IMMUNOLOGICAL CROSS-REACTIONS OF SULFOBENZYLPENICILLIN

KANJI TSUCHIYA, MASAHIRO KONDO, TOKIKO OISHI
and KIYOSHI ISHIFUJI

Biological Research Laboratories, Central Research Division,
Takeda Chemical Industries, Ltd., Osaka, Japan

(Received for publication December 1, 1972)

Sulfobenzylpenicillin (sulbenicillin)-BGG conjugate was shown to be highly reactive with anti-sulbenicillin-BSA serum. This conjugate was, however, not reactive or was only slightly reactive with anti-benzylpenicillin- and anti-ampicillin-BSA sera produced in rabbits. The immunological specificity of sulbenicillin seems to be dependent on the acyl side chain of the molecule, while, its immunological cross-reactivity seems to be dependent on the 6-aminopenicillanic acid moiety in the structure of penicillin. Also, with other penicillin derivatives, similar evidence was obtained. Immunodiffusion studies revealed that two precipitin bands developed between several penicillin-BGG conjugates and anti-penicillin-BSA serum. Since these precipitin bands do not correlate with BGG and BSA used as carrier, the bands are probably formed by the specific immunological reaction between penicillin and anti-penicillin serum. Furthermore, specific reactivity of sulbenicillin is also demonstrated by the development of passive cutaneous anaphylaxis test.

Sulfobenzylpenicillin (sulbenicillin)* is a newly developed semi-synthetic penicillin with broad antibacterial activity. This penicillin is especially active against *Pseudomonas aeruginosa*.[1,2] The structural formula of sulbenicillin is similar to that of benzylpenicillin, ampicillin and carbenicillin. These penicillins differ in having $-SO_3$, $-H$, $-NH_2$ and $-COOH$, respectively at the α-position of the acyl side chain.

It is well known that benzylpenicillin and various semi-synthetic penicillins sometimes elicit hypersensitivity in clinical use. The antigenicity of the semi-synthetic penicillins is not only depended on the 6-aminopenicillanic acid moiety which is common to penicillins, but also strongly on the chemical nature of the side chain at the 6-position. Similar findings were observed regarding cephalosporin derivatives.[4,5]

In the present study, the immunological cross-reactivity between sulbenicillin and each of its related penicillins was examined using quantitative precipitin reaction, hapten-inhibition of precipitin, agar-gel precipitin reaction and passive cutaneous anaphylaxis.

Materials and Methods

Chemicals: Sulbenicillin and carbenicillin used in this study were supplied by Takeda Chemical Industries, Ltd., Osaka, Japan, ampicillin was by Wyeth Laboratories, U. S. A., 6-aminopenicillanic acid was by Royal Netherland Fermentation Industries, Holland, and benzylpenicillin was a commercial product of Takeda Chemical Industries, Ltd.

Bovine serum albumin (BSA) and bovine gamma globulin (BGG) were purchased from Armour Laboratories, Kankakee, Illinois and Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A., respectively.

* Formally named sulfocillin.

Preparation of Penicillin-protein Conjugate: One hundred mg of BSA or BGG was dissolved in 10 ml of physiological saline and 600 mg of a penicillin was added to these protein solution. The mixture was adjusted to pH 10 with 1 N NaOH. After 24-hour incubation at 37°C the reaction mixture was dialyzed at 4°C for 56 hours repeatedly against a large volume of physiological saline, pH 8.

Antisera: Rabbits weighing 2.5~3.5 kg of either sex were used. Ten ml of a penicillin-BSA conjugate was emulsified in 10 ml of complete FREUND's adjuvant. One tenth ml of the mixture was injected intradermally into 20 sites on the abdomen. A week later two ml of the emulsified mixture was intramuscularly injected. Thereafter, injections were repeated at weekly interval by the intradermal and intramuscular routes. One week after the fourth injection the rabbits were intravenously given 60 mg of penicillin alone dissolved in physiological saline as a booster. Four to ten days after the last injection of the penicillin the sera were prepared from the blood obtained by bleeding.

Quantitative Analysis of Precipitin Reaction: Following the method developed by HEIDERBERGER,[6] antisera were clarified by centrifugation of the above-mentioned sera at 10,000 r.p.m. for 60 minutes. Various amounts of penicillin-BGG conjugate dissolved each in 1 ml of 1/15 M phosphate buffer saline at pH 7.4 were added to 1 ml of each antiserum. After 2 hours of incubation at 37°C the mixtures were stored at 4°C for 48 hours. Each reaction mixture was then centrifuged and the precipitation thus obtained was washed three times with 2 ml of ice cold phosphate buffer saline. The amount of protein in the precipitate was assayed by the method of LOWRY[7] (modification of CIOCULATEAU's method) and was calculated as BGG.

Quantitative Hapten-inhibition of Precipitin Reaction: Methods developed by PAULING *et al.*[8] were used. Increasing amounts of a hapten dissolved in 0.5 ml of phosphate buffer saline at pH 7.4 were added to 0.5 ml of antiserum. After 2-hour incubation at 37°C, a maximum reaction producing dose on homologous precipitin reaction of penicillin-BGG conjugate dissolved in 1 ml of phosphate buffer saline at pH 7.4 was added to the hapten-antiserum mixture. After a further 2-hour incubation at 37°C and subsequent 48-hour storage at 4°C, the resultant precipitate was washed and assayed for protein by the method described above.

The inhibitory effect of hapten was calculated by comparing with the amount of protein in the precipitate of the hapten-containing and non-containing tubes.

Immunodiffusion Analysis: Methods developed by OUCHTERLONY[9] were used. Analysis was done in 0.8 % agar (Special agar B, Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 0.85 % NaCl and 0.01 % NaN_3.

Passive Cutaneous Anaphylaxis Test: The method developed by OVARY[10] was used. Antisera were injected intradermally into the skin of guinea pigs, and a mixture of penicillin-BGG conjugate and 1 % Evans blue solution was challenged by the intravenous route 6 hours after sensitization. Fifteen to 30 minutes after challenge, areas of dye leakage were measured in size from two distinct directions.

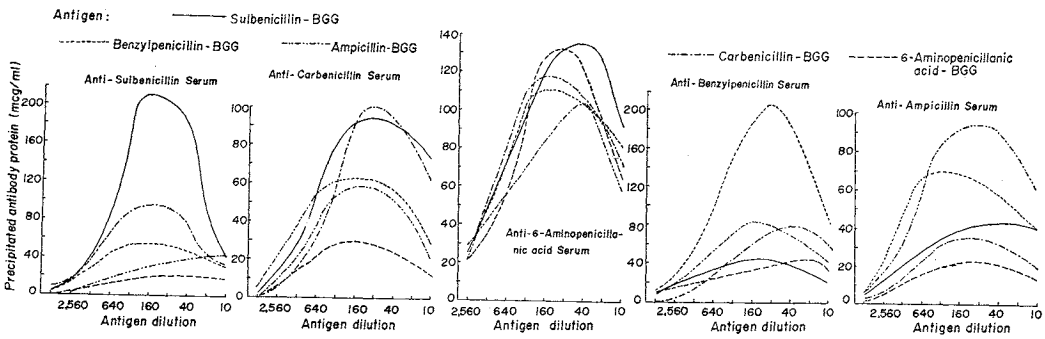
Results

1. Cross-reactivity between Various Anti-penicillin-BSA Sera and Penicillin-BGG Conjugates

The results of quantitative precipitin analysis of rabbit anti-penicillin-BSA sera by homologous and heterologous penicillin-BGG conjugates are shown in Fig. 1 and the relative values of precipitable antibody and antigen complex at a point of maximum precipitation are summarized in Table 1. In all the cases of anti-penicillin-BSA sera tested, the strongest reaction was always observed between homologous antigens and antibodies.

Anti-sulbenicillin-BSA serum showed a strong reactivity against sulbenicillin-BGG conjugate. Carbenicillin-BGG conjugate reacted moderately with anti-sulbenicillin-BSA serum and the other

Fig. 1. Cross reaction of quantitative precipitation of penicillin-BGG conjugates with various anti-penicillin sera



three conjugates showed weak reactivity. Consequently, the percentages of precipitable antibody and antigen complex of the antiserum were 26.2, 20.0, 45.0 and 11.9 against benzylpenicillin-, ampicillin-, carbenicillin- and 6-amino-penicillanic acid-BGG conjugates, respectively, based on the whole amount precipitated against homologous antigen at a maximum reaction.

Table 1. Cross reactivity of quantitative precipitation of sulbenicillin, benzylpenicillin, ampicillin, carbenicillin and 6-aminopenicillanic acid-BGG conjugates with various antisera (%)

Antiserum	Antigen (penicillin-BGG conjugate)				
	SB-PC	PC-G	AB-PC	CB-PC	6-APA
Anti-SB-PC	100*	26.2	20.0	45.0	11.9
Anti-PC-G	22.1	100	39.3	41.0	21.6
Anti-AB-PC	45.3	65.4	100	37.4	32.9
Anti-CB-PC	94.0	61.5	58.0	100	31.0
Anti-6-APA	101.9	84.0	79.1	89.4	100

SB-PC: Sulbenicillin. PC-G: Benzylpenicillin. AB-PC: Ampicillin. CB-PC: Carbenicillin. 6-APA: 6-Aminopenicillanic acid.

* Relative value of precipitable antibody and antigen complex at point of maximum precipitation, taking the absolute rate of homologous system as 100.

Against anti-benzylpenicillin-BSA serum, 41.0 and 39.3% of maximally precipitable antibody and antigen complex of homologous system were precipitated by carbenicillin- and ampicillin-BGG conjugates, respectively. On the other hand, only 22.1% of precipitable antibody and antigen complex was precipitated by sulbenicillin-BGG conjugate.

Against anti-ampicillin-BSA serum, 65.4% of precipitable antibody and antigen complex at maximum precipitation in homologous system was precipitated by benzylpenicillin-BGG and by the other three antigens 32.9~45.3% of precipitable antibody and antigen complex at maximum precipitation in homologus system was precipitated.

Against anti-carbenicillin-BSA serum, carbenicillin- and sulbenicillin-BGG conjugates showed mutually equivalent reactivity. Ninety-four per cent of precipitable antibody and antigen complex at maximum precipitation in the homologous system was precipitated by sulbenicillin-BGG conjugate. On the contrary, 61.5, 58.0 and 31.0% of precipitable antibody and antigen complex at maximum precipitation in the homologous system were precipitated by benzylpenicillin-, ampicillin- and 6-aminopenicillanic acid-BGG conjugates, respectively.

Against anti-6-aminopenicillanic acid-BSA serum, all the test antigens showed strong reactivity. Heterologous antigens were found to the precipitate of precipitable antibody and antigen complex at maximum precipitation in the homologous system in the range from 79.1 to 101.9%.

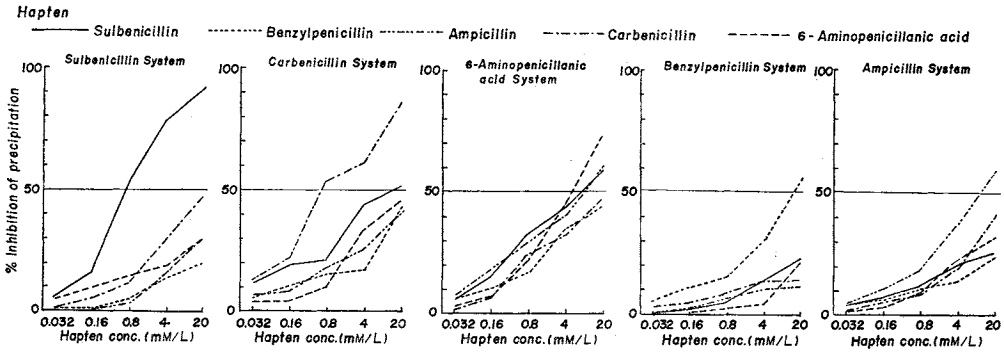
Each anti-penicillin-BSA serum was shown the strong reaction with BSA but no precipitate was

produced by addition of BGG into anti-penicillin-BSA serum. Also, no reaction was observed between penicillin-BGG conjugate and anti-BSA serum.

2. Inhibition of Precipitin Reaction by Hapten

A quantitative hapten inhibition test was carried out using each hapten in concentrations from 0.032 to 20 mM/liter on various anti-penicillin-BSA sera. As shown in Fig. 2, precipitation of anti-

Fig. 2. Hapten inhibition of precipitation of various antisera by sulbenicillin, benzylpenicillin, ampicillin, carbenicillin and 6-aminopenicillanic acid-BGG conjugates



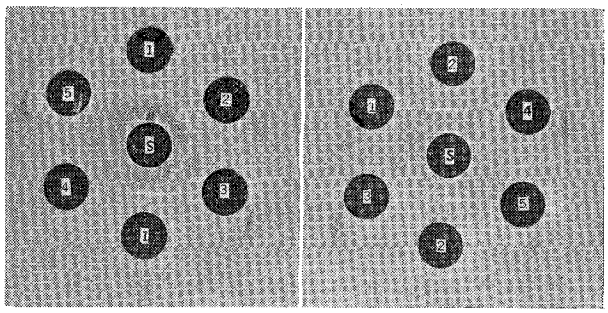
sulbenicillin-BSA serum against sulbenicillin-BGG conjugate was inhibited most effectively by sulbenicillin and the inhibition of precipitation by benzylpenicillin was the weakest. Furthermore, the precipitation in the benzylpenicillin-, ampicillin- and carbenicillin-systems was inhibited most effectively by homologous penicillin. In contrast, in the 6-aminopenicillanic acid system all the test haptens were almost equal in inhibitory activity.

3. Immunodiffusion Test

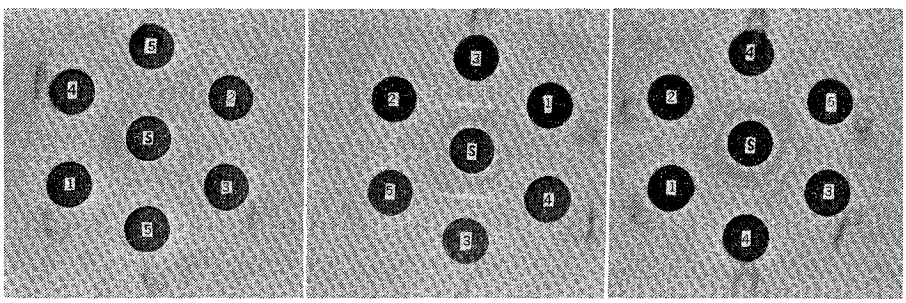
Immunodiffusion test was performed between various anti-penicillin-

Fig. 3. Agar precipitation of penicillin-BGG conjugates with anti-penicillin serum

S: Anti-penicillin serum (1:1)
1: Sulbenicillin-BGG conjugate (1:20)
2: Carbenicillin-BGG conjugate (1:20)
3: Benzylpenicillin-BGG conjugate (1:20)
4: Ampicillin-BGG conjugate (1:20)
5: 6-Aminopenicillanic acid-BGG conjugate (1:20)



Anti-sulbenicillin serum Anti-carbenicillin serum

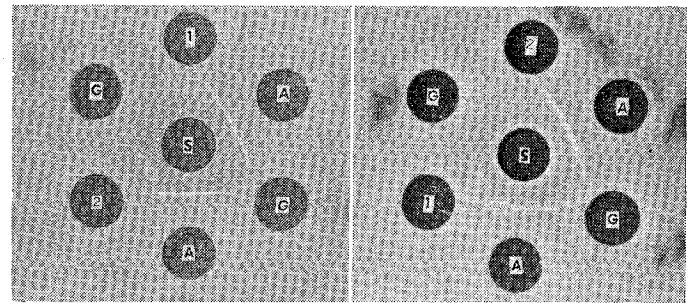


Anti-6-aminopenicillanic acid serum Anti-benzylpenicillin serum Anti-ampicillin serum

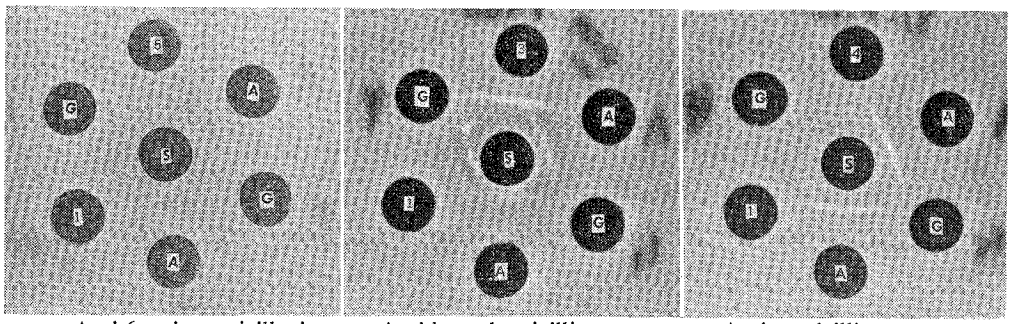
BSA sera and penicillin-BGG conjugates. Two distinct precipitin bands were developed within 72 hours at 4°C. As can be seen in Fig. 3, when antiserum was filled into the center hole, the inner precipitin band fused to all the test antigens, although the reaction between anti-penicillin-BSA serum and 6-aminopenicillanic acid-BGG conjugate was very weak. The outer precipitin band formed a spur between homologous and heterologous antigen, although no precipitin band was formed between anti-sulbenicillin-BSA serum and benzylpenicillin-, ampicillin- and 6-aminopenicillanic acid-BGG conjugates, between anti-carbenicillin-BSA serum and 6-aminopenicillanic acid-BGG conjugate, between anti-benzylpenicillin-BSA serum and sulbenicillin- and 6-aminopenicillanic acid-BGG conjugate between anti-ampicillin-BSA serum and sulbenicillin- and 6-aminopenicillanic acid-BGG conjugate and between anti-6-aminopenicillanic acid and all test antigens (Fig. 3).

Fig. 4. Agar precipitation of penicillin-BGG conjugates, BSA and BGG with anti-penicillin serum

S : Anti-penicillin serum (1:1)
A: Bovine serum albumin (1,000 mcg/ml)
G: Bovine gamma globulin (1,000 mcg/ml)
1 : Sulbenicillin-BGG conjugate (1:30)
2 : Carbenicillin-BGG conjugate (1:30)
3 : Benzylpenicillin-BGG conjugate (1:30)
4 : Ampicillin-BGG conjugate (1:30)
5 : 6-Aminopenicillanic acid conjugate (1:30)



Anti-sulbenicillin serum Anti-carbenicillin serum



Anti-6-aminopenicillanic acid serum Anti-benzylpenicillin serum Anti-ampicillin serum

The precipitin band produced between anti-penicillin-BSA sera and BSA and the two precipitin bands produced between anti-penicillin-BSA and penicillin-BGG conjugate crossed (Fig. 4). From these observations, two precipitin bands seem to be produced by specific immunological reactions.

4. Passive Cutaneous Anaphylaxis Test

Passive cutaneous anaphylaxis in the skin of guinea pigs was observed between various concentrations of anti-penicillin-BSA sera and penicillin-BGG conjugates. The guinea pigs were treated intradermally with anti-sulbenicillin-BSA serum in the dilution range from 1:10 to 1:1,000 and they were challenged intravenously with sulbenicillin-BGG conjugate in the dilution range from 1:5 to 1:200. The largest dye leakage was obtained at the site of injection of the ten fold-dilution of antiserum and by the challenge of 20-fold diluted antigen. The same observations were made by

passive cutaneous anaphylaxis test between each anti-penicillin-BSA serum and the homologous penicillin-BGG conjugate. The optimum dose of benzylpenicillin-, ampicillin-, carbenicillin- and 6-aminopenicillanic acid-BGG conjugates was 10 times, 20 times, 20 times and 20 times dilution, respectively. Using a concentration of antiserum which showed an area of dye leakage of 15 mm in diameter and an optimum dose of antigen, the cross-reactions were examined. As shown in Table 3, the test penicillins may be classified into two groups. By intravenous challenging injection of sulbenicillin-, carbenicillin- and 6-aminopenicillanic acid-BGG conjugates, little or no dye leakage was observed at the site of the intradermal injection of anti-benzylpenicillin- and anti-ampicillin-BSA sera, but anti-sulbenicillin-, anti-carbenicillin- and 6-aminopenicillanic acid-BSA sera reacted with all the antigens. On the other hand, benzylpenicillin- and ampicillin-BGG conjugates produced a strong reaction of the same degree at the injection site of all the test anti-penicillin-BSA sera.

Table 2. Passive cutaneous anaphylaxis of sulbenicillin-BGG conjugate with anti-sulbenicillin serum

Antiserum	Antigen (penicillin-BGG conjugate)					
	1:5	1:10	1:20	1:50	1:100	1:200
1: 10	15.0	15.0	15.8	14.8	13.5	0
1: 20	13.5	13.5	14.0	11.0	11.0	0
1: 50	12.0	11.0	13.3	11.3	9.3	0
1: 100	11.3	11.0	11.5	10.8	9.8	0
1: 200	10.0	8.8	10.5	9.0	8.8	0
1: 500	8.0	0	8.0	0	0	0
1:1000	0	0	0	0	0	0

Each value represents the average size (mm) of dye leakage at the antiserum injection sites

Table 3. Cross reactivity of passive cutaneous anaphylaxis of sulbenicillin, benzylpenicillin, ampicillin, carbenicillin and 6-aminopenicillanic acid-BGG conjugates with various antisera

Antiserum (1:10)	Antigen (penicillin-BGG conjugate)				
	SB-PC (1:20)	PC-G (1:10)	AB-PC (1:20)	CB-PC (1:20)	6-APA (1:20)
Anti-SB-PC	15.8	12.3	15.0	12.5	15.3
Anti-PC-G	0	14.8	13.8	7.8	0
Anti-AB-PC	0	11.0	16.8	0	0
Anti-CB-PC	14.5	14.5	15.5	15.5	10.0
Anti-6-APA	14.5	14.5	15.0	16.8	17.0

Each value represents the average size (mm) of dye leakage at the antiserum injection sites
SB-PC: Sulbenicillin. PC-G: Benzylpenicillin. AB-PC: Ampicillin. CB-PC: Carbenicillin. 6-APA: 6-Aminopenicillanic acid

Discussion

Benzylpenicillin, ampicillin and carbenicillin are similar in chemical structure to sulbenicillin, and 6-aminopenicillanic acid is the basic structure of these semi-synthetic penicillins. Therefore, these compounds were selected as the reference compounds in the present study.

In the quantitative precipitin test, a weak reaction was observed between sulbenicillin and benzylpenicillin, but a strong reaction was observed between sulbenicillin and carbenicillin. Also, moderate cross-reactions were obtained among benzylpenicillin, ampicillin and carbenicillin. Furthermore, the results of hapten-inhibition test on the sulbenicillin system correlate well with those given by the quantitative precipitin test. With the other penicillin systems, the strongest inhibition of precipitin reaction was produced by the homologous hapten. Although 6-aminopenicillanic acid showed a strong reactivity with anti-6-aminopenicillanic acid system, it showed weak reactivity with several anti-penicillin sera. Furthermore, the test penicillins and 6-aminopenicillanic acid showed a strong reactivity with anti-6-aminopenicillanic acid system. These findings suggest that the immunological specificity of penicillin depends on the acyl side chain moiety of the penicillin molecule, and the weak cross-reactions among the penicillins tested may depend on

the 6-aminopenicillanic acid moiety. These results were similar to those obtained in the study on benzyl-, phenoxymethyl-, phenoxyethyl-, dimethoxyphenyl-, methylphenylisoxazolyl- and aminobenzyl-penicillins by HORIUCHI and SHIBATA.[3]

ATSUMI *et al.*[11] found that anti-benzylpenicillin antibody can be fractionated into five antibody populations having different binding properties. In this study, several anti-penicillin sera reacted weakly with 6-aminopenicillanic acid, although they reacted strongly with homologous penicillin. Accordingly, the antibody production by the acyl side chain moiety in penicillin seems to occur more readily than with the 6-aminopenicillanic acid moiety.

In passive cutaneous reaction test, no reaction was observed between sulbenicillin-, carbenicillin- and 6-aminopenicillanic acid-BGG conjugates and anti-benzylpenicillin- and anti-ampicillin-sera.

The experimental observations reveal that chemically related penicillin derivatives show immunologically somewhat different antigen-antibody reaction patterns due probably to different acyl moieties. Such being the case, intradermal skin test for detection of penicillin allergy due to the penicilloyl antigenic determinant, should be performed with the penicilloyl derivative of the penicillin to be used for therapy and reliance not placed wholly on the benzyl penicilloyl derivative as is current practice.

It was noteworthy that we have observed two precipitin bands between anti-penicillin-BSA sera and penicillin-BGG conjugates under the experimental condition used, although the penicillin used was chemically pure. Further study on this point is under progress and will be reported separately.

References

1) TSUCHIYA, K.; T. OISHI, C. IWAGISHI & T. IWAHI: *In vitro* antibacterial activity of disodium α-sulfobenzylpenicillin. J. Antibiotics 24: 607~619, 1971
2) YAMAZAKI, T. & K. TSUCHIYA: *In vivo* antibacterial activity of disodium α-sulfobenzylpenicillin. J. Antibiotics 24: 620~635, 1971
3) HORIUCHI, Y. & K. SHIBATA: Immunochemical studies on the antigenic bindings of benzylpenicillin and five synthetic penicillins with proteins. Int. Arch. Allergy 28: 306~320, 1965
4) SHIBATA, K.; T. ATSUMI, Y. HORIUCHI & K. MASHIMO: Immunological cross-reactivities of cephalothin and its related compounds with benzylpenicillin. Nature 1966: 419~420, 1966
5) KUWAHARA, S.; Y. MINE & M. NISHIDA: Immunogenicity of cephazolin. Antimicr. Agents & Chemoth. -1970: 374~379, 1971
6) KABAT, E. A. & M. M. MAYER: Experimental immunochemistry. 2nd Ed. pp. 22~96, Charles C. Thomas, Springfield, Illinois, 1961
7) LOWRY, O. H.; H. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 193: 265~275, 1951
8) PAULING, L.; D. PRESSMAN & A. L. GROSSBERG: The serological properties of simple substances. 7. A quantitative theory of the inhibition by haptens of the precipitation of heterogenous antisera with antigens and comparison with experimental results for polyhaptenic simple substances and for azoproteins. J. Amer. Chem. Soc. 66: 784~792, 1944
9) OUCHTERLONY, O.: Diffusion-in-gel methods for immunological analysis. Progr. Allergy 5: 1~78, 1958
10) OVARY, Z.: Immediate reactions in the skin of experimental animals provoked by antibody-antigen interaction. Progr. Allergy 5: 459~508, 1958
11) ATSUMI, T.; K. NISHIDA, Y. KINOSHITA, K. SHIBATA & Y. HORIUCHI: The heterogenicity of combining sites of anti-benzylpenicilloyl antibodies obtained from individual rabbits: Fractionation of antibodies with a specific immuno-adsorbent. J. Immunology 99: 1286~1293, 1967