

Chemical approaches of penicillin allergy—III

Isolation of the penicillin-free carrier receptor protein (CRP) on a polymeric 7-deoxy penicillin analogue template and its role in penicillin immunogeneses in rabbit and man

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Abstract. Specific penicillin-carrier receptor proteins (CRP) have been isolated from the sera of penicillin allergic rabbits and human subjects in the unconjugated native state in electrophoretically homogeneous form by employing a synthetic polymeric affinity template containing the 7-deoxy analogue of penicillin G. The synthesis of the 7-deoxy analogue has been described. In this affinity system the antipenicillin-antibody is desorbed by 0.9M thiourea and the CRP in 8M urea. The CRP after incubation with penicillin is converted into the full-fledged antigen. Studies on the origin of CRP and the nature of antibody as well as comparative studies on the properties of the rabbit antibody and those of antibodies elicited by a BSA-BPO conjugate are reported.

Keywords. Penicillin allergy; immunology; receptor; affinity chromatology; antigen-antibody interaction; hapten.

1. Introduction

The status of the chemical nature of the penicillin antigen has been the subject of considerable controversy in the last decade. The prevalent opinion (Levine 1960) regarding the formation of the penicillin antigen has been centered around a reactive derivative of penicillin (benzyl penicillenic acid) BPE (1) in a preliminary step, followed by the coupling of this derivative to epidermal proteins either through the benzyl penicilloyl group (BPO II) (figure 1) to give the amide of benzyl penicilloic acid (III) or through the thiazolidine to give a mixed disulphide (IV) (figure 2). Further work by DeWeck *et al* (1968) and Parker *et al* (1962) implicated the involvement of a polypeptide (V and VI) impurity in commercial preparations as the major antigen (figure 3). On the other hand, Batchelor *et al* (1967) and Stewart (1967) isolated proteinaceous impurities in commercial preparations of penicillin and demonstrated their antigenicity in penicillin sensitive individuals.

In our previous publications (Bhattacharyya *et al* 1974, 1975) the isolation of specific carrier receptor proteins (CRP) from the serum of allergic rabbits and from both penicillin sensitive and in-sensitive human subjects (Mandal 1977) by the use of hydrophobic affinity template chromatography have been described. However, the design of these templates was such that the CRP would be bound to the penicillin on the template covalently-presumably by the opening of β -lactam of penicillin by an ϵ -amino group (possibly of lysine) and the CRP could be detached from the

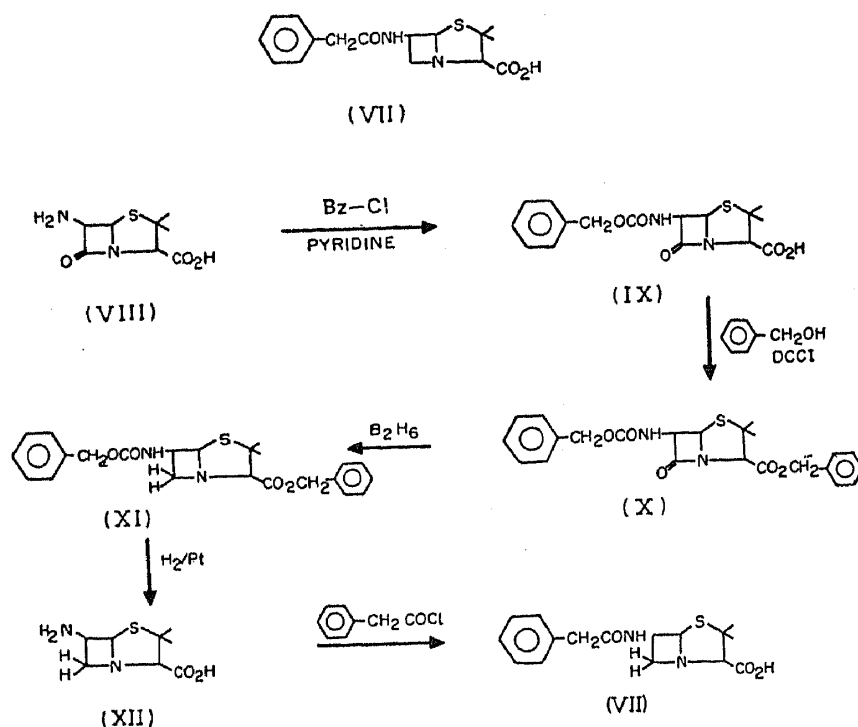


Figure 4. Schematic representation of the synthesis of 7-deoxy penicillin.

3.3. Reduction of β -lactam carbonyl in (X) with diborane

Into a stirred solution of benzyl ester (X) (1 g) in dry tetrahydrofuran (50 ml) at -10°C , diborane gas, generated externally by the addition of boron trifluoride etherate into sodium borohydride, was bubbled. Nitrogen gas was used to carry the diborane gas into reaction mixture. The reaction mixture was stirred for 4 hr and was left overnight at room temperature under nitrogen atmosphere. The boron complex was decomposed by adding saturated ammonium chloride solution and the compound was extracted into ether. Ether extract was purified by TLC. Yield 0.5 g. The compound (XI) was identified by IR and NMR. IR $\nu_{\text{max}}^{\text{neat}}$ 3300, 2950, 1601 cm^{-1} . NMR δ_{max} (CDCl_3): 7.3 (s, 10H, ArH), 4.9 (d, 1H, C_6H), 4.5 (q, 4H BZ CH_2), 4.5 (s, 1H, C_3H), 3.8 (m, 3H, C_7H_2 , C_5H), 1.6 (d, 6H, $\text{C}_2(\text{CH}_3)_2$). M^+ at 426.

3.4. Hydrogenolysis of XI

The reduced compound (XI) (0.5g) was dissolved in 50 ml rectified spirit and hydrogenolysed over platinum at atmospheric pressure for about 2 hr. The catalyst was filtered and the solvent evaporated to obtain 7-deoxy 6-amino penicillanic acid (XII) (0.3 g). It was recrystallised from water to get a pale yellow solid melting at $185-188^\circ\text{C}$. IR $\nu_{\text{max}}^{\text{nujol}}$ 3350, 3250, 2950, 1720, 1520 cm^{-1} . Found: C, 47.6, H, 7.01, N, 13.6, S, 15.9%. Calculated for $\text{C}_8\text{H}_{14}\text{N}_2\text{O}_2\text{S}$: C, 47.52, H, 6.91, N, 13.8, S, 15.8%.

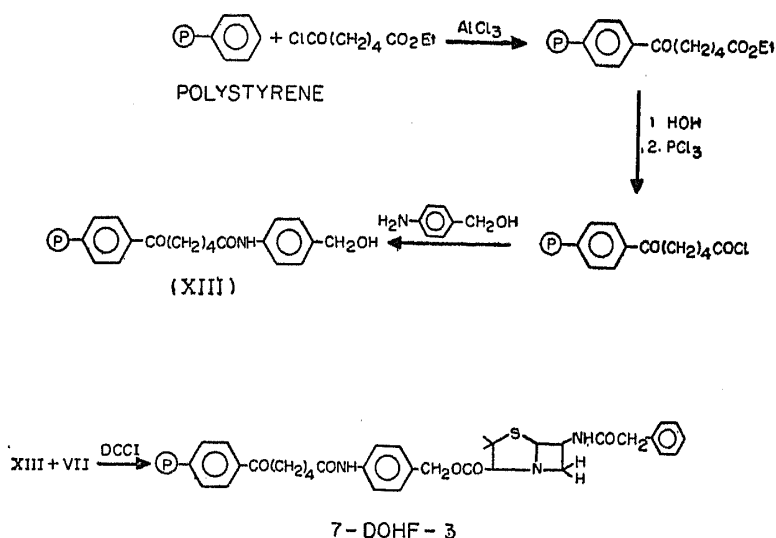


Figure 5. Synthesis of polymeric benzyl alcohol and condensation of 7-deoxy penicillin with polymeric benzyl alcohol.

3.5. Acylation of (XII) with phenyl acetyl chloride

To a stirred solution of (XII) (1.0 g) in 10 ml dry pyridine, phenyl acetyl chloride (0.75 g) was added slowly keeping the temperature below 15°C by cooling in ice. The solution was stirred for 1 hr at room temperature. The resulting 7-deoxypenicillin (VII) was worked up by neutralising the pyridine solution with 6N hydrochloric acid and extracting the compound (VII) into ether, and the ether extract was washed with water and evaporated. Yield of (VII) 1.2 g. Found: C, 62.0, H, 6.0, N, 8.01, S, 9.3%. Calculated for $C_{16}H_{20}N_2O_3S$ C, 62.8, H, 5.9, N, 8.1, S, 9.2%.

3.6. Coupling (VII) with polymeric benzyl alcohol (XIII) (Fig. 5)

To a stirred solution of (VII) (1.0 g) in 50 ml of dry dimethyl formamide, dicyclohexylcarbodiimide (0.8 g) was added and the solution was stirred for $\frac{1}{2}$ hr at room temperature. Polymeric benzyl alcohol (XIII) (10.0 g) was added in small lots (0.5 g each) over a period of 2 hr. The mixture was then stirred at room temperature for a further 36 hr.

The mixture was filtered and the dicyclohexylurea was washed with warm ethanol (150 ml).

The polymer was dried in vacuum. Yield of polymer 7-DOHF3 10.9 g. Found: C, 83.5, H, 7.3, N, 2.59, S, 1.95%. Calculated: C, 83.3, H, 7.2, N, 2.66, S, 2.01%.

4. Immunological methods

The method of immunization of rabbits with benzyl penicillin has been described in earlier communication (Bhattacharyya *et al* 1975).

4.1. Fractionation of rabbit antisera in the analogue polymer

A suspension of the 7-DOHF3 (2.0 g) was stirred with 5 ml of antiserum in 10 ml

Table 1. Eluates from 7-DOHF3 polymer against the results of the various experiments to characterise the protein.

Eluate	Qty. of protein $\mu\text{g/ml.}$	Homogeneity in electrophoresis (SDS)	Immuno- ^a electrophoresis	Immuno- ^b electrophoresis	Double ^a diffusion in agar	Double ^b diffusion in agar	Hemagglutination
PBS	Bulk	—	—	—	—	—	—
0.75 M phosphate buffer	260	—	—ve	—ve	—ve	—ve	1.10
0.9M thiourea	97	3	—ve	—ve	—ve	—ve	1.640
4M urea	80	2	—ve	+ve	—ve	+ve	1.20
8M urea	70	1	—ve	+ve	—ve	+ve	—

^a before incubation with penicillin.

^b after incubation with penicillin followed by dialysis.

phosphosaline (0.05 M, pH 7.2) for 30 min at 4°C. The polymer was filtered and the unadsorbed proteins were washed with two 10 ml portions of 0.5 M phosphate buffer, by first stirring the polymer with buffer for 10 min at 4°C following by filtration.

The polymer was eluted with 0.75M phosphate buffer (pH 7.2, 10 ml) 0.9M thiourea (20 ml) 4M urea and 8M urea. All the eluates were dialysed separately against double distilled water in the cold for 48 hr. The dialysates were lyophilised. The hemagglutination, immunodiffusion and immunoelectrophoresis patterns of the various fractions are summarised in the table 1.

It was found that the desorption of the proteins followed a sequential pattern with the antibodies being cleaved first (0.9M thiourea) followed by the receptor protein (CRP) (i.e. 4M and 8M urea). However, the antibodies in the 0.9M thiourea were contaminated with a small quantity of a protein which persisted in the 4M and 8M urea. Further, the CRP (free from penicillin) did not show a precipitin reaction against antiserum. However, on incubation with penicillin at 37°C for 1 hr and subsequent dialysis (to remove the excess penicillin) the conjugate gave a clear precipitin reaction against antiserum. In later experiments elution with 4M urea was avoided.

4.2. Immunization of rabbit with BSA-penicillin conjugate

Preparation of BSA penicillin conjugate

Benzyl penicillin G (500,000IU) and bovine serum albumin (50 mg) were dissolved in PBS (6 ml) and incubated at 37°C for 24 hr. The pH was maintained between 6.5–7.0 during the course of the reaction. The mixture was dialysed against phosphate buffer (PBS) (0.01M, pH 7.0) for two days with occasional change of buffer. The colourless nondialysable solution was then lyophilised after centrifugation at 5,000 rpm for 10 min stored frozed at -18°C .

4.3. Immunization procedure

Rabbits (male and female) were immunized by injecting the BSA-penicillin conjugate according to the procedure as described in earlier communication (Bhattacharyya

et al 1975). Injections were given subdermally twice a week for two weeks. The injection contained 3 mg of BSA-penicillin conjugate in saline solution. The dosage was increased by 1 mg/rabbit in every subsequent injection. After 15 days when antiserum showed a high titre of antibodies a booster dose of 6 mg of BSA-penicillin conjugate in saline was injected intravenously into the ear vein. The animal was bled 7 days later as well as after the booster injections for ascertaining the progress of antibody formation by following the hemagglutination titre of the sera.

4.4. Purification of antibody

Antisera were similarly fractionated with 7-DOHF3 polymer. 0.9M thiourea eluate was tested for hapten specificity in hemagglutination. Disc. electrophoresis of the 0.9 M thiourea eluate in tris-Hcl buffer (pH 8.5) was carried out to find out the homogeneity of antibodies in each case.

4.5. Comparison of the antibodies elicited by BSA-penicillin conjugate with those elicited by penicillin alone

The 0.9M thiourea eluate of BSA-penicillin conjugate antiserum (225 μ g) was loaded in the first tube (a) and the 0.9M thiourea eluates of antiserum elicited by penicillin alone (225 μ g) was loaded in the second (b). In the third a 50 : 50 mixture of antibodies elicited both by penicillin alone and BSA-penicillin conjugate was loaded (c). Gel electrophoresis was done for this set of three tubes simultaneously maintaining identical conditions. All the eluates showed identical mobility (shown in figure 6).

4.6. Determination of nature of antibody

Molecular weight of antibodies was determined by SDS gel electrophoresis by Laemmli's (1970) method with the help of several marker proteins. The mobilities were plotted against the known molecular weights on a semi logarithmic scale. The antibody was characterised as belonging to complement fixing IgG class of immunoglobulin with a molecular weight of 150,000 and containing two sub units one corresponding to the heavy chain (molecular weight 50,000) and the other to the light chain (Molecular weight 23,500) obtained after treatment with SDS-Mercapto ethanol at high temperature.

4.7. Estimation of CRP

Determination of amount of CRP in rabbits before immunization and both in human allergic and non-allergic patients

Before immunization rabbit sera and human (allergic and non-allergic) sera were similarly fractionated separately and analysed for CRP content. The 8M urea fraction was estimated for protein by the method of Lowry and characterised by immunoelectrophoresis, immunodiffusion and gel electrophoresis after incubation with penicillin. The amount of CRP varied from one rabbit to the other ranging between 12 μ g/ml of rabbit sera. The corresponding amount of human CRP in allergic and non-allergic human subjects were 70 μ g/ml and 34 μ g/ml respectively.

5. Results and discussion

If one has to choose between the two possible methods of attachment of CRP with penicillin at biological pH, mechanistically the most likely one would be, through the β -lactam carbonyl cleavage. All the polymers reported previously (Bhattacharyya *et al* 1974, 1975) had the β -lactam carbonyl which is an integral structural component of the penicillin molecule. If the above choice has any relevance, the best strategy to trap the penicillin-free native CRP from the sera of allergic animals is to present a polymeric structure with all the structural features or dimensions of the penicillin molecule more or less intact, but without the β -lactam carbonyl. One of the obvious molecules which will meet the above criteria is the 7-deoxy penicillin.

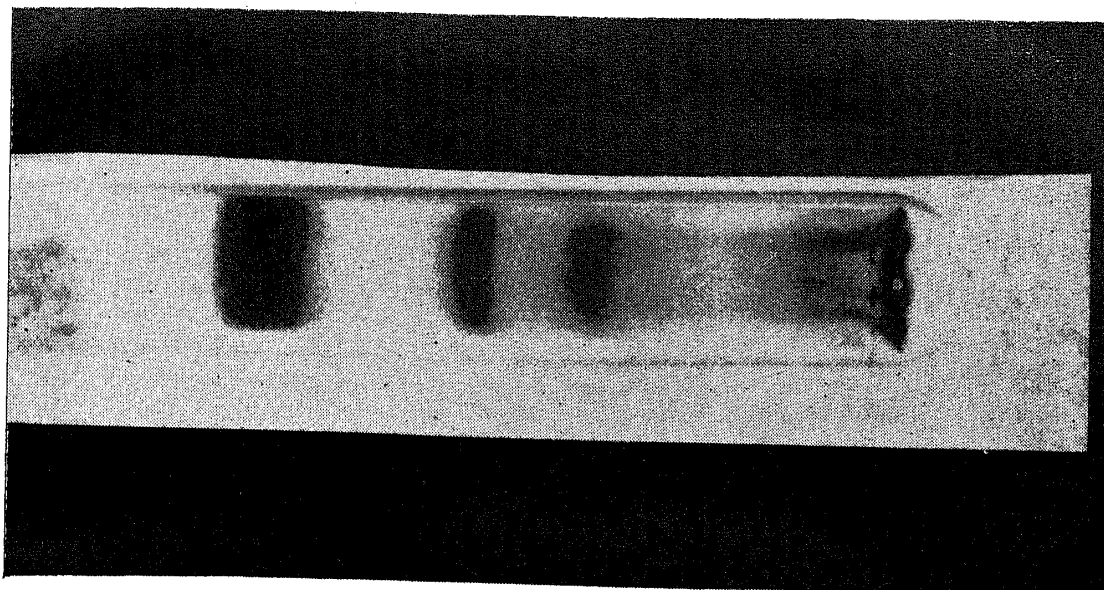
7-deoxy penicillin was synthesised in the present work for the first time from the protected 6-carbobenzoxy amino benzyl ester which was obtained in two steps from 6 amino penicillanic acid involving reaction with benzyloxycarbonyl chloride in pyridine followed by esterification with benzyl alcohol and dicyclohexyl carbodiimide. The compound (X) obtained in 66% yield (based on 6-aminopenicillanic acid) was identified, through the characteristic infrared and NMR spectra. The presence of intact β -lactam, the ester and the amide groups could be seen from the IR bands at 1780, 1740 cm^{-1} . The protons of the aromatic system, the benzylic protons, the protons of carbons, 5, 3 and 6, the gemdimethyl group were identified in the NMR spectrum. The aromatic protons appeared as a singlet of 10 proton intensity at 7.3 ppm (δ) the 5, 6 protons appear as doublets at 5.0 ppm and 5.5 ppm respectively. The proton at carbon 3 appears as a singlet of one proton intensity at 4.5 ppm, the benzylic protons appear as a double doublet of four proton intensity at 4.5 ppm and the gemdimethyl group is split into two peaks at 1.6 ppm (δ) of 3 proton intensity each.

The doubly protected 6 amino penicillanic acid derivative was very conveniently reduced to the 7-deoxy derivative in high yields taking advantage of the preferential reduction of di-substituted amides by diborane. The resulting 6-carbobenzoxy-amino 7-deoxy penicillanic acid benzyl ester obtained in 65% yield was fully characterised by IR, NMR and mass spectral data. The reduction of the carbonyl group at C-7 was indicated by the absence of the IR absorption at 1780 cm^{-1} and also by the upfield shift of the C₆ methine proton and the appearance of a multiplet at 3.8 ppm corresponding to the C-7 methylene. Further, the mass spectrum had an M⁺ peak at 426 corresponding exactly with the expected molecular weight of the deoxyderivative.

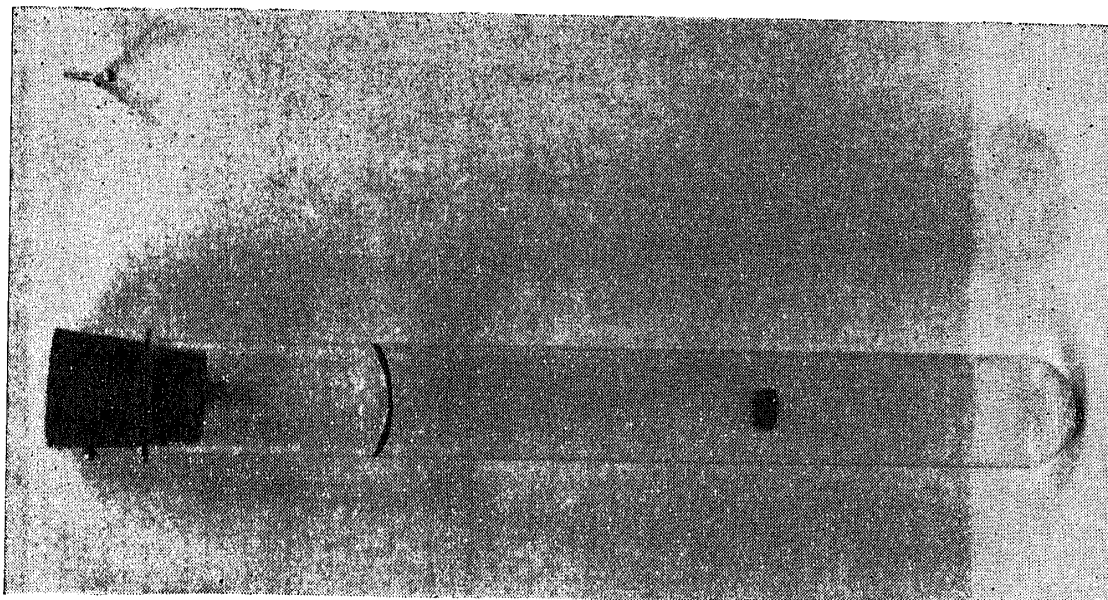
The protecting groups were removed by hydrogenolysis over platinum and the 7-deoxy penicillin obtained from a reaction with phenylacetyl chloride was coupled to the polymer with dicyclohexyl carbodiimide.

The sera from the allergic rabbits were fractionated on this polymer by absorption and sequential desorption according to the general scheme outlined in the experimental section. The antibodies were eluted in the 0.9 M thiourea and the penicillin free receptor in the 4M and 8M urea fractions. In later experiments elution with 4M urea was omitted.

The CRP from the 8M urea eluate after incubation with penicillin, and subsequent dialysis to remove excess penicillin gave a clear precipitin band in immunodiffusion and immunoelectrophoresis against the antiserum and showed a single band in disc gel electrophoresis (figure 7). However, the CRP without prior incubation with

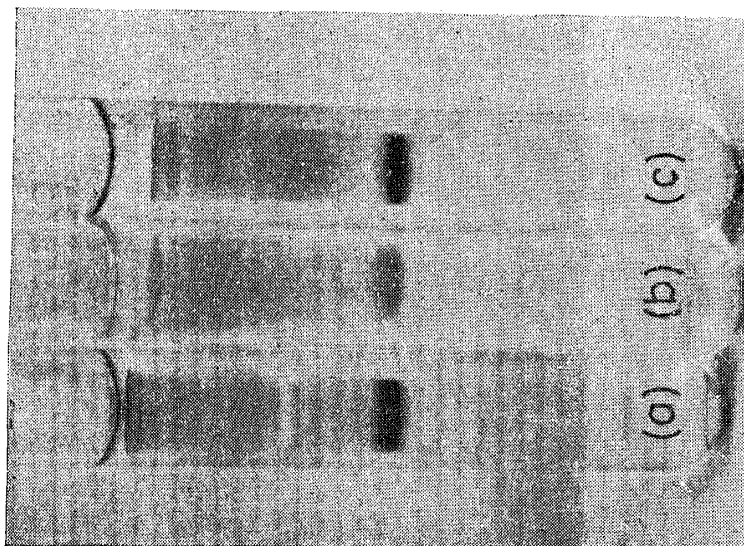


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(Captions in page 12)



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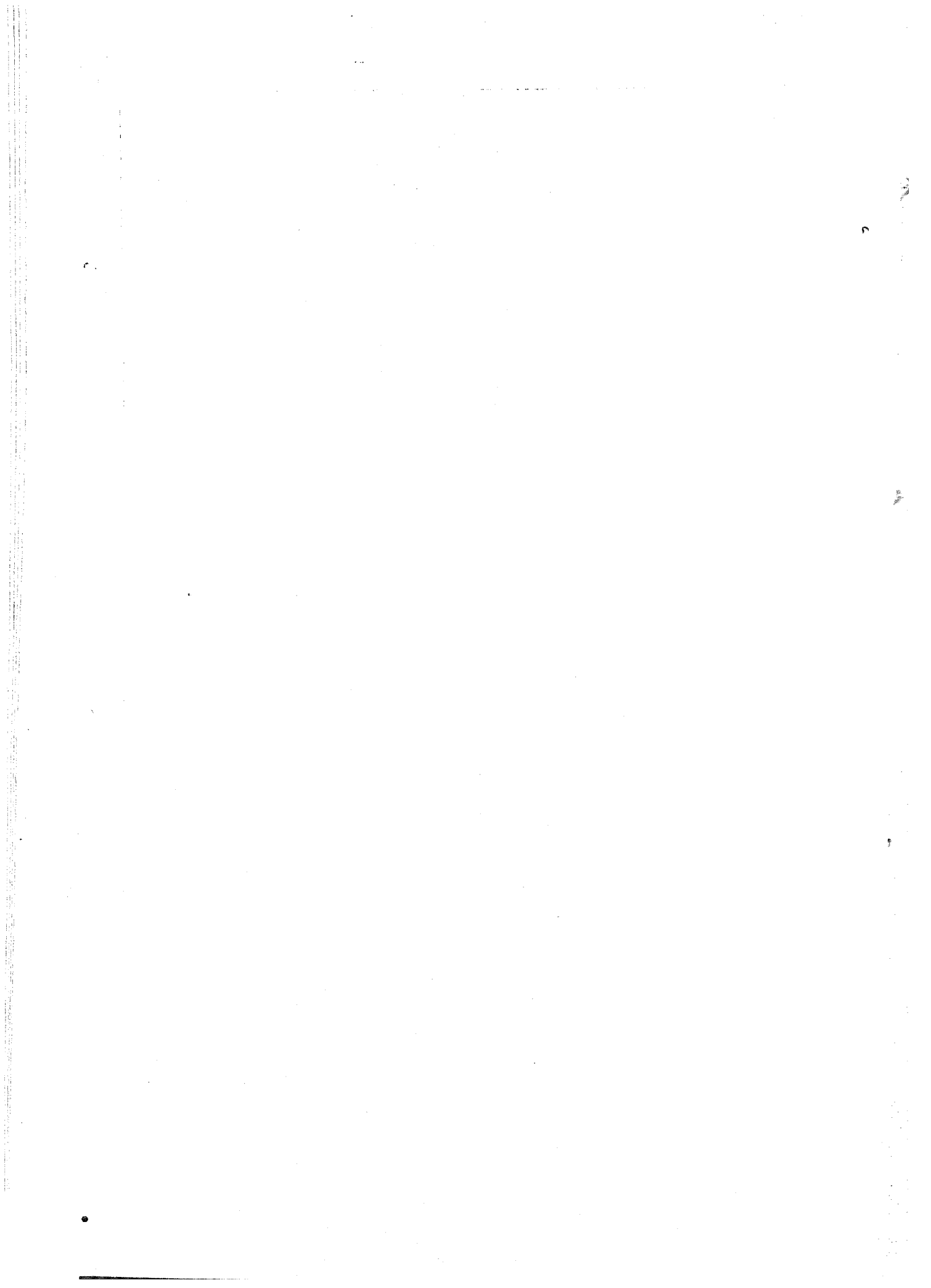


Table 2. Amounts of carrier-receptor protein (CRP) in different rabbits during the course of immunization.

Rabbit	No. of weeks	Amount of CRP μ g/ml
F	Before immunization	12
	5	34
	10	70
M	Before immunization	50
	5	78
	10	96

M and F represent the male and female rabbit respectively.

penicillin did not give a precipitin band. These results confirm the fact that 8M urea fraction was the CRP free from penicillin and only on incubation with penicillin, the complete antigen is formed. Thus the hypotheses that the carrier receptor protein binds covalently to penicillin by the opening of the β -lactam ring, has been confirmed in the present work. Furthermore, the earlier hypotheses of Levine (1963) and Nishida *et al* (1972) stating that the entire molecule of penicillin offers binding sites to a protein have been confirmed by the fact that the CRP was eluted only with a strong denaturing agent such as 8M urea.

The isolation of CRP before immunization from all rabbits indicates that it is a constitutive protein. During the course of immunization with benzyl penicillin the amount of CRP increased considerably in all the rabbits. Table 2 presents the amount of CRP in different rabbits during the course of immunization.

It is interesting to note that in preimmunized animal 0.9M thiourea gave three bands (figure 8). Obviously there may be some serum proteins which have decided affinity for penicillin and perhaps play a role in penicillin binding. Since the 0.9M thiourea fractions did not give any positive hemagglutination test they may not play a role in the antigen-antibody reaction. After immunization these eluates gave only one electrophoretically homogeneous protein, which is the antibody synthesised in response to the antigen as evident from its hemagglutination test and reaction with CRP penicillin conjugate. Whether the penicillin binding protein in non-immunised animal have any relationship to the penicillin binding liver protein (Korngath *et al* 1976) was not investigated further.

The presence of CRP in unsensitized rabbits and a normal human subject does not prove that CRP-penicillin conjugation is necessarily a pre-requisite for development of allergy. It is possible that non-sensitive individuals do not have the B cells or T cells with surface antibodies which can recognise the CRP-penicillin conjugate as antigen. However, these speculations remain to be verified at a chemical level. The main emphasis of the work is to study in details the formation of CRP-penicillin or CRP-analogue conjugates and the mechanism of its binding with rabbit IgG antibody. There is still a possibility that the quantity of CRP in the sera may also be a determining factor in penicillin allergenesis. The rabbit which had more receptor protein before immunization showed a more rapid response to penicillin than the rabbits having a smaller amount of CRP in the sera.

The molecular weight of human allergic CRP as estimated by comparative disc electrophoresis with marker proteins to be 43,000.

An identical mobility of antibodies proves that two different antigens containing the penicillin moiety give rise to the same or very similar antibody. This result

establishes one important point—irrespective of the nature of antigen, the haptenic structure is the major determinant in the antibody response. Although the antibody is the IgG class here it may be argued that possibly the reaginic antibodies, IgE, may also be biosynthesised on the same principle. Different allergens, such as BPO conjugate, penicilloylated proteins or polymer carrying a common haptenic structure may possibly elicit the same or very similar reaginic antibodies. Out of these the CRP-penicillin conjugate may form an important missing allergen.

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Caption for figures 6, 7 and 8

Figure 6. Polyacrylamide gel electrophoretic patterns of the 0.9M thiourea eluates from the 7-DOHF3 affinity column. (a) Elicited by BSA-penicillin conjugate. (b) Elicited by benzyl-penicillin only. (c) 50:50 mixture of both types of 0.9M thiourea eluates.

Electrophoresis was carried out in 7.5% acrylamide gel in tris-glycine buffer, pH 8.5 at room temperature. Gels were stained with coomassie blue.

Figure 7. Polyacrylamide gel electrophoretic patterns of the 8M urea eluates from the 7-DOHF3 polymer. Electrophoresis was carried out in 7.5% acrylamide gels in β -alanine-acetic acid buffer of pH 4.3 at room temperature. Gel was stained with coomassie blue.

Figure 8. Polyacrylamide gel electrophoretic patterns of the 0.9M thiourea eluates from the 7-DOHF3 affinity column. Electrophoresis was carried out in 7.5% acrylamide gels in tris-glycine buffer of pH 8.5 at room temperature. Gel was stained with coomassie blue.