

ORIGINAL ARTICLES

Antigenicity of the tetracyclines

Jose T. Queng, M.D.,* C. Dean Dukes, Ph.D., and
John P. McGovern, M.D., Houston, Texas

Specific antisera to oxytetracycline were produced in rabbits. The antibody was shown to be in the globulin fraction by DEAE-Sephadex separation. Its activity was demonstrated by tanned-cell hemagglutination, hemagglutination-inhibition, and the Schultz-Dale smooth muscle contraction phenomenon. Weakly reactive antibodies of broad specificity were produced to tetracycline, chlortetracycline, and demethylchlortetracycline. By tanned-cell hemagglutination these latter antisera of low titer were found to cross-react with all four tetracyclines.

The "tetracyclines" include four related compounds commonly used in antimicrobial therapy, namely, chlortetracycline, oxytetracycline, tetracycline, and demethylchlortetracycline (Fig. 1). The increasing clinical use of these potent antibiotics has resulted in a number of "reactions" being reported in the literature.¹⁻⁸ These reactions have been considered to be allergic in nature and, generally, of the immediate type of hypersensitivity. Patch and intracutaneous tests with the suspected tetracyclines have been nondiagnostic in determining the presence or absence of hypersensitivity in patients.^{2, 4, 7, 8}

Hypersensitivity to the tetracyclines, though often suspected clinically, has not been substantiated by significant laboratory evidence to date. This study was undertaken to investigate the antigenic activity of the tetracyclines, to determine whether circulating antibodies to the tetracyclines can be produced

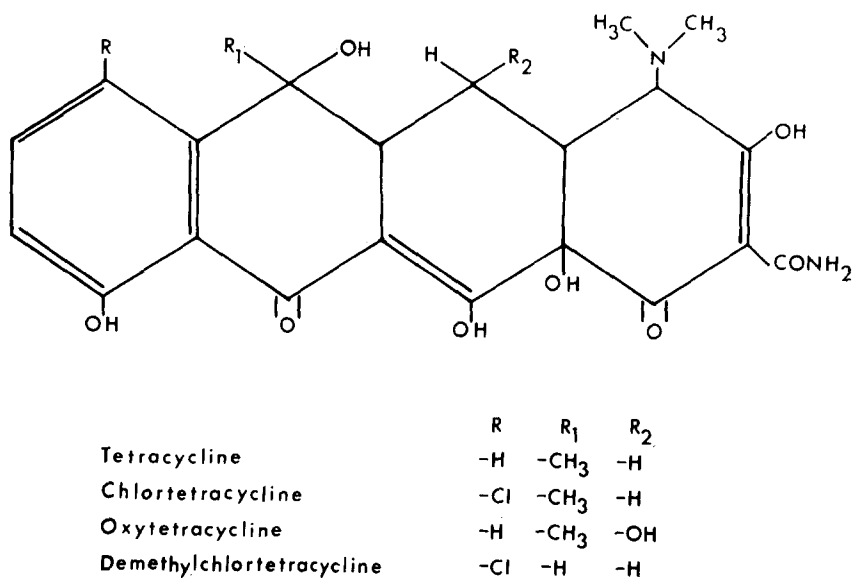
From the Department of Microbiology and Pediatrics, Baylor University College of Medicine.

Supported by Training Grant T1-A1-47-05 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, United States Public Health Service; and the Texas Allergy Research Foundation.

Presented before the Twentieth Annual Meeting of the American Academy of Allergy at San Francisco, Calif., Feb. 8-12, 1964.

Received for publication June 25, 1964.

*Fellow in Pediatric Allergy, Departments of Microbiology and Pediatrics, Baylor University College of Medicine.

**Fig. 1**

Schematic representation of the basic tetracycline structure showing the loci of variations between the different compounds.

in experimental animals, and to establish whether such antibodies can be detected by laboratory procedures.

MATERIALS AND METHODS

Animals

Fourteen New Zealand albino rabbits weighing 2,300 to 3,000 grams each, at the start of the experiments, were used.

Antigens

The following tetracyclines: Aureomycin HCl, chlortetracycline (Lot No. 501-721B-37-2; 99.5 per cent pure); Declomycin HCl, demethylchlortetracycline (Lot No. 571-896B-160-5; 99.9 per cent pure); tetracycline HCl (Lot No. 509-723B-50-2; 100 per cent pure); and oxytetracycline (Lot No. 6227, National Formulary Reference Standard), were used throughout this study.*

The solutions utilized in all four courses of administration were prepared daily, immediately before injection to minimize in vitro degradation. In addition the solutions employed in the various antibody detection tests were used within a few minutes of the time of preparation.

Immunization procedures

Four rabbits were immunized with chlortetracycline (CT), four with tetracycline (TC), three with oxytetracycline (OT), and three with demethylchlortetracycline (DMCT).

*These purified products were supplied by Lederle Laboratories.

tetracycline (DMTC). For the first immunization, each antigen (30 mg. of CT, OT, and TC, and 20 mg. of DMTC) was dissolved in a minimal amount of 5 per cent NaHCO_3 solution, diluted to 1.0 ml. with distilled water, and emulsified in 1.0 ml. of complete Freund's adjuvant (Difco) for injection. The emulsions were injected intramuscularly on alternate days for a total of 5 injections.

After a two-week rest period, each rabbit received a second immunization with the corresponding tetracyclines. The same amount of each antigen, dissolved in 2.5 ml. of buffered isotonic saline, pH 7.4, was injected intramuscularly every other day for 3 injections.

The third immunization was given after a three weeks' rest period. The antigens were prepared as for the first immunization and injected intramuscularly every other day for 5 injections.

The final immunization was given after a rest period of five weeks. The antigens were prepared as in the second immunization except that the dose was reduced to half the previous amount. Injections were administered, subcutaneously, daily for five days.

The rabbits were bled from the ear vein seven days after the last injection in each of the four immunizations.

Hemagglutination tests

A previously described tanned-cell hemagglutination technique was used.⁹ Fresh sheep erythrocytes, washed three times with isotonic saline, were resuspended in buffered saline, pH 7.2, to a cell concentration of 2.5 per cent. Equal volumes of the erythrocyte suspension and a 1:22,000 solution (w/v) of tannic acid were mixed, incubated in a water bath at 37° C. for 10 minutes, centrifuged, and washed with buffered saline, pH 7.2, followed by unbuffered isotonic saline. The cells were then resuspended to a concentration of 2.5 per cent with buffered saline, pH 6.4.

The appropriate tetracyclines in buffered saline, pH 7.2, were added to the tanned-red blood cell suspension to a final concentration of 2.5 mg. of antigen per milliliter of red blood cells. After incubation at 37° C. for 30 minutes, the tanned cells with adsorbed antigen were centrifuged and washed twice with normal rabbit serum diluted 1:200 in isotonic saline. The cells were resuspended to a final concentration of 1.5 per cent in a 1:200 dilution of normal rabbit serum.

Antisera were first heated at 56° C. for 30 minutes to inactivate complement and adsorbed with fresh sheep erythrocytes. Twofold serial dilutions of the test serum, starting at 1:10, were placed in tubes in 0.5 ml. amounts and 0.05 of antigen-treated tanned cells added. The tubes were allowed to stand at room temperature for two hours and overnight at 5° C. Controls included antigen-treated tanned cells mixed with 1:100 dilutions of normal rabbit serum and antigen-treated tanned red cells with preimmunization sera diluted serially. Readings were made at the end of two hours at room temperature and checked after 16 hours at 5° C. followed by two hours at room temperature.

Hemagglutination cross-reactions were done by using different tetracyclines as antigen for each antisera.

Hemagglutination inhibition tests

Sera from rabbits No. 514 and No. 518, immunized with OT, were used in this test. Serial twofold dilutions of the antisera, starting with a 1:10 dilution, were placed in tubes in 0.5 ml. amounts. Serial twofold dilutions of OT in 0.05 ml. amounts, starting with 2.0 mg. per milliliter, were then added to the dilutions of antisera. These mixtures were incubated at room temperature for one hour and overnight at 5° C. before adding 0.05 ml. of antigen-treated tanned red cells prepared as in the hemagglutination tests. The procedure was later modified by incubating the dilutions of antisera and the antigen for only 15 minutes at room temperature before adding the antigen-treated tanned red cells suspension.

Hemagglutination inhibition tests also were carried out using CT, DMCT, and TC as antigens against the anti-OT rabbit sera.

Column chromatography

The anion exchanger, diethylamino-ethyl (DEAE)-Sephadex A-50* was prepared for use according to the procedure described by Pharmacia. The prepared material was equilibrated with 0.07M sodium chloride solution before packing to a depth of 30 cm. in 10 mm. diameter chromatographic columns for fractionation of rabbit anti-OT serum No. 518. Eluates were collected in 3 ml. volume, and stepwise elution was accomplished by the addition of 0.12M, 0.17M, 0.27M, and 0.38M sodium chloride solutions at regular intervals. Optical density of individual eluates was determined in a Beckman DB Spectrophotometer at 280 m μ .

The eluate from each peak in the fractionation was dialyzed against 0.425 per cent sodium chloride for 24 hours and rendered isotonic by the addition of 1.25 per cent sodium chloride solution. Pooled fractions from the first two peaks were treated the same way except for concentration to one third the original volume. Individual fractions, as well as the pooled fractions, were tested for antibody activity by tanned-cell hemagglutination and PCA tests.

Passive cutaneous anaphylaxis (PCA)

The technique described by Ovary¹⁰ was employed. Albino guinea pigs weighing 200 to 300 grams were used to test anti-OT rabbit sera and chromatographic fractions of the same antisera.

Schultz-Dale tests

One-centimeter-long sections of terminal ileum from normal guinea pigs were used for determinations of smooth muscle contraction. Washed ileal segments were suspended in Tyrode's solution in a 4 ml. bath and attached to an appropriate recording device. Quantities of OT in excess of 0.1 mg. were found to be excessively irritating to the test ileum. Consequently, 0.1 mg. OT was employed as the standard antigen concentration.

*A product of Pharmacia Fine Chemicals, Inc., New Market, N. J.

RESULTS

Hemagglutination reactions

Table I shows the antibody responses obtained following the different series of immunizations as measured by tanned-cell hemagglutination. Subsequent to the first immunization, only four rabbits possessed hemagglutinating antibody, and these sera were of relatively low reactivity. Following the second series of injections, using antigen *without* adjuvant, six rabbits responded with antibody production. Two of the three rabbits receiving oxytetracycline had titers of 1:640 and 1:320, which increased during the next two immunizations. Seven rabbits produced variable and weak responses, while five rabbits failed to produce demonstrable antibody during the course of the experiment.

Cross-reaction between the tetracyclines

To determine the presence or absence of cross-reactivity, each serum was tested with its homologous antigen as well as with the three heterologous tetracyclines (Table II). Antisera obtained following the injection of tetracycline, chlortetracycline, or demethylchlortetracycline exhibited hemagglutinating titers against all four tetracyclines. The two high-titer antisera produced by immunization with oxytetracycline gave hemagglutinating titers only when tested against the homologous antigen. One low-titer serum from an oxytetracycline rabbit did exhibit cross-reactivity with all four tetracyclines.

Hemagglutination inhibition

In order to determine the specificity of the induced antibody, hemagglutination inhibition studies were done employing the two high-titer oxytetracycline antisera. Both oxytetracycline and the heterologous antigens were used as in-

Table I. Hemagglutinating titers of sera of rabbits immunized with the different tetracyclines. See text for dosage and time intervals

Rabbit number	Antigen injected*	Immunization			
		I	II	III	IV
500	CT	0†	0	0	0
502	CT	10	10	0	×‡
504	CT	20	20	10	20
506	DMCT	0	0	0	0
508	DMCT	0	0	0	0
510	DMCT	0	0	20	0
512	DMCT	10	80	40	10
514	OT	160	320	1,280	5,120
516	OT	0	0	0	20
518	OT	320	640	1,280	640
520	TC	0	0	0	20
522	TC	0	0	0	0
524	TC	0	0	0	0
526	TC	40	0	0	0

*CT, chlortetracycline. DMCT, demethylchlortetracycline. OT, oxytetracycline. TC, tetracycline.

†Hemagglutination titer (reciprocal).

‡×, Rabbit died.

Table II. *Titers and cross-reactivity of rabbit antisera against the different tetracyclines*

Serum No.	Antigen injected†	Antigen tested†			
		CT	DMCT	OT	TC
504	CT	40*	40	40	40
512	DMCT	10	10	10	10
514	OT	0	0	5,120	0
516	OT	10	20	20	20
518	OT	0	0	640	0
520	TC	40	20	20	20

*Hemagglutination titer (reciprocal).

†CT, chlortetracycline. DMCT, demethylchlortetracycline. OT, oxytetracycline. TC, tetracycline.

Table III. *Inhibition of the hemagglutinating activity of rabbit anti-oxytetracycline serum by varying concentrations of oxytetracycline*

Inhibitor OT* (mg./ml.)	Antibody titers									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120
2.0	+	+	+	—	—	—	—	—	—	—
1.0	+	+	+	—	—	—	—	—	—	—
0.5	+	+	+	+	+	+	—	—	—	—
0.25	+	+	+	+	+	+	+	—	—	—
0.125	+	+	+	+	+	+	+	+	—	—
0.0625	+	+	+	+	+	+	+	+	—	—
None	+	+	+	+	+	+	+	+	+	+
Control (NRS)†	—	—	—	—	—	—	—	—	—	—

*OT, Oxytetracycline.

†NRS, Normal rabbit serum.

hibiting antigens. Table III shows the inhibiting effect of different concentrations of oxytetracycline on the hemagglutinating ability of one anti-oxytetracycline sera. None of the other three tetracyclines demonstrated this inhibiting effect.

Column fractionation of rabbit anti-OT serum

Serum No. 514, having a hemagglutinating titer of 1:5,020, was fractionated on DEAE-Sephadex A-50 using different concentrations of sodium chloride as eluent. Six separate peaks were obtained as shown in Fig. 2. Each tube of eluate corresponding to each peak was tested for the presence of antibody activity by hemagglutination or PCA techniques. No activity was detected in these individual fractions. However, when the eluates from the slopes of each of the first two peaks were pooled, dialyzed, concentrated, and rendered isotonic, both pooled fractions exhibited tanned-cell hemagglutination antibody titers.

The first two peaks obtained with DEAE-Sephadex fractionation are generally considered to contain the gamma globulin fractions. To confirm the presence of gamma globulin in these fractions, the pooled concentrates were tested against sheep antirabbit gamma globulin serum by the Preer technique. Distinct bands of reaction were observed at 24 hours.

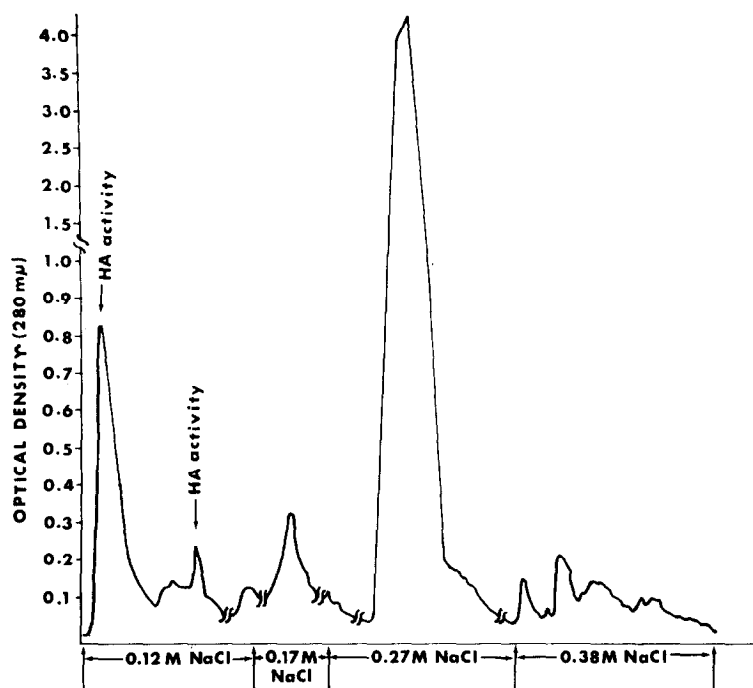


Fig. 2

DEAE-Sephadex separation of anti-oxytetracycline rabbit serum No. 514. Tanned-cell hemagglutinating activity was found in the areas eluted by 0.12M NaCl.

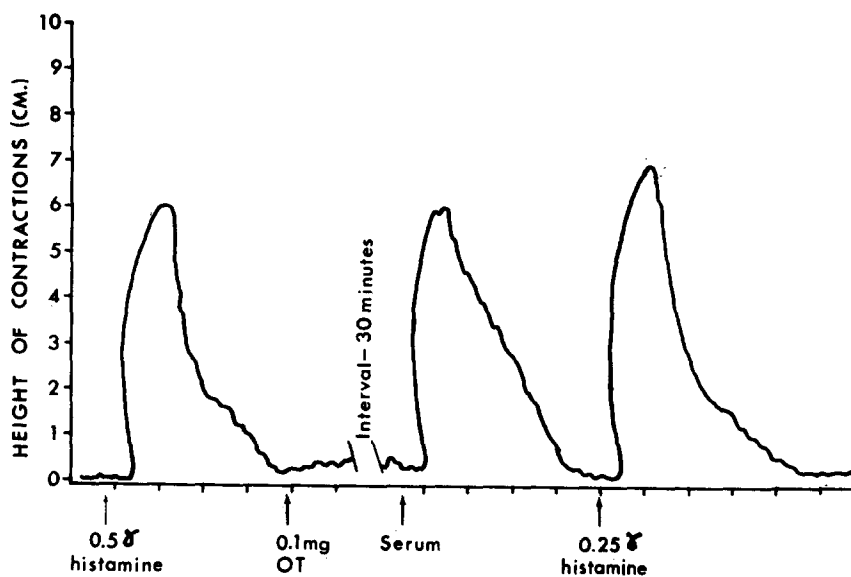
Schultz-Dale reactions

When guinea pig ileum in a 4 ml. Tyrode's solution bath was incubated for 30 minutes with 0.1 mg. of OT and challenged at the end of this period with 0.5 ml. of 1:5 dilution of the specific antiserum, a typical histamine-like contraction was observed (Fig. 3). Reversing the order of addition of antigen and antiserum did not alter the results. Incubation of 0.5 ml. of a 1:10 dilution of the anti-OT rabbit serum with the guinea pig ileum for 30 minutes followed by challenge with 0.1 mg. OT, produced a contraction comparable to that elicited by 0.25 meg. of histamine (Fig. 4).

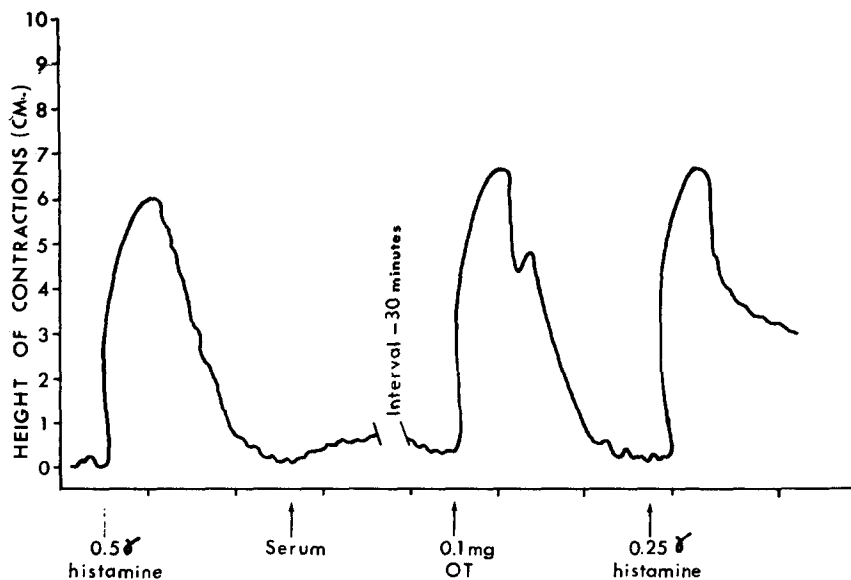
When the above tests were repeated with each of the three other tetracyclines, no response was observed. This was expected because the antiserum used (No. 514) did not cross-react with the other tetracyclines in the tanned cell hemagglutination test.

Other immunologic tests

Passive cutaneous anaphylaxis tests carried out on both anti-OT serum and the pooled chromatographic fractions were negative in spite of the presence of hemagglutinating antibodies in these test materials. Different concentrations of OT varying from 5 mg. to 40 mg. per guinea pig were employed without success.

**Fig. 3**

Condensed tracing of Schultz-Dale reactions of guinea pig ileum in response to oxytetracycline and anti-oxytetracycline serum No. 514.

**Fig. 4**

Condensed tracing of Schultz-Dale reactions of guinea pig ileum in response to oxytetracycline and anti-oxytetracycline serum No. 514.

DISCUSSION

Clinically, there is a high incidence of allergic response to drugs and chemicals of low molecular weight. The immunologic responses produced by these low molecular weight substances may present a variety of reactions similar to those obtained with pure antigen.¹¹ There has been general acceptance of the concept that low molecular weight substances must react or conjugate in vivo with body protein to form complete antigens. Synthetically produced conjugates such as penicillinate, penicilloyl- and aspiryl-conjugates^{12, 13, 14} have been demonstrated to be highly antigenic for rabbits and guinea pigs. However, the production of antibodies to penicillin derivatives has not required, routinely, the formation of in vitro conjugates.⁹

Antisera specific to oxytetracycline were produced without resorting to artificial in vitro conjugation of the antigen to protein. However, the tetracyclines do bind to plasma proteins, as shown by Kunin and co-authors.¹⁵ The different analogues were found to bind to plasma proteins in the following proportions: chlortetracycline, 47 per cent; demethylchlortetracycline, 41 per cent; tetracycline, 24 per cent; and oxytetracycline, 20 per cent. With the intensive immunization procedures employed, impurities common to several preparations or mutual degradation products might account for the low-titer cross-reactivity encountered in many of the test sera. The marked specific reactivity of the two high-titer anti-oxytetracycline sera was surprising in view of the close structural similarity of the four compounds being investigated. Sufficient differences do exist in the chemical and physiologic activities¹⁵ to give credence to immunologic specificity for one analogue.

REFERENCES

1. Welch, H., Lewis, C. N., Weinstein, H. I., and Boeckman, B. B.: Severe Reactions to Antibiotics, a Nationwide Survey, *Antibiotic Med. & Clin. Therap.* **4**: 800, 1957.
2. Peek, S. M., and Feldman, F. F.: Sensitivity Reaction to Aureomycin, *J. A. M. A.* **142**: 1137, 1950.
3. Bell, G. O., and Rubitsky, H. J.: Hypersensitivity to Aureomycin, Report of a Case, *Lahey Clin. Bull.* **7**: 136, 1951.
4. Bedford, P. D.: Idiosyncrasy to Aureomycin, *Brit. M. J.* **1**: 1428, 1951.
5. Sakamoto, A.: Anaphylactoid Reaction to Intramuscular Tetracycline Hydrochloride, Report of a Case, *California Med.* **84**: 55, 1956.
6. Burnstein, F.: Sensitivity to Aureomycin, Report of a Case, *Am. J. Ophth.* **33**: 973, 1950.
7. Kutscher, A. H., Budowsky, J., Lane, L. L., and Chilton, N. W.: Reactions Following the Use of Terramycin Troches: a Controlled Study, *J. ALLERGY* **23**: 177, 1952.
8. Johnston, T. G., and Cazort, A. G.: Serum Sickness Reaction with Cyanosis Following Terramycin, *Antibiotics & Chemotherapy* **3**: 481, 1953.
9. Wagelie, R. G., Dukes, C. D., and McGovern, J. P.: Antigenicity and Cross Reactivity of 6-Aminopenicillanic Acid and Penicillin G, *J. ALLERGY* **34**: 489, 1963.
10. Ovary, Z.: Immediate Reaction in the Skin of Experimental Animals Provoked by Antibody-Antigen Interaction, *Progr. Allergy* **5**: 459, 1958.
11. Eisen, H. N.: Hypersensitivity to Simple Chemicals, in Lawrence, H. S., editor: *Cellular and Humoral Aspects of the Hypersensitive States*, New York, 1959, Hoeber-Harper, p. 89.
12. Farah, F. S., Kern, M., and Eisen, H. N.: Specific Inhibition of Wheal-and-Erythema Response With Univalent Haptens and Univalent Antibody Fragment, *J. Exper. Med.* **112**: 1211, 1960.
13. Parker, C. W., deWeck, A. L., Kern, M., and Eisen, H. N.: The Preparation and Some Properties of Penicillenic Acid Derivatives Relevant to Penicillin Hypersensitivity, *J. Exper. Med.* **115**: 803, 1962.
14. Weiner, L. M., Rosenblatt, M., and Howes, H. A.: The Detection of Humoral Antibodies Directed Against Salicylates in Hypersensitive States, *J. Immunol.* **90**: 788, 1963.
15. Kunin, C. M., Dornbush, A. C., and Finland, M.: Distribution and Excretion of Four Tetracycline Analogues in Normal Young Men, *J. Clin. Invest.* **38**: 1950, 1959.