$L\text{-}T_4$, and thyroprotein was 1.3 times as active (based on 1% $L\text{-}T_4$ content) as $L\text{-}T_4$.

In a comparison of subcutaneous and oral administration of these hormones in the male and using the subcutaneous administration as 100%, in two trials, $L\text{-}T_4$ orally was 96.2 and 88.8% as active. $L\text{-}T_3$ was 67.5% as active and thyroprotein, 58.2%. In the females, in two trials, $L\text{-}T_4$ was 40.7 and 68.7% as effective orally; $L\text{-}T_3$ was 55.9% as effective orally; and thyroprotein was 62.1% as effective. Since the oral effectiveness of thyroprotein in male and females is about 60%, it is suggested that this value be used in estimating the amount of thyroprotein to add to a poultry ration to equal the thyroxine secretion rate.

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Factors Influencing Growth Inhibition of *Mycoplasma pneumoniae* By Immune Sera.* (32391)

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Recognition of the importance of *Mycoplasma pneumoniae* as a cause of human respiratory disease has stimulated the development of a variety of methods for detecting a serologic response to this agent(1). Each technique reveals a characteristic response pattern depending on its ability to detect the different immunoglobulins (Ig)(2). Of the biologic properties demonstrable in sera following infection or immunization with *M. pneumoniae,* only growth inhibition appears to derive significantly from all 3 classes: IgM, IgA and IgG. Growth inhibition (GI) has been quantitated directly by determining a decrease in numbers of viable organisms in the presence of homologous antiserum and indirectly by metabolic-inhibition techniques (3,4,5,6). This phenomenon has received particular attention because of its specificity and its tendency to persist in serum for prolonged periods. However, GI methods generally have been technically difficult to perform and standardize, and have been utilized infrequently. To provide insight into the mechanisms of immune inhibition, the dynamics of the GI reaction and variables affecting its expression were studied.

Materials and methods. All procedures utilized a broth medium(7) composed of 70% Difco PPLO broth, 20% unheated horse serum, 10% yeast extract, 1% dextrose, 0.004% phenol red and 1000 units of penicillin G/ml. The broth base was pretested for ability to

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support optimal growth of *M. pneumoniae* and a single lot was used for all experiments. Horse serum was pretested by the supplier for ability to promote optimal growth of mycoplasmas.

Normal sera. Human, rabbit and hamster bloods were allowed to clot overnight at room temperature before separation of sera. Horse and precolostrum calf sera (Robbin Laboratories, Chapel Hill, N. C.) were obtained from single donors and kept at 4°C for 24 to 48 hours until delivered to this laboratory; separate lots were divided into aliquots and frozen at −20°C until needed. Pooled lyophylized guinea pig complement (Baltimore Biological Laboratories) was reconstituted and stored at −70°C until used.

Immune sera. Three-week and six-month convalescent sera were obtained from an adult patient with *M. pneumoniae* pneumonia. Detailed studies of the antibody content of these sera are presented elsewhere(2). Rabbit antisera to *M. pneumoniae* and 7 other mycoplasma species (Table I) were prepared by

TABLE I. Specificity of *M. pneumoniae* Growth Inhibition.

Mycoplasma antisera*	*M. pneumoniae* inhibited (log_2) †	
	Pre-immune sera	Immune sera
M. pneumoniae	0	10
M. fermentans	0	0
M. salivarium	0	0
M. hominis	1	0
M. pharyngis	1	0
M. gallisepticum	0	0
M. canis	0	0
M. pulmonis	0	0

* Pre- and post-immunization sera of rabbits given listed *Mycoplasma* antigens.

† Number of 2-fold dilutions inhibited by 1:10 antisera compared to control without antisera.

intravenous inoculation of broth-grown organisms as described previously(2).

Organisms. M. pneumoniae, strain Mac, was cultured at 35°C on a rotary shaker for 4 days; aliquots were stored at −70°C. Replicated plate counts of this material averaged $10^{7.5}$ colony-forming units (CFU)/ml.

Titration procedures. Microtiter "U" plates (Cooke Eng. Co. #220-24, supplied in sealed polyethylene bags) were used as received for all experiments. Equal volumes (0.05 ml) of antiserum and organism dilutions were added to the wells, followed by 0.05 ml of diluent. Plates were sealed with cellophane tape and incubated at 37°C. Fermentation of glucose in the medium, reflected by a decrease in pH, served as an index of growth; the validity of this observation was easily checked by microscopic observation of mycoplasma "spherules" in the wells. End points were taken as the highest dilution showing at least 0.5 pH change from the control. The color differences were intensified by puncturing the tape over each well 1-2 hours before reading.

Results. Dynamics of M. pneumoniae *growth inhibition.* Immune inhibition was presumed to be the result of the interaction of 3 major variables: number of organisms, concentration of antibody, and time. A fourth reactant, heat-labile accessory factor, generally held constant by incorporating fresh serum in the culture medium, will be considered below. To evaluate the kinetics of the GI reaction a series of checkerboard titrations was prepared employing 2-fold dilutions of organisms and immune sera. Growth of the organism was observed over a 14 day period of incubation; representative experiments with rabbit antiserum and human convalescent serum are shown in Fig. 1. A linear

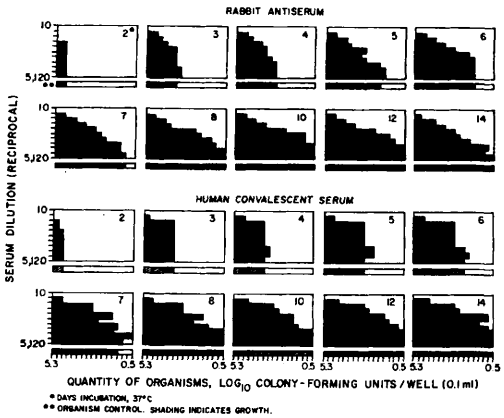

FIG. 1. Dynamic aspects of *Mycoplasma pneumoniae* growth inhibition test. Successive figures show daily stages of checkerboard titrations performed in microtiter plates.

relationship between the organism and serum dilution endpoints was established by the 5th day and, although further incubation expanded the range of endpoints, the slope of this line remained constant. Both rabbit

and human serum experiments showed a similar temporal progression although the latter was marred by an early growth breakthrough at the 1:40 and 1:80 serum dilutions. By plotting the logarithm of the number of organisms inhibited against the logarithm of the serum dilutions, the quantitative relationship of these two variables was determined. Using data from day 7 (Fig. 1) the slope of the regression line was approximately 2.0 for both sera, as demonstrated in Fig. 2. Thus the serum dilution endpoint, as

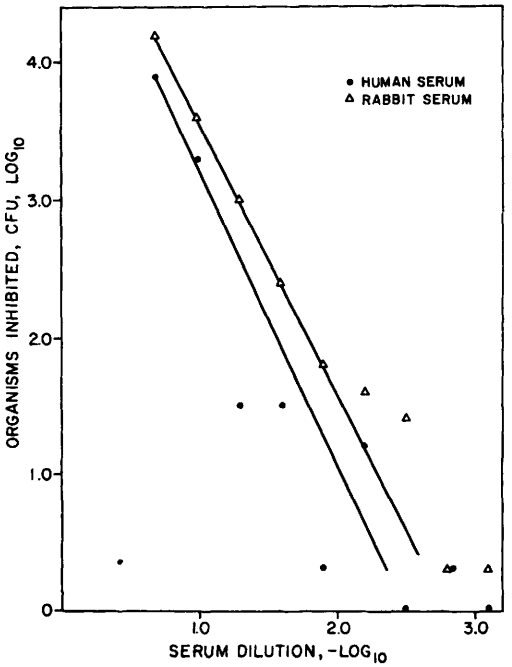

FIG. 2. Quantitative relationship between amount of *M. pneumoniae* inhibited and serum dilution. Coordinates derived from readings on 7th day of incubation (see Fig. 1).

well as the time required for full development, was inversely proportional to the initial dose of organisms in each well.

The above data indicated that growth inhibition could be expressed in two ways: either as the highest dilution of serum inhibiting a constant number of organisms or as the maximum number of organisms inhibited by a constant amount of antiserum. These alternative methods for measuring GI antibody were compared by applying both to a random group of 23 human and 9 rabbit anti-*M. pneumoniae* sera. Fig. 3 compares results ob-

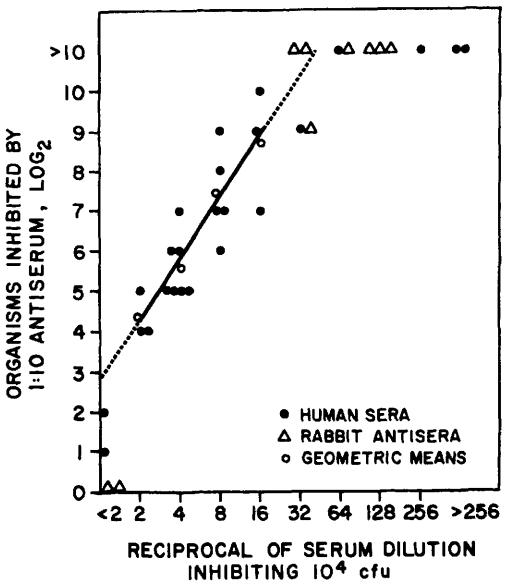

FIG. 3. Correlation of results obtained by alternative methods for measuring growth inhibition of *M. pneumoniae* by immune sera.

tained by titering 2-fold organism dilutions against a constant 1:10 serum (ordinate), and serial 2-fold dilutions of the same sera against a constant 10^4 CFU inoculum (abscissa). The coordinates of these paired determinations describe a straight line, the slope of which is 1.5. Thus, within a limited range especially suited to human convalescent sera, dilution of antigen in the presence of a constant amount of antiserum provided an advantage in sensitivity. However, the obverse method, serum dilution-constant antigen, was better suited to titration of hyperimmune sera.

Specificity. To determine if specificity had been forfeited by the gain in sensitivity afforded by the organism-dilution technique, *M. pneumoniae* was titered against rabbit antisera to 5 human mycoplasmas and 3 additional fermentative species as listed in Table I. These data indicated that growth inhibition was produced by the homologous combination only.

In another experiment 14 different strains of *M. pneumoniae* were titered against the same reference human antisera to determine if variations in serum inhibitory capacity could be detected. The history of these organisms ranged from recent isolates to high

passage laboratory strains. All strains were inhibited to the same degree when the antigen pools were adjusted to yield equivalent numbers of viable organisms per unit volume.

Heat labile serum factors. Growth inhibition of *M. pneumoniae* in broth media has been shown to require the presence of heat-labile serum factor. Both guinea pig and horse serum have been employed for this purpose(6). However, attempts in this laboratory to utilize commercial guinea pig "complement" even at high dilutions revealed inhibition of growth in control wells. Because of this observation, different animal sera were screened for natural inhibitors to *M. pneumoniae*. Fresh or heated (56° × 30 min) normal animal sera were serially diluted in heated or freshly prepared complete broth medium and each dilution was inoculated with 10^4 CFU of organisms. As shown in Table II, fresh horse serum contained little or no inhibitory property, but other animal sera required heat-inactivation to permit optimal growth. In the case of guinea pig serum, heating of the broth medium was also required to reduce the inhibitory effect significantly. This suggested the presence of specific antibody; the remaining heat-stable inhibitor was found to be sodium azide which is present in commercial complement. In view of the above findings, horse serum, pretested for its ability to support growth inhibition, has been used to prepare all media for the GI procedure.

Since fresh sera from unimmunized animals may contain heat labile inhibitors for *M. pneumoniae,* these factors also would be expected in immune sera. For this reason heat inactivation is necessary before testing serum for GI antibody. However, heating, as well as aging, was found to reduce the inhibitory activity of immune sera. The ability of fresh horse serum (accessory factor) to reactivate specific and nonspecific inhibition by human and rabbit sera was evaluated by studying the effect of heat on combinations of these components. As shown in Table III,

TABLE III. Effect of Heat-Labile Serum Factors on Growth Inhibition of *M. pneumoniae.*

Serum*	Time (wk)	Heated†	CFU *M. pneumoniae* inhibited, $\log_2$ Fresh medium‡	Heated medium†
Hyperimmune rabbit	0		8	8
	2	No	10	6
	4		>11	>10
	0		0	0
	2	Yes	7	0
	4		9	1
Convalescent human	3	No	8	5
	24		>11	>11
	3	Yes	2	0
	24		8	0

* 1:10 dilution in heated broth medium.
† 56° × 30 min.
‡ Prepared with 20% fresh horse serum.

the presence of accessory factor restored the specific inhibitory activity of immune sera but did not reactivate non-specific inhibitors.

Reproducibility. Definition and control of the variables outlined above made it possible to assess the reproducibility of the GI assay. For these experiments replicate titrations were performed using a single antigen pool, a human convalescent serum and a rabbit antiserum. (Test sera were heat inactivated and

TABLE II. Effect of Fresh Normal Sera on Growth of *M. pneumoniae.*

Serum	Source	Reciprocal of serum dilution inhibiting 10^4 CFU organisms: Fresh serum in fresh medium*	Heated† serum in fresh medium	Heated serum in heated medium
Horse	3 donors	2‡	<2	<2
Pre-colostrum calf	2 "	16‡	<2	<2
Rabbit	Pool of 6	32	4	<2
Hamster	" " 6	8	2	2
Guinea pig	Lyophilized pool§	128	64	16

* Complete PPLO broth culture medium containing 20% fresh horse serum.
† 56° × 30 min.
‡ Mean of determinations on each donor.
§ Commercial product contains 0.1% sodium azide.

titrations were performed in broth supplemented with fresh-frozen horse serum). Fifteen titrations of each of the two immune sera against a constant quantity of organisms revealed a standard deviation of 0.8 tube (<1 $\log_2$ serum dilution). Using the same human serum at 1:10, 30 titrations of the organism pool were performed to evaluate the constant serum-varying organism technique. The capacity of this serum to inhibit growth also had a standard deviation of 0.8 tube (<1 $\log_2$ CFU). Thus, under the conditions defined, a random variation in results exceeding a 4-fold difference in endpoints by either method would occur less than once in 20 tests.

Discussion. Eaton has suggested that the inhibition of *M. pneumoniae* by specific antiserum is analogous to virus neutralization. However, unlike many viruses, neutralization of *M. pneumoniae* infectivity requires complement-like factors which are available *in vivo,* but must be added to *in vitro* tests(8). The kinetics of virus neutralization have been studied by Tyrell and Horsfall, and others, who found a linear relationship between the logarithm of the quantity of virus neutralized and logarithm of the serum dilution endpoint. The slope of the regression line ranged between 1.0 and 4.0, depending on the virus and the methodology employed(9). Observations on the quantitative aspects of *M. pneumoniae* growth inhibition have not been reported previously. In the present study a linear relationship between log CFU *M. pneumoniae* inhibited and log antiserum dilution was demonstrated, the regression ratio being 2.0. Thus, a 2-fold change in serum dilutions affected the final expression of the test to the same degree as a 4-fold change in amount of antigen.

These findings imply that a GI assay based on titration of antigen against constant serum would be twice as sensitive as the more conventional serum titration methods for expression of antibody activity. Indeed, a comparison of results by both methods performed on randomly selected sera showed this advantage to hold in practice. This increase in sensitivity, gained without sacrificing specificity, has facilitated detection of GI antibody in human and hamster sera and in dilute fractions from chromatographic columns(2).

The enhancing effect of fresh serum on immune inhibition of *M. pneumoniae* was first demonstrated in tissue culture(10). Subsequently, it has been observed that inhibition of growth in artificial media is also dependent on heat labile serum factors(6). In the present study the fresh horse serum used to supplement growth medium was found to serve as an ideal source of these factors. Most other animal sera, including guinea pig, required heat-inactivation to remove nonspecific inhibitory activity, thus making them useless as a source of accessory factor. These observations provide an explanation for the failure of rabbit serum to support growth of *M. pneumoniae* as reported by others(6,11). In fact, it has been possible to grow *M. pneumoniae* in Medium 199 supplemented with heat inactivated rabbit serum and dialyzed yeast. This material has been used to immunize rabbits without producing antibodies to heterologous proteins.

Summary. Studies on growth inhibition of *M. pneumoniae* revealed three interdependent major variables: number of organisms used in titrations, amount of antiserum, and time of test incubation. A linear relationship between the logarithms of the number of organisms inhibited and the antiserum dilution was observed in which the regression ratio was 2.0. Heat-labile factors in normal sera, required for inhibition of *M. pneumoniae,* were readily provided by incorporating fresh frozen horse serum into the growth medium. Other animal sera could not be used because of the presence of heat-labile inhibitors; commercial guinea pig "complement" additionally contained 2 heat-stable inhibitors, specific antibody and sodium azide. Control of the factors indicated provided means of producing immune inhibition measurements of varying sensitivity which were reproducible within a 4-fold range at the 95% confidence level.

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Clearance Studies of Insulin and Nonsuppressible Insulin-Like Activity (NSILA) in the Rat Liver.* (32392)

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Two components of insulin-like activity (ILA) have been described in serum. One is that fraction readily identifiable by immunoassay(1); the other is that fraction of serum, nonreactive with insulin specific antiserum and, hence, "nonsuppressible" on bioassay(2,3,4). This latter component has been studied extensively by investigators using widely differing methods of preparation and assay(2,3,4). This fraction has been called "bound" insulin(4), "atypical" insulin(3) and nonsuppressible insulin-like activity (NSILA)(2,5). Recent investigations in our laboratory(6,7,8) support the initial postulate of Kipnis and Stein(9) that these forms of ILA are probably identical. Because of the lack of indisputable evidence readily identifying this component as insulin or one of its metabolites, we prefer the terminology of Froesch *et al*(2,5), nonsuppressible insulin-like activity (NSILA).

Studies of the quantitation of NSILA in diabetes mellitus and following oral glucose administration have yielded inconsistent and confusing results(2,3,10). In most instances, major changes in NSILA have not been demonstrated in humans. Previous work has failed to establish whether or not NSILA is cleared in the circulation(11). Therefore, these investigations were undertaken to determine whether the liver removes NSILA from the circulation and to compare its removal rate with that of crystalline insulin.

Methods. NSILA was prepared from pork plasma because of the lack of availability of sufficient human or rat plasma. When dialyzed and assayed directly at 2.5% concentration in the isolated fat cell bioassay(12, 13), pork plasma contained 60 μU NSILA/ml. After preparative chromatography on Dowex 50(4,8,13), the lyophilized NSILA preparation contained 250-300 μU/mg.

Intact livers weighing 14-15 g from male Sprague-Dawley rats (450 g), fed *ad libitum*, were perfused cyclically according to the method of Miller *et al*(14). Heparinized rat blood, diluted 1:3 with Ringer's solution (Hematocrit = 17%), was used; the total perfusate volume was 80 ml. Blood flow through the liver was maintained at 25-30 ml/minutes, and the perfusions terminated after 4 hours. Viability of the preparation was assessed by the appearance of the liver and bile output. Sampling was done from the hepatic effluent and not more than 10 ml (12%) were removed during any single perfusion.

After a one-hour control period, crystalline pork insulin (Lilly 25 U/mg) was added to the perfusate to give an initial concentration of 2000 μU/ml. Serial samples were taken throughout three hours. In additional experiments, 100 mg porcine NSILA (300 μU/mg) were introduced into the perfusion bath and serial sampling performed as above.

Samples from the 2 insulin perfusions and

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