells were subjected on their viability. The mean percent viability of the cells, as determined by the trypan blue exclusion test, was 87.7 prior to washing and incubation as compared to 86 after incubation. Therefore, washing of the cells and incubation at 37C for 2 hours did not seem to affect their viability.

TABLE III. Statistical Comparisons.

Group and treatment	Group comparisons	X^2 values
I 1:1 ratio AgAb	I *vs* II	No signif. diff.
II 10:1 ratio AgAb	I *vs* III	($P<.05$)
III Antigen only	II *vs* III	($P=.05$)
IV HBSS*	III *vs* IV	No signif. diff
V Observation	V *vs* III & IV	" " "
VI Normal, non-tolerant	I & II *vs* III & IV I & II *vs* VI	$P=.014$ $P<.01$

* Hanks' Balanced Salt Solution.

In order to avoid possible loss of viability of the cells through excessive manipulations, additional washing after *in vitro* exposure to the antigen-antibody complexes or to antigen was omitted. The risk of conferring passive immunity to the recipient mice through injection of rabbit antibody present in the fluid phase of the cell suspensions was thought to be minimal, since the rabbit antibody should have been neutralized by antigen present in equimolar or greater concentration. The failure to detect immunity among the recipient mice one week after splenectomy confirmed the belief that the injected rabbit antibody did not interfere with the interpretation of the results.

According to the model of Eisen and Karush(1) it should be possible to terminate tolerance by introducing preformed antibodies so as to shift the antigen:antibody ratio from excess antigen to a more nearly equimolar ratio, which should favor the formation of immunogenic, bimolecular AgAb complexes. Attempts to terminate tolerance by administration of antibody have been reported with varying results. Mitchison(5) was able to terminate tolerance to turkey erythrocytes in chickens by passive administration of specific

antibody. Hasek and Puza(6) reported similar findings in ducks tolerant to erythrocytes of ducks of a different genus. In contrast, Weigle was unable to terminate tolerance to bovine serum albumin (BSA) in rabbits by passive administration of antibody (7) or by injection of antigen-antibody precipitates formed at equivalence(8). Friedman's(9) attempts to terminate tolerance to *Shigella* soluble antigens in mice resulted in only a temporary suppression of the tolerant state when antibody was passively administered. The mice reverted to the tolerant state as soon as the passive antibody was catabolized.

The successful termination of tolerance to erythrocytes by passive immunization(5,6) and the failure to terminate tolerance to soluble antigens by similar means(7,9) may be related to a fundamental difference between the nature of tolerance to particulate antigens and that of tolerance to soluble antigens. Friedman(9) suggested that *Shigella* soluble antigens may establish an intracellular depot in the tolerant animal and become protected from interaction with antibody. Conceivably, intracellular erythrocytic antigens may be rapidly degraded. In the latter case, maintenance of tolerance would depend exclusively on the persistence of circulating antigen, which would be accessible to passively injected antibody.

In line with Eisen's and Karush's model(1), the failure to terminate tolerance by passive administration of antibody may have resulted from failure to establish the antigen:antibody ratio which would result in formation of sufficient bimolecular complexes to stimulate antibody formation. While the quantities of antigen and antibody used by Weigle(7) would have resulted in an antigen:antibody molecular ratio of approximately 2:1, after correction for decay of antibody during the 2-day interval between administration of immune serum and of antigen, residual antigen in the tolerant rabbits may have resulted in a larger antigen excess, especially if the antigen or its determinants persisted in intracellular depots. The same reasoning applies to Friedman's work(9), with the additional uncertainty as to the numbers of different antigens present in the *Shigella* extract and the quantity of antibodies injected. Weigle(8) also failed to terminate tolerance to BSA in rabbits by injection of BSA-rabbit anti-BSA precipitates made at equivalence and emulsified in Freund's complete adjuvant. Since precipitates formed at equivalence are known to contain more antibody than antigen on a molecular basis, they would not be expected to induce immunity according to Eisen's and Karush's model.

In the present work the problem of residual antigen was circumvented by removing the immunologically competent splenic cells from the excess antigen environment. The cells could then be treated *in vitro* under environmental conditions in which the antigen:antibody ratio could be accurately measured. The immunologically competent cells exposed *in vitro* to the appropriate concentrations of antigen-antibody complexes presumably took up the complexes and, after having been returned to the host, produced antibody which in turn formed bimolecular complexes with the residual antigen and induced other immunologically competent cells to produce specific antibody against the tolerated antigen. Thus, when the antibody concentration was sufficiently high to establish the proper antigen:antibody ratio, tolerance was broken.

Antigen-antibody mixtures in molar ratio of 10:1 were as effective in terminating tolerance as were the equimolar mixtures (Table II). The rationale for inclusion of 10:1 antigen:antibody mixtures among the experimental treatments stems from the work of Segre and collaborators, (10,11,12,13) who reported that injections of mixtures of antigen with small quantities (presumably less than equimolar) of corresponding antibody were more effective in stimulating antibody formation in colostrum-deprived piglets than were injections of antigen alone.

The ability of antigen-antibody complexes prepared in antigen excess (10:1 ratio) to induce immunity in tolerant mice may appear to contradict Eisen's and Karush's postulate that Ag_2Ab complexes do not immunize but induce tolerance. However, the two types of complexes, Ag_2Ab and AgAb, should be in equilibrium with one another and with free

antigen in the mixture. If both free antigen and Ag_2Ab complexes are incapable of being taken up by the immunologically competent cells, as maintained by Eisen's and Karush's model, their presence and concentration should have no influence on the chance of the AgAb complexes to encounter immunologically competent cells. Such chance should be dependent only on the relative numbers of immunologically competent cells and of AgAb complexes, and on the concentrations of both cells and complexes. In a small reaction volume as used in this work (0.27 ml) the concentration of both cells and complexes was high. The number of immunologically competent cells was unknown, but it was smaller than the total number of splenic cells (approximately 10^8). The quantity of HSA used for *in vitro* exposure was 100 μg. This quantity corresponds to 10^{15} molecules of HSA (molecular weight 60,000). Even if only 1% of the molecules of HSA had formed bimolecular AgAb complexes, there would have been 10^{13} such complexes in the reaction mixtures. Even assuming that all the spleen cells were immunologically competent, which was obviously not the case, there would have been 10^5 AgAb complexes per cell. Therefore, collision between inductive complexes and inducible cells should have been a likely and frequent event when the cells were exposed to the mixtures made at an antigen:antibody ratio of 10:1.

The spontaneous loss of tolerance in 2 of 5 mice of Group V would seem to indicate that tolerance of the principal mice was waning, thus detracting significance from the results obtained in Groups I and II. However, mice of Group V were not splenectomized and cannot be regarded as the appropriate controls for groups I and II. Mice of Groups III and IV, in contrast, were splenectomized and treated in all other respects as the animals of Group I and II, but their spleen cells were not exposed to the antigen-antibody complexes. Groups III and IV, therefore, should be regarded as the appropriate controls for Groups I and II. We have no explanation for the high rate of spontaneous loss of tolerance in Group V. In later work, 7 mice were treated as those of Group V, except that they received a fourth subcutaneous injection of 20 mg HSA at 5 weeks of age. All other treatments and tests were done as described for Group V (Table I), but they were delayed 2 weeks. All 7 mice were tolerant throughout the 13-week period.

The results reported here appear consistent with the assumptions of Eisen and Karush (1) that bimolecular AgAb complexes may be necessary for immunization and that there are cells susceptible to immunization in a tolerant animal, once they are removed from their environment and exposed to the appropriate antigenic stimulation.

Summary. Eisen and Karush(1) proposed a model for the extracellular control of immunity and tolerance based on the assumption that bimolecular antigen-antibody (AgAb) complexes stimulate antibody formation, while trimolecular Ag_2Ab complexes induce immunologic tolerance because they are not taken up by the immunologically competent cells. The model predicts that it should be possible to initiate an immune response in cells from tolerant animals provided they are stimulated with the appropriate AgAb complexes. Newborn NIH white mice were made tolerant to human serum albumin (HSA). At 7 weeks of age, single-cell suspensions were prepared from the individual spleens of the tolerant animals and exposed *in vitro* to HSA and rabbit anti-HSA mixed in 1:1 or 10:1 molecular ratios, to HSA only, or to a salt solution. The spleen cells were then injected intraperitoneally in the same mouse from which they had been removed. After challenge with HSA in incomplete Freund's adjuvant, the mice were tested by the tanned-cell hemagglutination procedure and by measurement of the rate of HSA elimination. Within 2 weeks following challenge, tolerance was broken in 55% of the mice whose cells had been exposed to AgAb complexes. In contrast, almost 90% of the mice whose cells had been treated with antigen only or with saline remained tolerant.

1. Eisen, H. N., Karush, F., Nature, 1964, v202, 677.
2. Boyden, S. V., J. Exp. Med., 1951, v93, 107.
3. Stavitsky, A. B., J. Immunol., 1954, v73, 360.

4. Morrison, S. L., Terres, G., ibid., 1965, v94, 667.
5. Mitchison, N. A., Immunology, 1962, v5, 359.
6. Hasek, N., Puza A., in Mechanisms of Immunological Tolerance, N. Hasek, A. Lengerová, N. Vojtíšková, Ed., Academic Press, Inc., New York, 1961, p257.
7. Weigle, W. O., J. Immunol., 1964, v92, 113.
8. ———, J. Exp. Med., 1962, v116, 913.
9. Friedman, H., J. Immunol., 1965, v94, 921.
10. Segre, D., Kaeberle, M. L., ibid., 1962, v89, 782.
11. ———, ibid., 1962, v89, 790.
12. Myers, W. L., Segre, D., ibid., 1963, v91, 679.
13. Locke, R. F., Segre, D., Myers, W. L., ibid., 1964, v93, 576.

Received June 29, 1966. P.S.E.B.M., 1966, v123.

Purification of Propionin, an Antiviral Agent from *Propionibacteria.* (31463)

S. RAMANATHAN, G. READ AND W. CUTTING
Department of Physiology and Pharmacology, School of Medicine, University of Hawaii, Honolulu

Extracts of *Propionibacterium freudenreichii* exhibit antiviral activity against Columbia SK virus *in vivo* and *in vitro,* and against vaccinia virus *in vitro*(1,2). The active agent(s) is referred to as propionin. A microcrystalline substance, active primarily against vaccinia virus *in vitro,* has been separated and is called propionin A; it seems to be a minor constituent(3). The purification of the agent(s) active against Col. SK virus is described in this report.

Materials and methods. 1. Source of effluents: The starting materials for these studies were effluents prepared from *Propionibacterium freudenreichii* grown in agitated culture in an enriched medium. The bacteria were separated from the medium by centrifugation and then subjected to moderate heating (80°C) to release their contents.

2. Virus host system in mice: Fractions of propionin were injected subcutaneously 2 hours after intraperitoneal inoculation of LD_{70-90} of Col. SK virus (brain adapted) and continued twice a day for 4 days; observation was continued for an additional 5 days. Ten or 20 mice were treated and 40 mice were controls in each experiment.

3. Purification methods: Precipitation with organic solvents, gel filtration(4), descending paper chromatography(5), dialysis, ion-exchange chromatography(6), and precipitation at different pHs were used.

Experimental and results. Precipitation with organic solvents: A preliminary purification was effected by precipitating the active component by addition of different organic solvents to the source effluent. The activity of the precipitate was correlated with the polarity of the solvent combination, expressed as the dielectric constant. The various precipitates obtained at different dielectric constants were redissolved in water and tested for activity against Col. SK in mice (Fig. 1).

About 40% of the inactive material could be removed at a dielectric constant of 55 (50% methanol) and the subsequent precipitate obtained at a dielectric constant of 28 (one volume of methanol, 2 volumes of n-propanol and 2 volumes of acetone to 1 volume of effluent) contained about 10% of the solids and most of the activity. Precipitates at intermediate dielectric constants exhibited lesser degrees of activity.

Gel filtration on Sephadex G-25: The active precipitate, dissolved in water, was passed through a column of Sephadex G-25, whereon it resolved into 3 different fractions. The first fraction (brown in color) contained most of the activity, while the second fraction (red) and the final fraction (yellow) were toxic and probably inactive (Fig. 2). Similar results were obtained when the crude effluent, without organic solvent treatment was passed through the column. But it is preferable to remove the inactive material with organic solvent to improve the separation and prolong the life of the column. The dry weight of the active fraction is about 7% as against 45 to