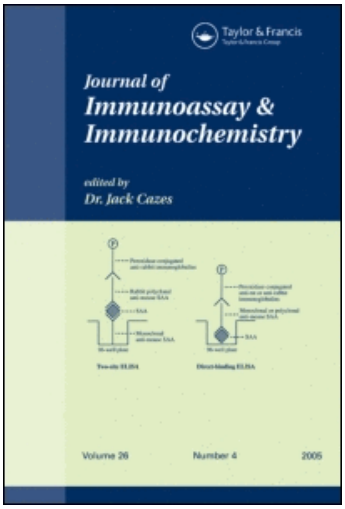

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TITRATION OF PENICILLOYL ANTIBODY BY ENZYME-IMMUNOASSAY: METHOD AND DATA ANALYSIS TO ASSIGN TITER

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ABSTRACT

Antisera to the hapten, penicilloyl, were titered in an indirect enzyme immunoassay with the marker enzyme, horseradish peroxidase. A multivalent solid-phase antigen for stable solid-phase immunocomplex adsorption and a 10 min peroxidase light-assay with photolabile ortho-phenylenediamine were features of the assay. The penicilloyl antisera titration curves formed the basis for an evaluation of 4 methods to assign titer. Titer was expressed as either the antiserum dilution^{-1} that produced an assigned absorbance, exhibited 50% maximal antigen-antibody binding, and equaled zero absorbance by extrapolation, or as absorbance at 10^{-3} antiserum dilution. The accuracy for extrapolated titer was improved with 2 or more solid-phase antigen concentrations. Absorbance at 10^{-3} antiserum dilution was most useful as a screen for relative titer and for specific titer from a standard curve generated with many test sera. The antiserum dilution may vary with a particular enzyme immunoassay. (KEY WORDS: Penicilloyl, Enzyme Immunoassay, Antibody Titer Units).

INTRODUCTION

The penicilloyl moiety has been termed the major antigenic determinant of penicillin (1, 2). Penicilloyl is formed from the covalent binding of the ruptured β-lactam ring of penicillin to primary amino groups in protein. Severe hypersensitivity

reactions to penicillin have encouraged the development of tests for antibody against penicilloyl as well as against the minor determinants in penicillin allergy.

The indirect ELISA method for antibody quantitation introduced by Engvall and Perlman (3) employed solid-phase antigen to bind specific antibody. The amount of solid-phase antigen-antibody complex was estimated with an enzyme labeled anti-immunoglobulin. Three solid-phase penicilloyl antigens which have been used in radioimmunoassay are penicilloyl E. coli acylase (4), penicilloyl bovine gamma globulin (5), and penicilloyl polylysine covalently attached to cellulose for the RAST test (6). Penicilloyl transferrin has been applied in an indirect ELISA (7).

In this study the indirect ELISA was performed with penicilloyl polylysine, a commercial product, and the marker enzyme, horseradish peroxidase (HRPO). The method measured penicilloyl antibody (Ab_p) produced in rabbits which had been immunized with penicilloyl proteins. Four methods were compared for the assignment of titer based on the antisera titration curves.

MATERIALS AND METHODS

Chemicals and their special sources were: penicilloyl polylysine (Cilligen) (2.9×10^4 ng/ml penicilloyl), benzylpenicillin, benzylpenicilloyl-ε-aminocaproate (pen-ε-cap), Tween-20, and ortho-phenylenediamine (OPD) (Sigma Chemical Co.,

St. Louis, MO); horseradish peroxidase conjugated to goat antirabbit IgG heavy and light chains (HRPO-conjugate) (Cappel Laboratories, Inc., Cochranville, PA); polystyrene tubes, 12 X 75 mm, No. 2052, (Falcon, Oxnard, CA); keyhole limpet hemocyanin (KLH) and penicillinase (Calbiochem, San Diego, CA).

Immunogens

Penicilloyl-substituted proteins were synthesized as described by Parker (8). KLH or rabbit IgG (1 g) and benzylpenicillin (12 g) were mixed for 16 h at $4^{o}C$ in 1 M carbonate, pH 10.4. Additional benzylpenicillin (6 g) was added and mixing continued 30 h. The product was dialyzed against 0.01 M phosphate buffered saline (PBS), pH 7.2, and concentrated by placement of the dialysis sacs in polyethylene glycol flakes.

The penicilloyl-proteins had no free benzylpenicillin detectable as penamaldate (9) following penicillinase treatment. The KLH immunogen had 29 mg Lowry protein/ml (10) and 3×10^{-9} mole penicilloyl/ml. The rabbit IgG immunogen had 34 mg Lowry protein/ml and 7.8×10^{-9} mole penicilloyl/ml.

Estimation of Penicilloyl as Penamaldate

The reaction of penicilloyl with p-chloromercuribenzoate to form a penamaldate mercaptide that absorbed maximally at 285 nm estimated penicilloyl (9). Sensitivity was enhanced by simple volume reduction (Fig. 1) and the product formed from 2, 3, and 4×10^{-5} M pen-ε-cap was stable at room temperature for 2 h.

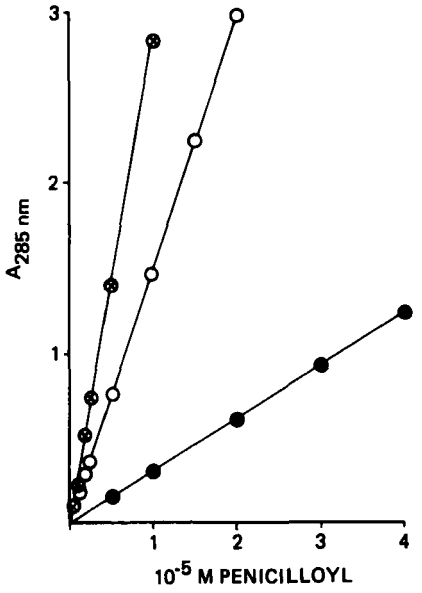

FIGURE 1. The change to greater sensitivity with volume reduction in the penamaldate method for penicilloyl estimation. Final volume: 5 ml ●, 2 ml O, and 1 ml ■. Conditions: 0.1 or 0.05 ml 15 mM p-chloromercuribenzoate in 0.05 M carbonate, pH 9.2; standard pen-ε-cap. Ten min after additions the absorbance was read.

Production of Penicilloyl Antisera (Ab_p)

Three groups of New Zealand white rabbits were immunized. The 100 series rabbits received 1 ml immunogen intravenously every 2 days for 9 injections. Six ml equal parts Freund's complete adjuvant and immunogen were administered subcutaneously (s.c.) on day 28. The 200 and 300 series received 8 ml equal parts Freund's complete adjuvant and immunogen s.c. A s.c. booster of 4 ml equal parts incomplete Freund's adjuvant and immunogen was given on day 21. The penicilloyl carrier was KLH for the 100 and 200 series and rabbit IgG for the 300 series.

The antiserum (Ab_p) was collected 2 to 3 weeks after the last injection.

HRPO-conjugate

The lyophilized HRPO-conjugate was dissolved in 5 ml H_2O, stored at -70°C, and used at a 1:500 dilution in PBS, 0.05% Tween-20 (PBS/T).

Solid-phase Cilligen

Tubes were coated with 1 ml 1:100 Cilligen dilution (290 ng penicilloyl) in 0.06 M carbonate buffer, pH 9.6, 0.02% NaN_3 at 37°C for 3 h. After 5 washes with 0.9% saline, 0.05% Tween-20 (S/T), the tubes were air dried, stored in sealed polyethylene bags at -20°C, and used within 3 months. However, sealed tubes containing coating solution and stored 3 weeks at 4°C also performed well in the enzyme immunoassay (EIA).

EIA for Penicilloyl Antibody Quantitation

Triplicate, 1 ml serial antiserum dilutions, in PBS/T, 0.02% NaN_3, and 0.1 ml 1:10 dilution of normal rabbit serum (NRS) in buffer were added to tubes coated with Cilligen, diluted 1:100. Tubes were covered and incubated at 37°C for 90 min in a shaking water bath. After 5 washes with S/T, 1 ml of HRPO-conjugate was added and the tubes incubated 2 h at 37°C. Following 5 S/T washes, 1 ml of H_2O_2-OPD substrate (2.2 mM OPD, 6.1 mM H_2O_2, in 0.1 M Na citrate, pH 6) was added at 5 sec intervals. The HRPO activity was stopped after 10 min with addition of 0.1 ml 12 N

H_2SO_4 at 5 sec intervals and the absorbance was determined at 492 nm (or 490 nm). Absorbances > 2 were diluted with 1.1 N H_2SO_4. Background tubes were run with 1 ml buffer in place of Ab_p to determine nonspecific adsorption.

For greater accuracy, the assay was repeated with the 1:250 dilution Cilligen-coated tube.

RESULTS

Characterization of Ab_p

The rabbit sera were titered for Ab_p by passive haemagglutination (PHA) (11) with sheep red blood cells and by EIA. In the detection of Ab_p, EIA was more sensitive than PHA by a factor of 3.9 to 139 (Table 1). The KLH immunogen produced more Ab_p by s.c. than by intravenous injection or than rabbit IgG immunogen by s.c. injection.

TABLE 1

Comparison of Passive Haemagglutination (PHA) and Enzyme Immunoassay (EIA) for Determination of Penicilloyl Antibody Titer

Serum No.	Titer		Ratio EIA/PHA
	PHA	EIA	
101	1,280	5,000	3.9
103	2,560	14,000	5.5
202	5,120	710,000	139
203	1,280	13,000	10.2
204	2,560	335,000	131
301	20	500	25
302	20	1,000	50

The EIA titer was the reciprocal of the antiserum dilution calculated by extrapolation of the titration curve.

Coating the Tubes with Cilligen

In a solid-phase EIA for Ab_p quantitation, the solid-phase Cilligen should be stable and in excess without producing a high background (nonspecific adsorption of HRPO-conjugate) or steric hinderance to the immunochemical reactions. Experiments were run to determine the conditions that provided solid-phase Cilligen with these properties.

Time and temperature: Two Cilligen dilutions, 1:100 and 1:250, were tested for adsorption at 4°C and 37°C over 24 h in coating buffer (Fig. 2). Significant adsorption took place at 37°C but not at 4°C. The signal to noise ratio was also better at 37°C than at 4°C. Three h was the optimal coating time at 37°C. The pronounced dip in the curve with Cilligen diluted 1:100 at 4 and 5 h and less pronounced dip with Cilligen diluted 1:250 at 5 h may have been a contributory factor to apparent inversions observed later in the titration curves. After 24 h at 4°C, the amount of specific antigen-antibody complex was less than after 1 h at 37°C.

Background, a function of the solid-phase Cilligen: Background varied with the Cilligen concentration in the coating buffer (increased with increased Cilligen concentration) and with the batch of HRPO-conjugate. Attempts to post-coat or incubate with 0.5, 1, or 2% bovine serum albumin minimized background but diminished the specific antigen-antibody reaction. NRS at 1:500 dilution reduced background to a negligble value. Uncoated tubes had no background.

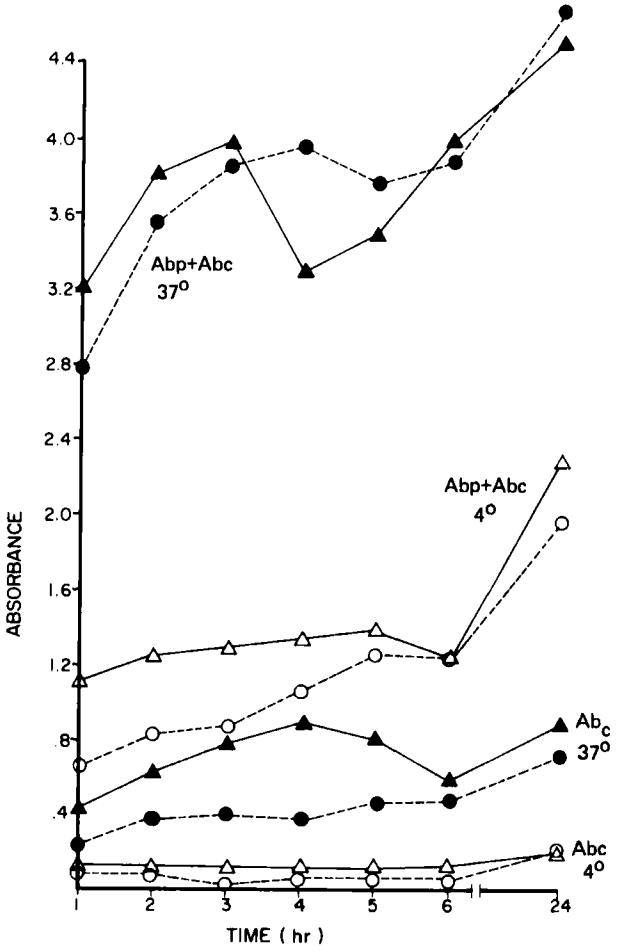

FIGURE 2. Incubation time, temperature and concentration for adsorption of Cilligen to solid phase.

Tubes were coated for the different time intervals at two Cilligen dilutions, 1:100 (Δ at 4°C, ▲ at 37°C) and 1:250 (O at 4°C, ● at 37°C) in 0.06 M carbonate, pH 9.6, 0.02% NaN_3. The maximum reaction product (Ab_p + Ab_c) and the nonspecific adsorption (Ab_c) were measured. No NRS was added to reduce nonspecific adsorption. The dilutions were Ab_p, 1:5000, and HRPO-conjugate, 1:500.

Monovalent penicilloyl as the solid-phase antigen: When benzyl-penicillin and pen-ε-cap were tested as potential solid-phase antigens, the time for adsorption was increased to 5 h. Benzylpenicillin is converted to penicilloyl under the alkaline coating conditions (9). Unlike Cilligen, benzylpenicillin did not promote nonspecific HRPO-conjugate adsorption; only a trace of specific binding was seen with the highest concentration of Ab_p. At a 1:500 dilution, the amount of Ab_p bound varied directly with the benzylpenicillin concentration in the coating buffer (data not shown).

Tubes coated with monovalent pen-ε-cap bound neglibible Ab_p at the highest Ab_p and pen-ε-cap concentrations. At lower concentrations of pen-ε-cap, Ab_p bound nonspecifically to the polystyrene; the amount bound varied directly with the Ab_p concentration. Some pen-ε-cap probably adsorbed at the high concentrations, since the nonspecific sticking of HRPO-conjugate was reduced.

Stability and Specificity of Penicilloyl Antiserum

Dose-response curves were similar for frozen and lyophilized Ab_p (Fig. 3). However, the frozen stock had the greater slope between 10^0 and 10^1 ng/ml penicilloyl and, therefore, would be preferred for a sensitive penicilloyl EIA.

The inhibition with pen-ε-cap verified penicilloyl as an antigenic determinant. Under the assay conditions, 10^5 ng/ml pen-ε-cap produced 100% inhibition. The dose-response curves

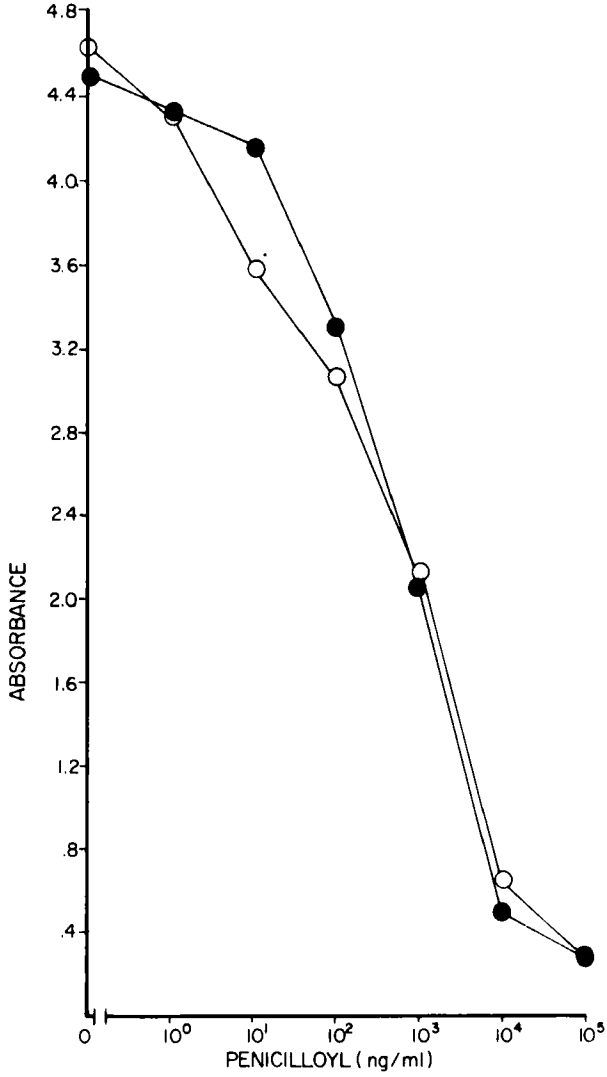

FIGURE 3. Evaluation of penicilloyl antiserum as a frozen and lyophilized stock.

Ab_p frozen O, lyophilized ●. A competitive EIA was performed with pen-ε-cap for 1.5 h at 37°C. The dilutions were: Ab_p $1:10^4$, NRS 1:500, and Cilligen 1:250. No corrections were made for substrate blank or background. HRPO assay time was 20 min.

exhibited (1) a mean C.V. of 6.4 with frozen Ab_p, and 7.7 with lyophilized Ab_p, (2) a ratio of maximum absorbance to background of 23:1, (3) a low, stable background and (4) overall linearity.

Antibody Titration to Determine Titer

Solid-phase Cilligen and the titration curve: Although the Cilligen concentration in the coating buffer influenced the Ab_p titration curve (Fig. 4), the linear segments appeared to

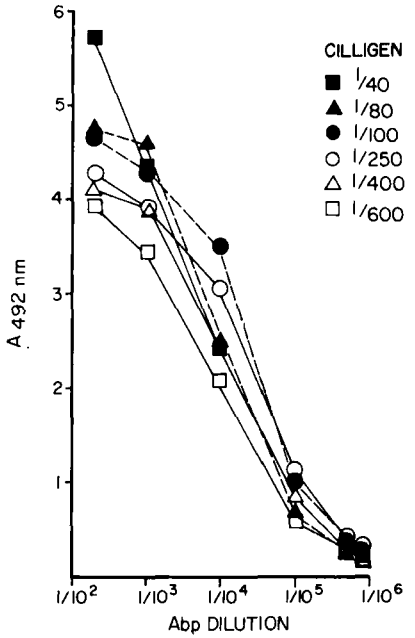

FIGURE 4. Effect of solid-phase Cilligen concentration on the titration of penicilloyl antibody by indirect EIA.

The assay conditions under Materials and Methods were followed. The absorbance was uncorrected for background (range 0.103 to 0.150, mean 0.124) or substrate blank (0.044). NRS was added. Dilution of Cilligen and the equivalent penicilloyl concentration in the coating buffer: 1:40, 725 ng/ml; 1:80, 362.5 ng/ml; 1:100, 290 ng/ml; 1:250, 116 ng/ml; 1:400, 73 ng/ml; 1:600, 48 ng/ml.

converge when extrapolated to background to a common titer. The more concentrated solid-phase Cilligen (dilutions 1:40 and 1:80) bound less Ab_p (at a dilution of $1:10^4$) than the lower Cilligen concentrations (dilutions 1:100 and 1:250). However, as Ab_p was diluted further the absorbance shifted to the expected order. A good dose-response was obtained even at the highest Cilligen dilution, 1:600. For example, the absorbance was 5.7 times higher than background at Ab_p diluted $1:10^5$, although the EIA extrapolated titer was 3.3×10^5 (204, Table 1). Moreover, precision was maintained with the dilute (1:600) solid-phase Cilligen since the C.V. was 4.7 at Ab_p dilution $1:10^4$ and the mean C.V. was 7.7.

The antisera and NRS were tested in the indirect ELISA for Ab_p by titration with 2 concentrations of solid-phase Cilligen (Fig. 5). At high antisera dilutions the curves had a distorted response due to interference from background. The linear segments of the curves were extrapolated to the substrate blank and the intercepts (at background) of the 2 curves for each antiserum were the titers as $dilution^{-1}$. The difference between the 2 intercepts measured error since the extrapolated titer was independent of solid-phase Cilligen concentration (Fig. 6A). The linear segments of the Ab_p titration curves exhibited parallelism which reinforced the placement of the extrapolated line if less than 3 points were on the linear part of the curve.

Change in antibody titer with a different HRPO-conjugate: The solid-phase Cilligen produced nearly superimposable linear

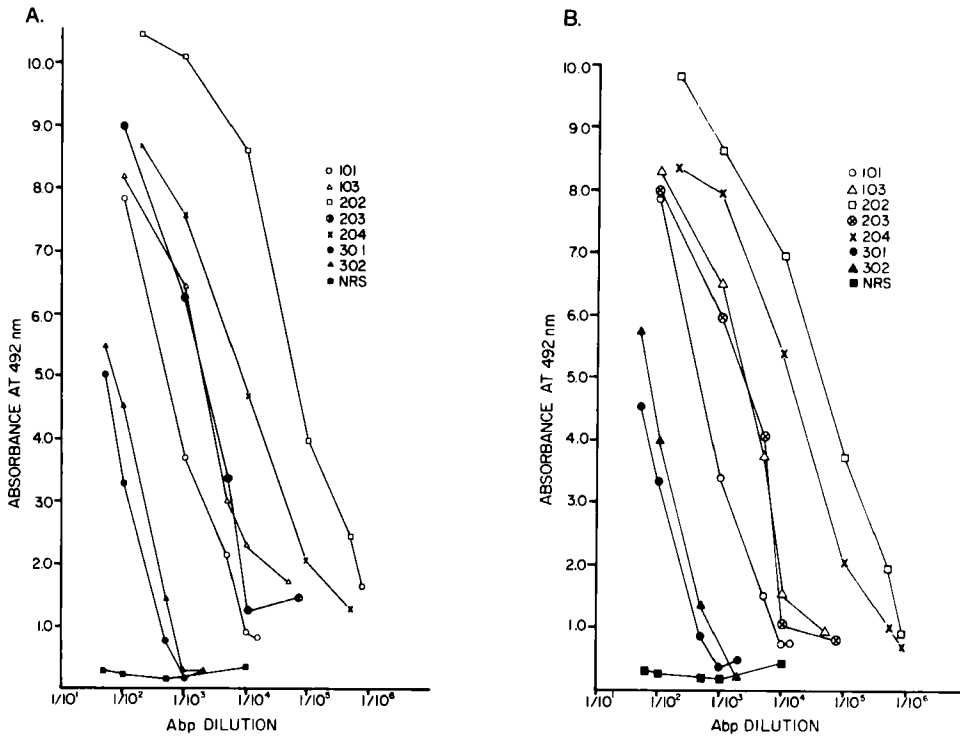

FIGURE 5. Titration curves for rabbit penicilloyl antisera. Serial dilutions were incubated in tubes coated with Cilligen, dilution 1:80 (A) and 1:250 (B). Refer to the graph for the identity of the rabbit sera. NRS was a control, ■. No correction was made for background or reagent blank. No NRS was added to the Ab_p dilutions.

extrapolations of the Ab_p titration curves (Fig. 5). However, in an earlier experiment with another HRPO-conjugate preparation the titrations gave extrapolated lines with marked slope differences. Moreover, a log increase in titer was obtained (Table 2).

The approximately log difference in titer could have been the result of a change in either Ab_p during storage or in the

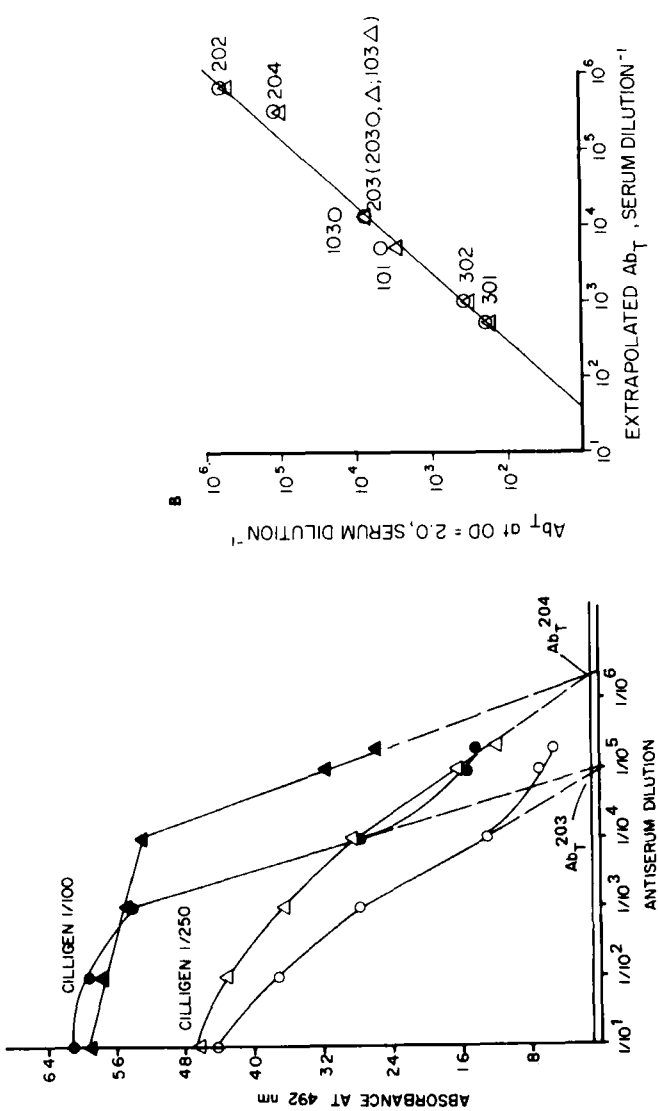

FIGURE 6. (A) Extrapolation of antisera titration curves with 2 solid-phase antigen concentrations to determine titer.

Tube coated with Cilligen at the dilutions, 1:100 (closed symbols) and 1:250 (open symbols) were used for titration of Ab_p 203 (O) and Ab_p 204 (Δ).

(B) The relationship between the titer determined at an arbitrary absorbance to the extrapolated titer.

Two solid-phase Cilligen coating dilutions were 1:80 (O) and 1:250 (Δ). Titer = Ab_T. Absorbance = O.D. 2.0.

TABLE 2

Effect of the Peroxidase Conjugate on Penicilloyl Antibody Titer

Ab_p serum No.	Titer (extrapolation to zero antibody) Reciprocal Dilution with Conjugate 1	Conjugate 2	F*
101	5×10^3	5.6×10^4	11.2
103	1.4×10^4	7.5×10^4	5.4
202	7.1×10^5	$> 10^7$	10 +
203	1.3×10^4	1.1×10^5	8.5
204	3.4×10^5	3×10^6	8.8
301	5×10^2	1.4×10^3	2.8
302	10^3	2.3×10^3	2.3

* F was the ratio of titer with conjugate 2 to titer with conjugate 1. Cilligen was diluted, 1:100 and 1:250, in the coating buffer.

HRPO-conjugate. Since the titer diminished relatively uniformly in the 7 antisera, and antisera should be stable 5 mo at -70°C, storage deterioration was unlikely. Repetition of the titration curves and with a higher Cilligen concentration produced the same titers as in Fig. 5. Although the experiments were done under isokinetic conditions with an excess of HRPO-conjugate, more HRPO activity was evident in the titrations that gave lower titers. Consequently, it appeared likely that the difference in the two assays reflected the change to a different lot of HRPO-conjugate. The HRPO-conjugate that produced higher titers may have had a higher affinity or had less labelled immunoglobulin. The other HRPO-conjugate may have had a higher molar ratio of HRPO to IgG that could hinder sterically the immunoreaction and yet the resultant immunocomplex would have a higher HRPO activity.

Avidity in the Ab_p titration curve: The Ab_p titration curves were examined for avidity and titer. As the Ab_p was diluted in the presence of solid-phase Cilligen concentrations that were initially in excess, the "resistance Ab_p exhibited to dilution" was assumed to vary directly with avidity. Theoretically, high avidity antibodies would maintain more solid-phase immunocomplex binding during dilution than lower avidity antibodies. This effect was more apparent with a low solid-phase Cilligen concentration (Fig. 5B). Titer determined the maximum absorbance and the dilution range that preceded the linear segment of the dose-response curve. Consideration of these 2 concepts suggested that Ab_p204 had a greater avidity than the higher titer Ab_p202 or than the lower titer Ab_p203 (Fig. 5B, 1:500 and 1:1000 dilutions Ab_p). This interpretation for the "resistance Ab_p exhibited to dilution" was supported by a competitive EIA with pen-ε-cap (G. K. Humphreys, unpublished work). The sensitivity for penicilloyl is limited by antiserum avidity and sensitivity was in the order Ab_p204 > Ab_p202 > Ab_p203.

DISCUSSION

Cilligen, a skin-testing compound for penicillin hypersensitivity in man (12), was a suitable solid-phase antigen for the quantitation of penicilloyl antibody in an EIA with the marker enzyme, HRPO. A negligible background and a brief 10 min enzyme assay were advantages over an EIA reported with penicilloylated transferrin (7) that had a higher background and required 1 h at 25°C (or 16 h at 4°C) for HRPO assay. Although

rabbit antisera were titered the protocol could be modified for penicilloyl antibody in other species and/or immunoglobulin class by substitution with the appropriate HRPO-labeled conjugate.

Monovalent, low molecular weight pen-ε-cap and benzylpenicillin were not useful as solid-phase antigens. These results were consistent with the use of macromolecules for adsorption. Small molecular weight compounds might serve as functional antigens if covalently attached by a molecular spacer arm to the solid-phase. However, the solid-phase multivalent Cilligen was expected to be superior to a monovalent penicilloyl for Ab_p assay. Competitions between solid-phase Cilligen and soluble monovalent or multivalent penicilloyl derivatives indicated that avidity increased with increased antigen valence (13). Even multivalent Cilligen required 3 h at 37°C for an adequate antigen coat since the specific immunochemical reaction was negligible with the Cilligen adsorbed 24 h at 4°C. Under the former conditions, a range of Cilligen concentration in the coating buffer of 725 to 48 ng penicilloyl/ml provided a sensitive Ab_p assay with the addition of NRS to eliminate a significant background (absorbance reduced, 1.3 to 0.09).

The assumption that the nonlinear change in the EIA titration curves in the low dilution range of Ab_p with limited solid-phase Cilligen was a useful indicator of relative avidity between common antisera would vary with the assay system. The final evaluation remained the dose-response curve.

A plot for Ab_p titration with 2 solid-phase Cilligen concentrations demonstrated the usefulness of the extrapolated

titer and its lack of dependence on solid-phase Cilligen concentration. If 2 or more concentrations of solid-phase Cilligen were used, the lines intersected at or near the same titer although the slopes varied. The highest solid-phase Cilligen had the steepest slope.

The titration of Ab_p and the detection limit for Ab_p changed with a change in the HRPO-conjugate. Variability in the HRPO-conjugate has prompted other groups to use an enzyme immunocomplex, like peroxidase-antiperoxidase (14) or alkaline phosphatase-antialkaline phosphatase (15), in place of a covalent enzyme-conjugate for EIA.

A 4 step ELISA that used a soluble antibody-alkaline phosphatase immunocomplex, the a-ELISA, has been reported to produce linear antibody titration curves in a log-log plot of absorbance against serum dilution^{-1} with a constant product of (absorbance) x (antibody dilution^{-1}) (termed ELISA units) (15). In the assay reported here, there was no constant product. For example, Ab_p204 (solid-phase Cilligen dilution 1:400) produced the following products over the linear range: 3,784, 24,230, and 87,080 (Fig. 4).

Comparison of four methods to estimate titer: The titration curves (Fig. 5) were analyzed by 4 methods in Table 3. Three methods designated titer as the antiserum dilution^{-1} and the fourth as the absorbance at Ab_p dilution 1:1000. The 4 methods required excess solid-phase Cilligen, excess HRPO-conjugate, and

TABLE 3

Four Methods of Analysis for Assignment of Titer to Penicilloyl Antisera and the Coefficient of Variation for the Methods

Serum No.	Absorbance: Extrapolated to 0 — 1:80 wall	1:250 wall	C.V.	Absorbance: 50 percent maximum — 1:80 wall	1:250 wall	C.V.
	Serum Dilution^{-1}					
101 O	7×10^3	5×10^3	23.6	8.9×10^2	7.5×10^2	12.0
103 Δ	2×10^4	1.4×10^4	25.0	2.9×10^3	3.8×10^3	19.0
202 □	10^6	7.1×10^5	24.0	5.5×10^4	4.5×10^4	14.1
203 ⊠	1.4×10^4	1.3×10^4	5.2	2.5×10^3	5×10^3	47.1
204 X	3.1×10^5	3.3×10^5	4.4	1.3×10^4	2.5×10^4	44.6
301 ●	4.4×10^2	5×10^2	9.0	1.4×10^2	1.4×10^2	0
302 ▲	10^3	10^3	0	2.4×10^2	1.9×10^2	16.5

Serum No.	Absorbance: 2.0 — 1:80 wall	1:250 wall	C.V.	Absorbance: at 1:10^3 serum dilution — 1:80 wall	1:250 wall	C.V.
	Serum Dilution^{-1}					
101 O	4.8×10^3	2.9×10^3	34.9	3.706	3.402	6.0
103 Δ	1.9×10^4	8×10^3	57.6	6.442	6.506	0.7
202 □	6.3×10^5	5.2×10^5	13.5	10.114	8.652	11.0
203 ⊠	7.6×10^3	8×10^3	3.6	6.276	5.962	3.6
204 X	1.2×10^5	1×10^5	12.9	7.590	7.958	3.3
301 ●	1.9×10^2	1.9×10^2	0	0.197	0.360	41.3
302 ▲	3.7×10^2	3.3×10^2	8.1	0.294	0.267	6.8

Titer was the reciprocal of the antiserum dilution that gave an absorbance with the solid-phase Cilligen dilutions, 1:80 or 1:250. Titer at an antiserum dilution of 1:1000 was in absorbance. The mean C.V. for 273 samples (91 triplicates) was 9.

parallelism in the segment(s) of the titration curves used in assigning titer.

(1) Titer defined by extrapolation as antibody dilution^{-1} at zero absorbance:

The main disadvantage was that 2 or 3 points were required on the linear segment of the titration curve. If parallelism

existed between the several curves, this reinforced placement of the extrapolated line. The extrapolated titer was independent of solid-phase Cilligen concentration; therefore, the intercepts of the titration curves with 2 or more Cilligen concentrations were at or near the same titer. For all 7 Ab_p's, extrapolated titers agreed well at different solid-phase antigens concentrations.

(2) Titer defined as antibody dilution^{-1} at an arbitrary absorbance, 2.0:

This method required that the arbitrary absorbance was on or near the linear part of the response curve. The absorbances with 2 concentrations of Cilligen were similar; therefore, the C.V.'s were low except for antisera 101 and 103 because their absorbances were on the nonlinear segment with Cilligen, 1:80 dilution. The titer by the arbitrary absorbance method cannot be used except for comparisons within one set of assay conditions.

The plot, arbitrary absorbance titer against extrapolated titer, was linear (Fig. 6B). Since the absorbance 2.0 was in the nonlinear part of the titration curve for Ab_p, 103 and 101 (Cilligen diluted 1:80), and for Ab_p204, these 4 points deviated from linearity. The curve only appeared independent of solid-phase Cilligen because, in this experiment, antiserum dilutions were similar at absorbance 2.0. If the arbitrary absorbance were greater, then the line would shift to a lower parallel line. A reduction in assigned absorbance would produce a higher parallel line. Although the method directly reflected the extrapolated

titer, more than 1 dilution of antiserum was necessary for the determination.

(3) Titer defined as dilution^{-1} of antibody at 50% maximum absorbance:

The titer assigned at 50% maximum absorbance by definition required the value for maximum absorbance. A high concentration of Ab_p established the 100% absorbance. This approach required that the Ab_p curves were parallel to each other throughout the range of their titration curves. The curves for Ab_p, 203 and 204, with both Cilligen concentrations lacked parallelism and consequently had high C.V.'s (44.6 and 47.1 respectively).

(4) Titer defined as absorbance at 1:1000 dilution of Ab_p (Note: The dilution required for titer estimation will vary with the particular system. Absorbance x 1000 is termed EIA units.):

The 3 previous definitions of titer expressed as dilution^{-1}, required 5 to 6 dilutions of antiserum. This definition required only a single dilution. Although Table 3 lists a low CV for the absorbances produced with the 2 Cilligen concentrations, this probably was unusual and was a theoretically invalid comparison. The more significant comparison was the relationship of absorbance at 1:1000 Ab_p dilution to the extrapolated titer (Fig. 7). The graph should theoretically produce a straight line if the titration curves at the standard dilution were linear and parallel. High titer and low titer Ab_p had an absorbance in the nonlinear range

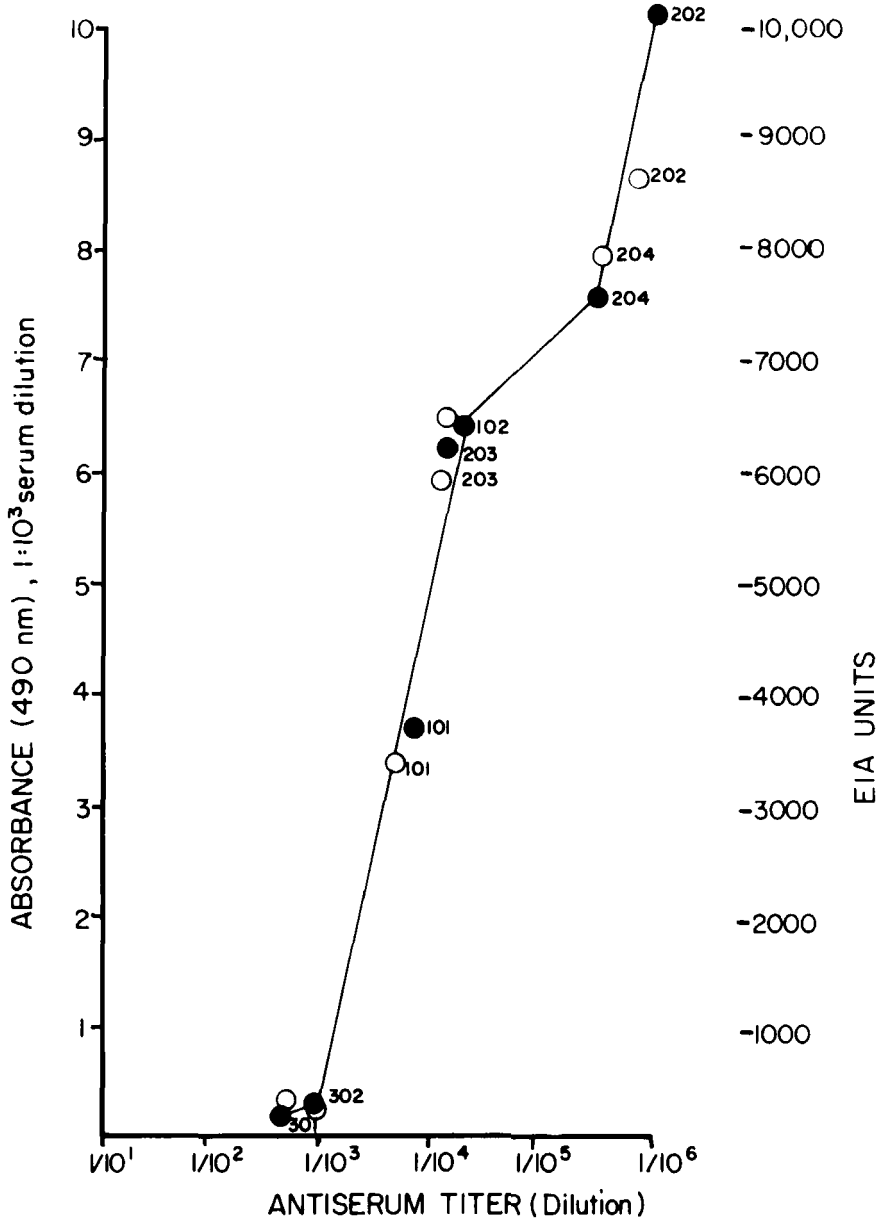

FIGURE 7. The relationship of the extrapolated titer to the absorbance at 1:1000 Ab_p dilution and to EIA units.
Two solid-phase Cilligen dilutions were used, 1:80 ● and 1:250 O. EIA units = (absorbance) x (1000).

of the titration curve. Therefore, the ratio of absorbance to titer declined for these Ab_p. For other systems or ranges of antibody concentrations, the arbitrary dilution would have to be adjusted to maintain a linear relationship with extrapolated titer. Two other groups (16, 17) have also found a correlation between ELISA endpoint titer and absorbance at 1:1000 dilution, using standard curves from 43 human antisera to Salmonella typhimurium (17) and from 41 human antisera to Toxocara (16). Absorbance at a single dilution conserves time, labor, and reagents, and provides a rapid means for screening the relative titer of similar antisera and, potentially, for reporting comparable interlaboratory titers.

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