STUDY OF PENICILLIN ANTIBODIES BY FLUORESCENCE POLARIZATION AND IMMUNODIFFUSION*

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In recent years, it has been shown that human allergic responses following administration of penicillin are usually correlated with the presence of circulating antibodies to derivatives of this antibiotic (1-5). The careful chemical studies by Levine *et al.* (6-9), De Weck (10-12), and Parker (13-15) have shown that these antibodies are most often directed against the penicilloyl determinant group, although other determinants may sometimes be involved. Antigenicity appears to be conferred upon this molecule following its *in vivo* conjugation with proteins derived from tissues (11, 16-18).

Antipenicilloyl antibodies have been evoked in experimental animals by several investigators (*e.g.*, 19-21). Direct immunization with penicillin G and some of its derivatives (12, 20, 22-24) or with penicilloyl-substituted proteins (3, 13, 20, 25) have both been successful. In the latter case, a heterologous protein has usually been used as the carrier (*e.g.*, human or bovine gamma globulin) (13, 25) although occasionally, homologous protein, rabbit serum albumin, has been utilized (20). In most of the studies dealing with this problem, the antibodies have been detected and assayed by passive hemagglutination technics (*e.g.*, 2, 4, 22, 25-27) using the free antibiotic as the source of the coating antigen. Evidence points to a chemical linkage of the penicilloyl moiety to the erythrocyte membrane (26). On only a few occasions have antipenicilloyl antibodies been tested by immunodiffusion technics (16, 24) and then only to a limited extent with rabbit antibodies evoked by injection of penicillin alone.

A new technic for the detection of antigen-antibody reactions which utilizes the principles of fluorescence polarization has been described by Dandliker *et al.* (28-30) and confirmed by others (31). Theoretical considerations, recently reported in detail by Dandliker *et al.* (30) indicate that this technic is particularly well suited to reactions involving small haptens, such as the penicilloyl group. It has a number of important advantages over the hemagglutination system. These include rapidity, simplicity, potential high sensitivity, ability to detect univalent as well as multivalent antibody, ability to yield data concerning the "avidity" and heterogeneity of the

antibodies and the lack of requirement of biologically variable indicators, such as erythrocytes or serum diluents.

The present study was undertaken to explore the usefulness of the fluorescence polarization method in the assay of penicilloyl antibodies and to correlate the results with more classical technics, primarily the immunodiffusion method. For most of this investigation, rabbit antisera to penicilloyl–rabbit serum albumin was utilized, since this complex is of the type which might be expected to occur *in vivo* as a result of injection of penicillin alone. Several rabbit antisera were also prepared to penicilloyl–bovine serum albumin, for comparison. In addition, human sera containing penicilloyl antibodies were examined.

Materials and Methods

Antigens.—Rabbit serum albumin (RSA) was obtained either from fresh whole rabbit serum by ammonium sulfate precipitation (33) or as a commercial product obtained by Cohn fractionation (Pentex, Inc., Kankakee, Illinois). The normal pooled human gamma globulin was stored frozen at −15°C as a 16 per cent aqueous solution (Squibb, New Brunswick, New Jersey).[1]

The following were commercial preparations: bovine serum albumin (BSA), Armour, Kankakee, Illinois, 4 x crystallized human serum albumin (Pentex); polylysine of average molecular weight 195,000 (Sigma Chemical Company, St. Louis), penicillin G (Lilly, Indianapolis, or gift from Pfizer, New York); 6-aminopenicillanic acid (gift from Bristol, Syracuse), and fluorescein isothiocyanate (Baltimore Biological Laboratories, Baltimore). Other aterials and reagents were either c.p. or reagent grade.

Penicilloyl–rabbit serum albumin (PRSA), penicilloyl–bovine serum albumin (PBSA) and penicilloyl-polylysine (PPL) were prepared by the direct condensation of the protein or polypeptide with penicillin G in alkaline solution by the method of Parker and Thiell (14). The products were preserved by lyophilization. A typical preparation of PRSA was found to contain 45 penicilloyl groups per molecule of protein, while the penicilloyl polylysine was 25 per cent substituted.

Haptens.—Haptens containing both the penicilloyl and the fluorescein group were synthesized.[2] Generally, the method involved the condensation of fluorescein isothiocyanate (FNCS) with a bifunctional or a polyfunctional amine. Subsequent condensation of the reaction products with an aqueous alkaline penicillin solution then produced substitution of further amino groups with penicilloyl residues. The coupling amines were 1.4 diaminobutane (DAB), lysine or polylysine (PL). The various haptens are denoted by abbreviations in which P stands for the penicilloyl group and F for the fluorescein group, e.g., the hapten made from FNCS, DAB, and penicillin is indicated by PDABF. Direct coupling of FNCS with 6-aminopenicillanic acid resulted in the hapten F-6APA. Details of the syntheses and analyses will be published elsewhere (44).

Determination of the number of penicilloyl groups substituted into the various protein molecules, or present in the hapten preparations was made by the *p*-hydroxymercuribenzoate method of Levine (7). The number of fluorescein groups contained in the synthesized haptens

was determined by integration of the area under the visible wave-length portion of the absorption band for fluorescein (30).

Antisera.—Four groups of animals were immunized.

Group I: Antisera were prepared in New Zealand albino rabbits (6 in La Jolla, and 4 in the Miami laboratories) by intradermal injection of penicilloyl–rabbit serum albumin (PRSA) in complete Freund's adjuvants (32) at multiple sites. Each dose contained 10 mg of PRSA in 1 ml of the final emulsion. After 2 to 3 doses at weekly intervals, booster immunizations were given 2 to 3 weeks apart, and the animals were bled from the ear artery 10 to 14 days after each booster. The total amounts of PRSA administered to each rabbit varied from 30 to 65 mg.

Group II: Three rabbits were similarly immunized with penicilloyl–bovine serum albumin (PBSA), but the quantity of PBSA given per dose was less (3 mg) and the total dose of PBSA was 18 to 35 mg.

Group III: Three rabbits received repeated doses of penicilloyl-polylysine by the same technic (total doses 20 to 45 mg).

Group IV: Five additional rabbits were immunized with penicilloyl–rabbit serum albumin by weekly intravenous injections on alternate days, of 5 mg in 1 ml, for thirteen doses (total of 65 mg). After a rest period of 2 months, the injections of PRSA in this group were resumed by the intradermal route as in Group I, for a total of 5 intradermal injections 1 month apart. The total amount of additional PRSA given intradermally was 50 mg.

Four coded samples of human sera were obtained through the courtesy of Dr. Paul P. Van Arsdel, Jr.[3] One was from a patient who previously had an allergic reaction to penicillin of the serum sickness type.

Rabbit gamma globulin was prepared by chromatography on DEAE-cellulose, using a column modification of the method described by Campbell *et al.* (46). The globulin fraction of human serum was prepared either as above, or by two successive precipitations at 50 per cent ammonium sulphate. The protein concentrations were determined by optical absorption at 280 mμ, assuming a value of 14 for $E_{1\ cm}^{1\ per\ cent}$ in the case of gamma globulin.

Fluorescence Polarization Technic.—The apparatus, technic, and calculations used for fluorescence polarization measurements were substantially the same as that described previously (29, 30). In many tests, readings were taken immediately after mixing the reactants, as well as after 5 to 10 minutes at room temperature. Since no consistent changes in the polarization readings were found upon standing for even longer periods of time (up to 60 minutes in some tests), final readings were made usually after 1 or 2 minutes.

Immunodiffusion and Hemagglutination.—The immunodiffusion technic employed was a micromodification (34) of the two directional plate method (35). The reactions were developed at room temperature and roughly five times as much volume of serum as antigen was used (about 25 μl *vs.* 5 μl). The 1.5 per cent bacto agar (Difco Laboratories Inc., Detroit) was dissolved in 0.15 M NaCl containing 0.01 M sodium phosphate at pH 7.5.

Absorption was usually carried out by adding the solid hapten or antigen to the serum, mixing, and storing at 4°C for 2 to 4 days with occasional stirring. After absorption, the sera were clarified by centrifugation, although it was found that this was not necessary.

The penicilloyl hemagglutination method of Van Arsdel (42) was employed, with slight modifications. Rabbit erythrocytes were used principally, instead of human, and all test sera were routinely absorbed 4 x with uncoated cells. The diluent consisted solely of normal rabbit serum and penicillin G was employed for the penicilloyl coating.

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[3] The authors are indebted to Dr. Van Arsdel for the specimens of human sera. These had been assayed for penicilloyl antibodies by the hemagglutination method in his laboratory.

RESULTS

Immunodiffusion.—The rabbit anti-PRSA revealed clearly defined and rather intense precipitation in immunodiffusion tests. All 10 rabbits of group I immunized with Freund's adjuvant emulsion responded with varying concentrations of detectable antibodies, while none of the 5 given intravenous injec-
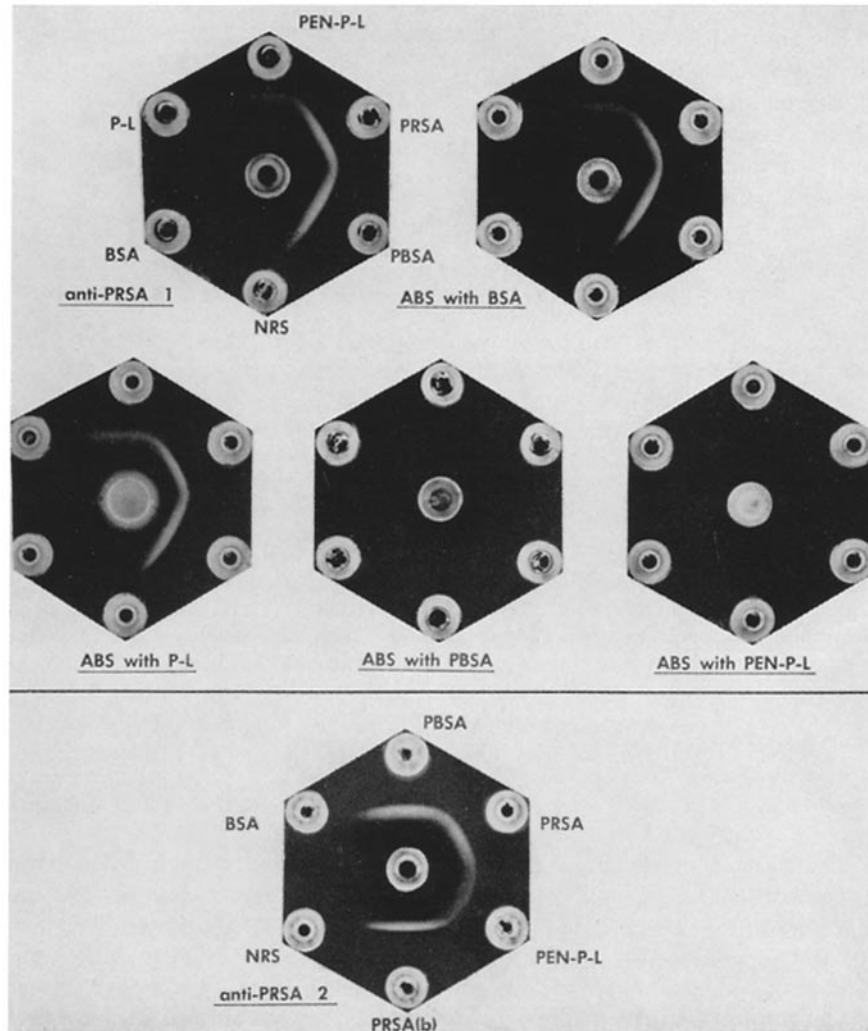

FIG. 1. Immunodiffusion reactions of rabbit antipenicilloyl–rabbit serum albumin (central wells), before and after absorption. Antigens (2 mg/ml). PEN-P-L, penicilloyl–polylysine; PRSA, penicilloyl–rabbit serum albumin (2 samples); PBSA, penicilloyl–bovine serum albumin; NRS, normal rabbit serum; BSA, bovine serum albumin; P-L, polylysine.

tions did. The bands which occurred showed "reactions of identity" when the antisera were simultaneously exposed to PRSA, PBSA, and penicilloyl-poly-lysine. These results are exemplified in Fig. 1, with two different antisera. It may be noted that spurs were not present between any of the three penicilloyl antigens, nor were they ever detected. Furthermore, reactions were never seen between the anti-PRSA and albumin in normal rabbit serum, bovine serum albumin, or polylysine.

The precipitin reactions of anti-PBSA sera were indistinguishable from those found with anti-PRSA and here, too, spurs were never seen. In addition, it was of interest that with anti-PBSA, reactions were never detected with bovine serum albumin itself, the heterologous penicilloyl carrier. These findings suggest that the high concentration of penicilloyl groups on the bovine albumin had essentially abolished the antigenic determinants of this protein molecule.
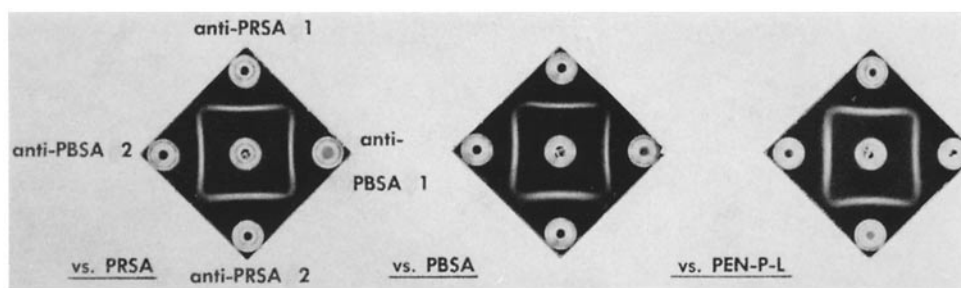

FIG. 2. Cross-reactions of anti-PRSA and anti-PBSA (peripheral wells) against the penicilloyl antigens (central wells). Antigens as in Fig. 1.

That the specificity in these cases was due to the penicilloyl moiety was further substantiated by absorption experiments. Each of two different potent anti-PRSA and two anti-PBSA sera were absorbed with PRSA, PBSA, penicilloyl-polylysine, RSA, BSA, or polylysine. After absorption, the sera were tested against the three penicilloyl antigens by immunodiffusion. In all cases where the antisera were exposed to penicilloyl proteins, the reactions against all were consistently negative, as exemplified in Fig. 1.

The specificities of the antibodies were also tested in the reverse manner, e.g., anti-PRSA and anti-PBSA in adjacent wells were exposed to PBSA. Typical results are shown in Fig. 2, where it may again be seen that only reactions of identity were noted, never any spurs. Similar results were obtained when PRSA or penicilloyl-polylysine were used as antigen and exposed to the two types of antisera.

All of the normal preimmunization control sera, and several sera from other control rabbits, consistently failed to reveal any detectable reactions with these antigens. Precipitin patterns similar to those seen above were found with the penicilloyl antigens and gamma globulin from immune sera, but none were

ever seen with normal gamma globulin. Bovine serum albumin and human serum albumin at a concentration of 30 mg/ml were completely negative in immunodiffusion assays with PRSA, PBSA, and penicilloyl-polylysine.

Titration of the antisera revealed that some could be diluted up to 1:32 and still produce an immunodiffusion precipitate. Similar assays of repeated bleedings of individual rabbits during the course of immunization suggested that the titers reached a peak early in the series and then maintained a fairly steady level in spite of further booster doses. When the antigens were assayed with undiluted antisera, as little as 0.023 mg/ml of PRSA could be detected and antigen excess effects were not found. The precipitin bands were only moder-

TABLE I

Antibody Response of Rabbits Immunized with Penicilloyl Antigens

Group	No. producing antibody/No. Injected	Total antigen injected	Range of titers *vs.* immunizing antigen by immunodiffusion
		mg	
I. PRSA intradermally, adjuvants	10/10	30 to 65	1:2 to 1:32
II. PBSA intradermally, adjuvants	3/3	18 to 35	1:2 to 1:32
III. Penicilloyl-polylysine, intradermally, adjuvants	0/3	20 to 45	—
IV. PRSA intravenously, then intradermally, adjuvants	0/5	65 i.v. 50 i.d.	—

For Groups I, II, and III, the antisera were obtained after 1 to 4 months of immunizations, while for Group IV, the injections and trial bleedings were maintained over a period of 1 to 7 months.

ately crisp in the early stages of development and, after several days, they became quite wide and diffuse over all ranges of concentration. In some instances, after more prolonged development, doubling and striations of the bands appeared. The significance of this is not clear, although it is known that some other single immune reactions behave in this manner (*e.g.*, see reference 36).

The responses of the various groups of rabbits are summarized in Table I. The failure of the intravenously injected animals to synthesize detectable antibody is of interest, and suggests the development of some type of immune unresponsiveness since subsequent intradermal doses in adjuvants failed to stimulate an antipenicilloyl response. Penicilloyl-polylysine proved non-antigenic in this series.

Immunological Specificity of the Fluorescent Penicilloyl Haptens.—After synthesis and purification of the fluorescein-tagged penicilloyl haptens, the identity

of their immunological determinants with the antibodies in anti-PRSA and anti-PBSA was revealed by absorption tests. These were carried out in a semi-quantitative way by serially diluting the haptens from 0.6 mM or 0.5 mg/ml, and adding to each dilution an equal amount of anti-PRSA or anti-PBSA. The latter were adjusted to a level just adequate to produce a definite precipitate by immunodiffusion. Controls consisted of the antisera diluted to the same extent in the diluent (buffered saline pH 7.0). The mixtures were agitated briefly, stored at 4°C for 3 to 5 days with occasional stirring, then tested against PRSA or PBSA by immunodiffusion. It was found that all of the haptens at high concentrations, with the exception of F-6APA, were capable of *completely* inhibiting precipitation of the antibodies in anti-PRSA or anti-PBSA sera. As little as 0.08 mM of PDABF, or 0.015 mg/ml of FPPL prevented the immuno-diffusion precipitin reaction. F-6APA failed to inhibit the reaction.

Appropriate control tests showed that the haptens were not non-specifically destroying antibody. The same haptens in the above concentrations were exposed to a suitable dilution of rabbit anti–bovine serum albumin. No diminution of the precipitating activity of the anti-BSA was noted by any concentration of the haptens used. These findings, together with the failure to absorb antipenicilloyl antibodies by F-6APA, clearly demonstrated the specific nature of the combination with the fluorescein–tagged penicilloyl haptens.

Fluorescence Polarization.—Tests with a pool of rabbit anti-PRSA and pre-immune normal rabbit gamma globulins with the various haptens are summarized in Fig. 3. The globulins were utilized at a concentration of 150 μg/ml, and it may be seen that the fluorescence polarization values were significantly increased only in the presence of antipenicilloyl gamma globulin with all of the haptens, except F-6APA. The failure of F-6APA to react by fluorescence polarization with the penicilloyl antibodies supports the above immunodiffusion data, and is in agreement with others regarding the specificities of these antibodies (19).

The sensitivity of the fluorescence polarization technic for detecting antibody activity against the penicilloyl haptens was tested by exposing serial dilutions of pooled immune gamma globulin to standard concentrations of PDABF, FPPL, and alpha fluorescein-epsilon penicilloyl-lysine (αFePL). Typical results are shown in Figs. 4 a to 4 c. As little as 5 to 10 μg/ml of immune gamma globulin was capable of producing a detectable increase in fluorescence polarization with the penicilloyl haptens. This was roughly equivalent to a whole serum dilution of approximately 1:2000, assuming that the gamma globulin represents 15 per cent of the total serum proteins. This value approaches the maximum for hemagglutination titers reported by others for potent animal or human antipenicilloyl sera (*e.g.*, 2, 3, 25–27). At even the highest concentrations, normal gamma globulin had no effect. It should be noted that the chromatographic serum fractions used represented total 7S gamma globulin, of which

only a portion was actually antipenicilloyl antibody. It is virtually certain that the limit of detectability is much lower if only antibody protein purified by dissociation methods is considered (see Table III).

Samples of gamma globulin prepared from several individual anti-PRSA and anti-PBSA rabbits were tested against PDABF. The results are summa-
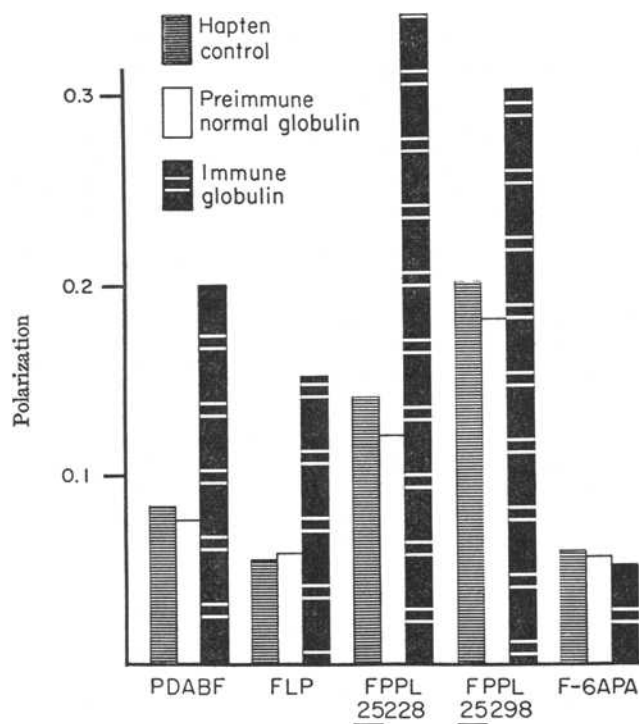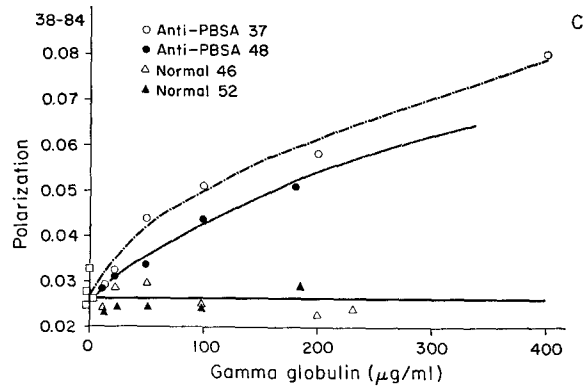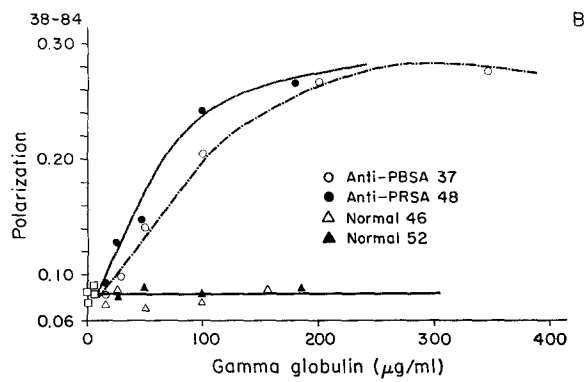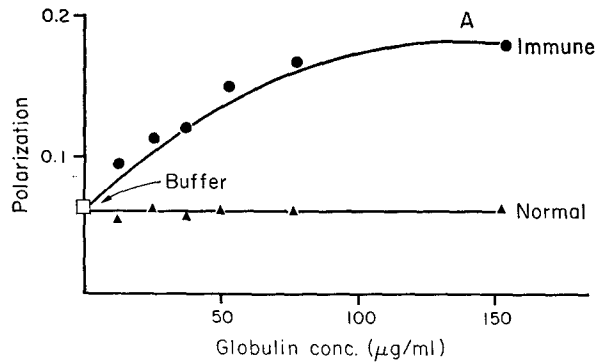

FIG. 3. Rabbit antipenicilloyl-RSA globulin and haptens, 3835. Fluorescence polarization measurements with pooled immune gamma globulin (antipenicilloyl–rabbit serum albumin), and normal rabbit gamma globulin (preimmune control). Haptens: PDABF, penicilloyl-diaminobutane-fluorescein; FLP, penicilloyl-lysine-fluorescein; FPPL, 2 different lots of fluorescein-penicilloyl-polylysine; F-6APA, fluorescein-6-aminopenicillanic acid.

rized in Fig. 5, and demonstrate the variations of the curves with antisera of differing potencies. Correlation with the immunodiffusion titers of whole anti-serum was only fair, but the latter titers were relatively inexact.

The results of testing human globulin solutions against PDABF are shown in Table II. It may be seen that the polarization values for the human globulins correlated well with the hemagglutination titers. Sample (1 abs) represented the globulin of serum 1 after it had been repeatedly absorbed with penicilloyl-coated erythrocytes. The separated globulin still agglutinated such

Figs. 4 a to 4 c. Titration of rabbit-antipenicilloyl antibody by fluorescence polarization with several penicilloyl haptens.

Fig. 4 a. Rabbit antipenicilloyl globulin and PDABF. Pooled anti-PRSA tested with penicilloyl-fluorescein diaminobutane (PDABF).

Fig. 4 b. Titration of rabbit gamma globulins with FPPL. Anti-PRSA and anti-PBSA tested with penicilloyl-fluorescein-polylysine (FPPL).

Fig. 4 c. Titration of rabbit gamma globulins with αFεPL. Anti-PRSA and anti-PBSA tested with α-fluorescein, ε-penicilloyl lysine.

erythrocytes to a low titer (1:16), but it revealed a substantial increase in the fluorescence polarization value. Control tests of normal and antipenicilloyl rabbit gamma globulin were simultaneously performed, as well as tests with *normal pooled human* gamma globulin. The latter, which did not reveal any antipenicilloyl activity, had previously been shown to be very rich in streptococcal and staphylococcal antibodies (36, 37).

In order to obtain information concerning the avidities, heterogeneities, and quantities of antibodies present in the rabbit and human globulin, complete
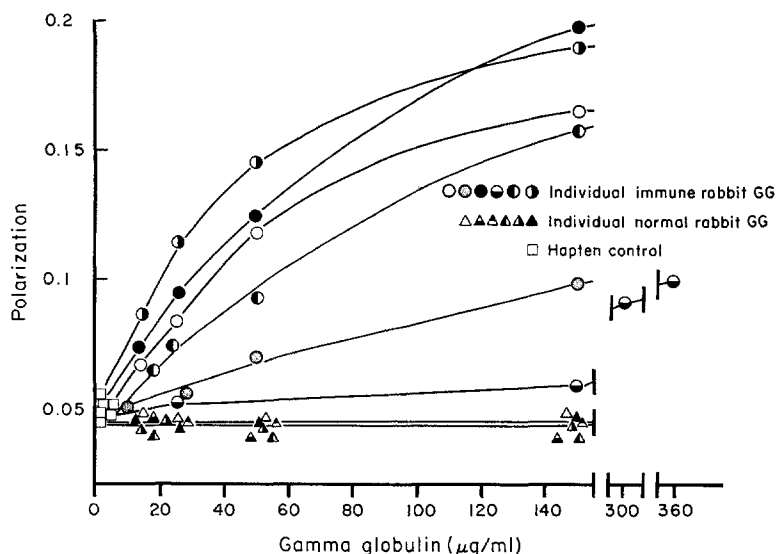

Fig. 5. Fluorescence polarization assays of individual rabbit antipenicilloyl gamma globulins vs. PDABF. O, ●, ◉ anti-PRSA, ◑, ◐, ◒ anti-PBSA. Three of the normal gamma globulin samples represent preimmune bleedings from animals furnishing the antibodies.

titrations were carried out at varying hapten and globulin concentrations. The results with PDABF are shown in Figs. 6 and 7. In each case, the upper three curves represent data for different concentrations of immune globulin and the lower-most solid points for a single concentration of normal globulin acting as a control. Experimental points, consisting of polarization as a function of molarity of hapten, are shown by symbols. From these data, using methods described previously (30), the best least square values of the thermodynamic parameters were calculated by digital computer. Using these parameters, the solid curves were then computer calculated. The degree of fit between theory and experiment can be expressed in terms of a percentage standard deviation (SD) defined by the expression:

$$\text{Per cent } \text{SD} = \frac{100}{N}\left[\sum_{i=1}^{N}\left(\frac{p_i - p_i \text{ calc.}}{p_i}\right)^2\right]^{\frac{1}{2}}$$

in which N is the total number of experimental values of the polarization p. For the rabbit and human systems, the values of per cent SD were 0.3 and 0.6, respectively.

The calculated values for avidities (Ko), heterogeneity (a), and antibody site concentration ($F_{b,max}$), together with the smallest antibody concentration readily detectable with this technic are shown in Table III. From the molar concentration of the binding sites, together with the globulin concentration in mg/ml, the proportion of antibody in the globulin preparations could be calculated. It represents the fraction of the total gamma globulin that is specifically reactive to the penicilloyl hapten. This was approximately 0.3 per cent

TABLE II

Fluorescence Polarization of Human and Rabbit Penicilloyl Antibody with the Penicillin Hapten PDABF

Globulin	Sample	Polarization	Hemagglutination
None	Hapten control	0.041	—
Human	1	0.097	1:512
	1 abs*	0.069	1:16
	3	0.050	Neg.
	4	0.051	Neg.
	Pooled normal		
	Gamma	0.048	Neg.
Rabbit	Immune	0.194	1:4,096
	Normal	0.042	Neg.

The globulin concentrations were adjusted to 0.15 mg/ml for the assays. Globulin 1 abs* represents the sample obtained from serum 1, after repeated absorption with penicilloyl-coated erythrocytes.

in the case of the human and about 3 per cent for the rabbit system (hyperimmunized animals). The data in Figs. 6 and 7 can also be used to calculate the sensitivity of fluorescence polarization for detecting antipenicilloyl antibodies. (See last column, Table III).

Fluorescence polarization tests with unfractionated *whole normal* rabbit sera always showed striking increases in polarization values. However, no significant differences were found between these, and the increases noted with potent *whole immune* serum. These effects occurred rapidly and were noted with all the haptens used, including the relatively large molecular weight FPPL. It seemed reasonable to suspect that non-specific binding of the haptens by some serum proteins might be involved. Since serum albumin is capable of binding many types of molecules non-specifically (*e.g.*, reference 38), tests were carried out on the polarization of some of the penicilloyl haptens with normal rabbit, bovine, and human serum albumins. The results are shown in Figs. 8 *a* and

8 *b* with two haptens. It may be seen that all of these albumins produced dramatic effects on the polarization values, indicating non-specific binding detectable at albumin levels as low as 150 μg/ml. The differences in the curves suggest that the binding is less marked with αFϵPL than with PDABF.
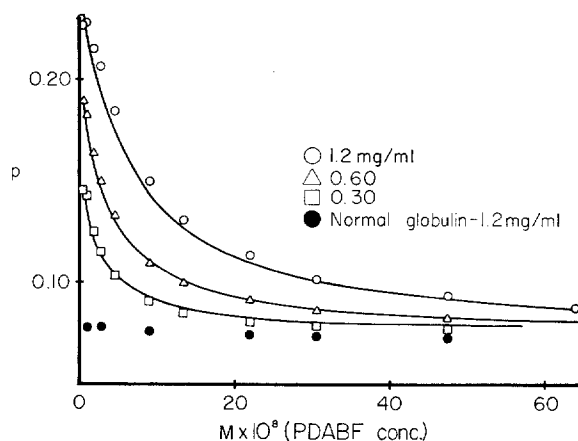

Fig. 6. Human antipenicilloyl globulin and PDABF. Titration curves used for determining avidity, amount, and heterogeneity of penicilloyl antibody in human gamma globulin from a single allergic patient. The hapten was PDABF and the globulin was obtained by DEAE chromatography. O, Immune globulin, 1.2 mg/ml; $\triangle$, immune globulin, 0.60 mg/ml; $\square$, immune globulin, 0.30 mg/ml; ●, normal globulin control, 1.2 mg/ml.

The points represent experimental values, and the solid curves were calculated by computer as described previously (30). Avidity (Ko) = 3×10^7 M^{-1}; heterogeneity (a) = 0.78, antibody site concentration (F$_{b,max}$ lowest curve) = 1.3×10^{-8} M.

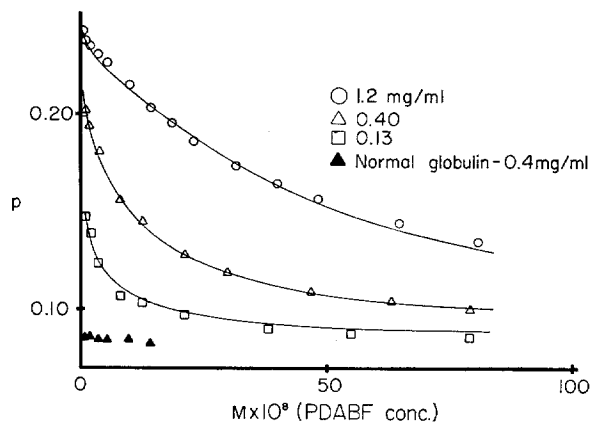Fig. 7. Data as in Fig. 6, for rabbit-antipenicilloyl gamma globulin from a single rabbit. O, Immune globulin, 1.2 mg/ml; $\triangle$, immune globulin, 0.40 mg/ml; $\square$, immune globulin, 0.13 mg/ml; $\blacktriangle$, normal globulin control, 0.4 mg/ml.

Avidity (Ko) = 9×10^6 M^{-1}; heterogeneity (a) = 0.71, antibody site concentration (F$_{b,max}$ lowest curve) = 4.3×10^{-8} M.

It seemed possible that this non-specific binding of the penicilloyl haptens by serum albumin (and possibly other non-antibody serum proteins) might be inhibited by conditions or substances which do not affect the reactions with specific antibody. It was found that increasing the pH from 7 to 10 decreased

TABLE III

Parameters for Antipenicilloyl Antibodies

Source	Avidity (Ko)	Hetero-geneity constant (a)	Binding site concentration (F b,max)	Globulin concentration	Antibody protein / total protein	Detectable anti-body concentration (μg/ml required to give $\Delta p = 0.01$)*
	(M⁻¹)		M	mg/ml		
Human	3.0×10^7	0.78	1.25×10^{-8}	0.30	0.0033	0.25
Rabbit	8.7×10^6	0.71	4.32×10^{-8}	0.13	0.027	0.44

* This quantity is a measure of the sensitivity of detection under the conditions used. A change in polarization of 0.01 is readily measurable.
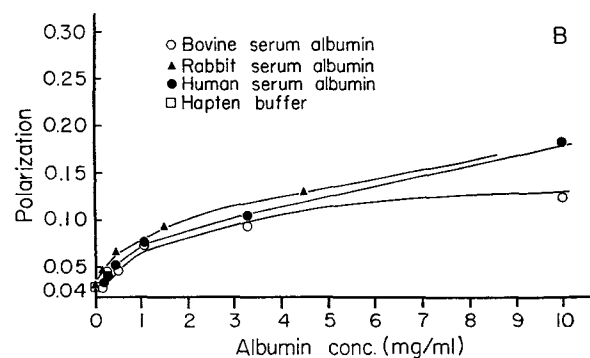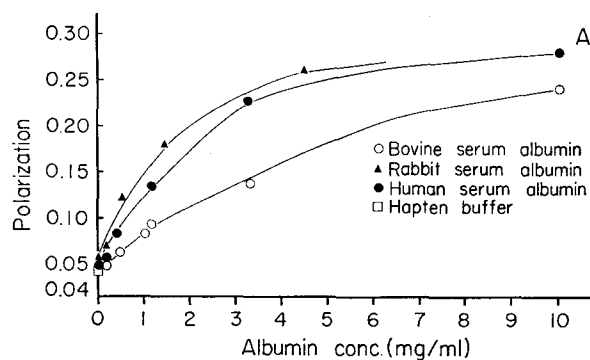
Figs. 8 a and 8 b. Non-specific binding by albumin. Fig. 8 a. Non-specific binding of serum albumin with PDABF; Fig. 8 b. Non-specific binding on serum albumin with αFεPL.

the non-specific binding considerably, with a somewhat smaller effect on the hapten-antibody interaction. In addition, elevation of the salt concentration to 0.5 m NaCl has an even more selective effect in depressing the non-specific binding, unmasking the specific reaction to some extent in whole sera (see Fig. 9). Preliminary tests suggest that certain straight chain aliphatic acid anions may be effective in eliminating the non-specific binding. It is planned to systematically explore combinations of these factors in the hopes of completely eliminating this interfering effect.
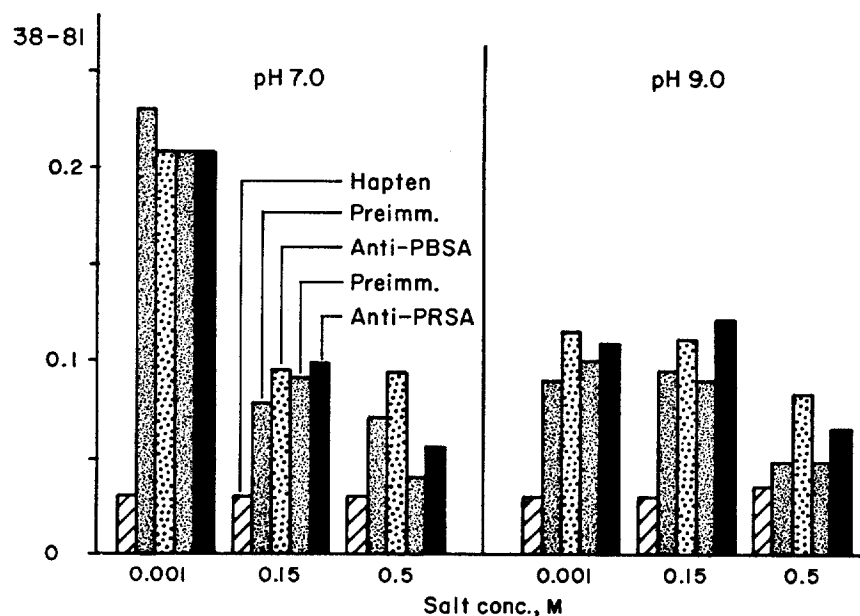

Fig. 9. Effect of pH and salt concentration on the non-specific and specific binding of alpha F-epsilon P-lysine with whole normal and immune serum.

Passive Hemagglutination.—Many of the published studies dealing with antibodies to penicilloyl and related determinant groups have utilized the passive hemagglutination technic with fresh erythrocytes. In most cases (*e.g.*, references 2, 4, 19, 22, 26), the red cells have been exposed to the parent compound, *e.g.*, penicillin G at an alkaline pH. Antisera containing penicilloyl antibodies are then capable of causing hemagglutination, but serum dilution titers of over 1:2000 have hardly ever been recorded, even with potent rabbit antisera (2, 3, 12). In some instances, the latter have been shown to possess rather substantial quantities of precipitating antipenicilloyl antibodies. Considerable efforts in this laboratory to develop a procedure employing fixed erythrocytes coated with penicilloyl-proteins were unsuccessful in yielding higher titers.

When the tanned fixed erythrocytes were coated with PRSA, PBSA, or PPL, the hemagglutination titers obtained with the sera used in Figs. 1 and 2 ranged from 1:160 to 1:1280, but the results were not adequately consistent from day to day. Control tests with the bovine serum albumin or the diphtheria toxoid systems proved that the erythrocytes were satisfactory for passive hemagglutination.

DISCUSSION

Potent rabbit antisera to the penicilloyl moiety were readily produced by immunization with penicilloyl conjugates of the homologous protein, rabbit serum albumin, as well as with penicilloyl–bovine serum albumin. In two directional immunodiffusion tests, the antibodies so produced were capable of precipitating each of these penicilloyl conjugates, in addition to a penicilloyl-polylysine complex. That the antibody specificity was the same in each case was indicated by the lack of spur formation when the several antigens were exposed to a single antibody, as well as when a single antigen was exposed to antibodies produced against heterologous and homologous protein penicilloyl complexes. Furthermore, absorption of any of the antisera with PRSA, PBSA, penicilloyl-polylysine, or the fluorescent penicilloyl haptens completely removed detectable gel precipitin reactions with *all* the penicilloyl polypeptides. The failure of the antisera to precipitate with the protein carrier, and failure of the carrier to absorb antibodies reacting with the penicilloyl protein complexes in any instance, strongly indicated that the penicilloyl determinant was essentially the only one present in the system. It was of interest to find that immunization of rabbits with the heterologous penicilloyl–bovine serum albumin did not result in any detectable antibodies with bovine serum albumin specificity. It seems likely that the large number of penicilloyl substitutions virtually eliminated the bovine albumin determinants. A somewhat similar situation has been recorded with BSA which has been heavily substituted with purinoyl groups (46).

The finding that penicilloyl-polylysine is capable of acting as a precipitating antigen is of some interest, as this substance is now widely used in skin tests for penicillin hypersensitivity (13, 39–42). The 3 rabbits receiving prolonged and intensive immunization with penicilloyl polylysine failed to reveal any detectable antibodies, however. This finding is in agreement with others, although Levine has demonstrated an immune response to such preparations in certain strains of guinea pigs, depending on the proportion of penicilloyl groups substituted (9, 43).

The antibody responses of the various groups of animals warrant further investigations. All animals injected by the intradermal route with Freund's adjuvants produced appreciable antibody, while those immunized intravenously followed by similar intradermal dosage, uniformly failed to produce any detectable responses. Whether an immune unresponsiveness, individual varia-

bility, or technical factors accounted for these differences in response of the several rabbit groups awaits further study.

The feasibility of detecting relatively small quantities of antipenicilloyl antibodies by the fluorescence polarization technic has been clearly demonstrated with several different types of penicilloyl haptens. As little as 0.4 μg/ml of antibody globulin was calculated to be capable of increasing the polarization over the base line value for the hapten alone. Normal rabbit gamma globulin had no detectable effect on the fluorescence polarization measurements at the highest protein concentrations tested, 1200 μg/ml.

Measurements on the globulin from a penicillin allergic patient revealed fluorescence polarization results quite similar to those seen with rabbit antibodies. Since fluorescence polarization measures not only the amount of antibody, but also its heterogeneity and combining affinity, it should now be possible to determine the clinical significance of these parameters in penicillin hypersensitivity. Suggestively, recent observations by Levine (47) indicate that high affinity antibodies to a dinitrophenyl hapten probably mediate delayed hypersensitivity in guinea pigs. Titers of antibody by the other methods used are not always correlated with the several manifestations of penicillin hypersensitivity (2).

Attempts to utilize fluorescence polarization with whole serum revealed that both normal (preimmune) and immune sera showed strong non-specific binding of the penicilloyl haptens. At pH 7.0, no significant differences between normal and immune sera were found with several of the haptens. That serum albumin was at least partly involved in this non-specific effect was clearly seen by tests with bovine, rabbit, and human albumins, acting on PDABF or αFePL. Studies aimed at reducing this interfering effect included tests under different environmental conditions. It was found that high pH, high salt concentration and certain aliphatic acid anions significantly decreased the non-specific binding. These findings indicate that it may be possible to create conditions which will eliminate this difficulty, so that only the specific immune reaction will be detected with whole serum. The overall data also suggests that the fluorescence polarization technic may be particularly useful in detecting antibodies to other small chemicals or drugs, such as procaine, picrylchloride and dinitrofluorobenzene.

SUMMARY

1. Antibodies prepared in the rabbit to penicilloyl–rabbit serum albumin or to penicilloyl–bovine serum albumin were demonstrated by immunodiffusion and absorption methods to be apparently directed exclusively against the penicilloyl moiety. The antibodies could precipitate heavily substituted penicilloyl polylysine, as well as each other, with "reactions of identity". All could mutually deplete detectable antibodies by cross-absorption.

2. Small haptens containing both fluorescein and the penicilloyl group were synthesized. They were also capable of completely absorbing the antipenicilloyl antibodies from rabbit antiserum, as evidenced by immunodiffusion tests. The haptens were used in fluorescence polarization tests with gamma globulin from normal and immunized rabbits, and from normal and allergic humans. The rabbit antipenicilloyl gamma globulins in concentrations as low as 5 to 10 μg of protein/ml could significantly increase the polarization of the haptens. Normal gamma globulin had no effect at the highest concentrations tested, 1200 μg/ml. In all tests, rabbit and human antibody reacted similarly.

3. Fluorescence polarization titration curves for both human and rabbit antipenicilloyl gamma globulins were analyzed by computer and the antibody concentrations, avidities, and heterogeneity constants were determined. For the human antibody, the latter two values were 3.0×10^7 M^{-1} and 0.78, while for the rabbit, they were 8.7×10^6 M^{-1} and 0.71. The data were employed to estimate the limit of sensitivity of fluorescence polarization for detecting antipenicilloyl antibodies. Under the conditions employed, this value was roughly 0.4 μg antibody/ml.

4. When the whole rabbit sera were tested for penicilloyl antibodies by fluorescence polarization, both normal (preimmune) and immune sera revealed striking and equivalent increases in polarization with the penicilloyl haptens. This non-specific binding was shown to be due at least in part to serum albumin. Indications were obtained that it might be significantly reduced by increasing the pH or the salt concentration of the medium, or by addition of certain anions.

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