

Elicitation of skin test reactions by complexes of penicillin or its derivatives with solubilized erythrocyte stroma receptors

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Two methods are described for reproducibly preparing from penicillin or its derivatives and solubilized erythrocyte stroma complexes which are capable of eliciting skin test responses in sensitive subjects or in producing passive transfer reactions with sera containing penicillin antibodies. The complexes maintain their biological activity for at least 27 months when stored at 4° C., and microgram quantities are active in provoking specific skin test reactions. The data suggest that both the penicillin and receptor components of these conjugates are heterogeneous. Besides the benzyl penicilloyl determinant, it appears that 2 or more additional penicillin derivatives also are present in these complexes. Thus they have been able to elicit passive transfer reactions when tests with either penicillin or penicilloyl polylysine were positive, and with 2 sera tests with the stroma complexes were repeatedly positive while the other antigens gave no response. The sensitized stroma had no demonstrable antibiotic activity. The presence of penicilloyl groups in the sensitized stroma could be confirmed by biochemical methods, but the nature of additional penicillin derivatives could not be ascertained. Further data suggest that different penicillin derivatives may attach to different red cell components. The myxovirus receptors are not involved.

Studies of passive hemagglutination reactions involving penicillinized erythrocytes have shown that unmodified red cells can bind penicillin and/or its derivatives so firmly that the drug is not eluted even by 25 washings.¹⁻⁴ The stroma of such washed cells is capable of adsorbing hemagglutinating antibodies specific for penicillinized erythrocytes.⁴ It appeared to be of interest to attempt to solubilize the complexes of penicillin or its derivatives with the erythrocyte stroma receptors and to study the biological activity of

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such complexes in eliciting skin test reactions. In addition, an attempt was made to identify by immunologic and biochemical means the nature of the penicillin derivatives bound to the stroma receptors and to obtain some preliminary data about the composition of the latter.

MATERIALS AND METHODS

Materials

Commercial potassium benzyl penicillin was used. Penicilloyl polylysine was prepared from penicillin by the method of Parker and Thiel,⁵ and additional material was purchased from the Sigma Chemical Company. Two or 3 times crystallized trypsin, chymotrypsin, papain, and RNase and once crystallized DNase were purchased from the Worthington Chemical Company. The sialidase used was a highly purified preparation⁶ obtained from *Clostridium perfringens* filtrates.*

Preparation of drug-stroma complexes

Howe's⁷ method for obtaining crude preparations containing influenza virus receptors in solution was tried initially. Subsequently 27 different procedures were evaluated in which the concentration of penicillin used for sensitizing the cells, the pH and ionic strength of the buffers used, and the extent of mechanical agitation at certain steps were varied. Based on this experience the following two procedures were adopted for use in the experiments reported in this paper, though precisely optimal conditions varied slightly depending upon the individual being evaluated.

Method I. A 0.15 ml. quantity of freshly prepared solution containing 1,000,000 units of benzyl penicillin per milliliter of saline was added to 2 ml. of heparinized human venous blood (20 units of heparin per milliliter). The mixture was incubated for 75 minutes at 37° and overnight at 4°. The cells were lysed by adding 10 ml. of water in the presence of 1.7 ml. of pH 4.45, 0.1M acetate buffer. The mixture was immediately placed in an ice bath for 15 minutes, and the stroma was then washed 3 times with pH 5.6, 0.01M acetate buffer. It then was resuspended in pH 8.0, 0.15M phosphate buffer and solubilized to the greatest extent possible by periodic mechanical agitation at room temperature during a period of at least 1 hour. Insoluble material was removed by centrifugation for 30 minutes at 1,600 G at 4°, the supernatant was diluted fivefold with saline (to avoid irritant skin reactions), and the solution sterilized by Millipore filtration. Control materials were prepared in the same manner except for omitting the addition of penicillin.

Method II. An alternate procedure, which was almost as satisfactory, involved preparing solubilized stroma from *unsensitized* red cells by the procedure just described. After washing the stroma once with the pH 5.6, 0.01M acetate buffer, the stroma was suspended in 3 ml. of the 0.15M, pH 8.0 phosphate buffer. To the suspension was added 0.3 ml. of a saline solution containing 1,000,000 units of penicillin per milliliter. After 1 hour incubation at 37°, the excess penicillin was dialyzed off at 4° into a large volume of pH

*Supplied by Dr. James Cassidy, Rackham Arthritis Research Unit, University of Michigan.

7.6, 0.15M phosphate buffer which was changed several times over a 2 day period. The sac contents then were centrifuged, diluted, and filtered as described previously. Control materials were prepared in the same manner except for omitting the addition of penicillin.

Testing procedures

Passive transfer (Prausnitz-Küstner) tests were carried out by the usual technique,⁸ which involved planting 0.1 ml. quantities of patients' sera intracutaneously on the recipient's back or arms and challenging the sites about 24 hours later with intracutaneous injections of 0.02 ml. quantities of penicillin-treated stroma preparations made from the recipient's own red cells. Benzyl penicillin was used in a concentration of 10,000 units per milliliter and benzyl penicilloyl polylysine (PPL) in 5×10^{-5} M strength for comparative tests. The average diameter of any wheals or erythematous reactions was recorded 20 minutes later. Controls included injecting the same sensitized stroma preparations into the recipient's normal skin or sites prepared with normal serum and injecting saline or unsensitized stroma preparations into sites prepared with patients' sera. The results to be reported are based on at least 2 sets of passive transfer tests performed on the same recipient at different times. Stroma preparations were stored in a sterile state at 4° C.; aliquots of sera were stored at about -20° C.

The nitrogen content of the stroma preparations was measured by the micro-Kjeldahl method.⁹ The procedure of Parker and colleagues¹⁰ was used to seek evidence for the presence of penicilloyl groups on the sensitized stroma by measuring changes in absorbance at 282 m μ following the addition of mercuric chloride. Antibiotic activity was measured by noting inhibition of growth of *Streptococcus faecalis* surrounding discs saturated with sensitized stroma.

RESULTS

Table I shows the reproducibility of results obtained with five different batches of penicillinized, solubilized stroma freshly prepared by Method I (described previously) and tested at different times. All these passive transfer tests were done on the same recipient with the same serum. The bracketed results indicate duplicate tests performed simultaneously, and it may be noted that the size of the skin response on testing with different lots of sensitized stroma varies no more than the results of duplicate tests. Negative control tests were obtained where sensitized stroma was injected into normal skin and sites prepared with normal serum and where saline and unsensitized stroma were injected into sites prepared with the allergic serum.

Table II shows results of testing different lots of stroma simultaneously on two occasions. It is seen that the different lots give passive transfer reactions of fairly similar intensity. More importantly, testing with serial three-fold dilutions of sensitized stroma on Feb. 7, 1967, showed that all 5 lots gave a titer of 1:3. The results also indicate that these preparations retain their activity after storage at 4° for at least 17 months. Subsequent experiments showed these materials still were active after 27 months. Similar controls were used as in the experiments reported in Table I.

Table I. *Reproducibility of preparing sensitized stroma*

Stroma lot	P-K test results with the same 1:2 serum at different times on the same recipient	
	Wheal	Erythema
A	7*	25*
B	{ 7 6	{ 20 20
C	{ 7 9	{ 20 25
D	{ 8 5	{ 25 20
E	{ 8 9	{ 21 10

*Average diameter in millimeters.

Table II. *Reproducibility of preparing sensitized stroma: Stability*

Lot	Date prepared	P-K test results with the same serum at the same time on the same recipient		
		Wheal	Erythema	Stroma titer
I. Tests May 3, 1966 (1:20 serum)				
F	7-7-65	9*	23*	
G	9-2-65	7	30	
H	1-20-66	8	20	
I	4-1-66	7	15	
II. Tests Feb. 7, 1967 (1:10 serum)				
G	9-2-65	12	20	1:3
H	1-20-66	12	27	1:3
I	4-1-66	9	20	1:3
J	9-8-66	10	23	1:3
K	11-30-66	11	30	1:3

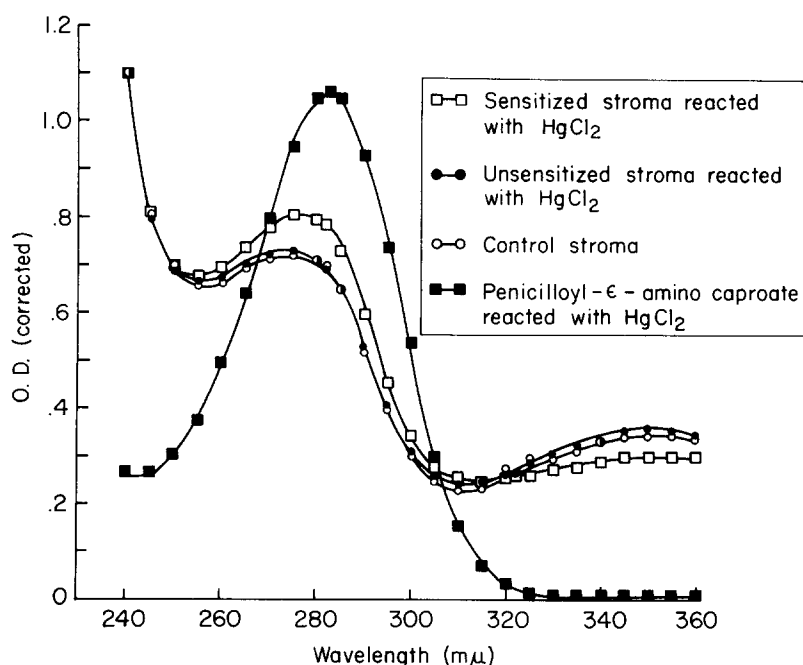
*Average diameter in millimeters.

Table III. *Solid and nitrogen content of solubilized stroma preparations*

	Weight (mg./ml.)	N (μg/ml.)	% N
Crude sensitized stroma	1.29	110	8.5
Crude unsensitized stroma	1.92	99	5.1
"Purified" sensitized stroma	2.66	242	9.1
"Purified" unsensitized stroma	3.40	299	8.8

Table III gives the solid and nitrogen content of typical stroma preparations. Almost all of the N was precipitable by phosphotungstic acid.¹⁴ It may be calculated from these data that with some sera less than 1 μg of solids or 0.05 μg nitrogen was capable of eliciting passive transfer reactions.

Fig. 1 shows the ultraviolet absorbance of these preparations. Curves were similar for control and for penicillinized stroma, the latter failing to show any detectable peaks, such as at 282 mμ for penamaldate or 322 mμ for penicil-

**Fig. 1**

Ultraviolet absorbance of solubilized erythrocyte stroma preparations.

lenate. After the addition of mercuric chloride,¹⁰ however, there was a definite increase in absorbance which was maximal at about 282 mμ. This suggests the presence of penicilloyl groups, a supposition supported by finding the expected Hg-penamaldete peak at 282 mμ after reacting penicilloyl ε-aminocaproate with HgCl₂ (Fig. 1). Furthermore, a shift from maximal absorbance at 282 mμ to 265 mμ was noted on reacting the HgCl₂-treated sensitized stroma with acid, again suggesting the development of penamaldete from penicilloyl groups. No increase in absorbance was obtained after adding HgCl₂ to control stroma.

Table IV summarizes the results obtained with 32 sera. Penicillinized stroma elicited passive transfer reactions with *all* sera which reacted to challenge with *either* benzyl penicilloyl polylysine (PPL), benzyl penicillin (Pen), or both. The relatively large number of sera reacting with penicillin, but not with PPL, may be related to the fact that in collecting these sera a special effort was made to obtain specimens from patients who had experienced anaphylactic-type reactions. Control tests with sensitized stroma injected into normal skin were negative. Sera from 2 individuals repeatedly reacted to challenge with sensitized stroma but not with PPL or benzyl penicillin. One of these also did not react to the minor determinants, sodium penicilloate or sodium penilloate.* This subject had experienced an erythe-

*Determined by Drs. B. B. Levine and A. P. Redmond.

Table IV. Results of P-K tests

No.	Test antigen		
	Sensitized stroma	Penicilloyl polylysine	Penicillin
3	+	+	+
3	+	+	-
8	+	-	+
2	+	-	-
16	-	-	-

Table V. Passive transfer neutralization test results

Serum	Challenged with equiractive amounts of:		
	Stroma	PPL	Pen
K. H.	10,000*	10	
K. C.	200		200
O. W.	250	25	

PPL = penicilloyl polylysine; Pen = penicillin.
*Units of penicillin required for passive transfer neutralization.

matous, cutaneous eruption 1 to 2 days after a penicillin injection more than 15 years ago.

Sensitized stroma could elicit passive transfer tests with serum dilutions of the same order of magnitude as PPL, the maximal titer of the sera tested being 1:160. When sites were challenged with serial dilutions of antigen, however, PPL could be diluted much more than sensitized stroma and still elicit skin reactions.

Further evidence for the heterogeneity of penicillin derivatives on red cell stroma was obtained by comparing the capacity of penicillin to neutralize passive transfer tests when the sites were challenged by equally reactive amounts of sensitized stroma, PPL, or penicillin. The latter requirement was met by doing preliminary passive transfer tests with each serum studied, challenging the sites with serial dilutions of the 3 antigens (sensitized stroma, PPL, or Pen) to determine the least amount of each required to elicit reactions with the particular serum in the dilution being used. Usually 4 to 10 times this amount of allergen was used for challenging the sites in the in vitro passive transfer neutralization tests. In performing the latter, the sera were incubated with the neutralizing material (i.e., penicillin) for one-half hour at room temperature before the mixtures were planted intracutaneously on the recipient. As shown in Table V, similar amounts of penicillin neutralized serum K.C. to challenge with sensitized stroma or to penicillin itself. With the other 2 sera, however, numerous tests repeatedly showed significant discrepancies in the amount of penicillin required for neutralization to challenge with stroma or PPL. This would suggest that determinants in addition to penicilloyl groups are reacting with the sera or that carrier specificity is playing a role. Numerous attempts to confirm this supposition by in vitro passive transfer neutralization tests employing penicilloyl ϵ -aminocaproate (PAC) yielded irregular results.

In many experiments, however, the highest hapten concentration (7×10^{-4} M PAC) failed to inhibit passive transfer sites challenged with sensitized stroma.

In an attempt to identify additional penicillin derivatives in sensitized stroma, this material was dialyzed against water, lyophilized, incorporated into KBr, and examined by infrared spectrophotometry. No difference was noted between the absorption pattern of sensitized and unsensitized stroma. A positive control employing penicillin incorporated into KBr showed the expected absorption bands.^{12, 13} It should be noted, however, that this biophysical method is less sensitive than some immunologic techniques for detecting penicillin products. Under the conditions of these observations, the minimal detectable concentration of benzyl penicillin was between 1.5 and 15 μ g per milliliter. Likewise, an attempt was made to identify penicillin in this sensitized stroma by hydroxylamine assay,¹⁴ but again the results were negative. The sensitized stroma in concentration used for skin testing also failed to show antibiotic activity against *Streptococcus faecalis*. The conditions of the tests allowed for the detection of less than 1.25 μ g of benzyl penicillin.

Some observations were made concerning the possible nature of the erythrocyte receptors with which penicillin or its derivatives may be reacting. Since the procedure initially followed for preparing stroma originally was designed to yield influenza virus receptors, a highly purified myxovirus receptor preparation¹⁵ was obtained for further study.* Following exposure to penicillin, however, this material was essentially inactive in eliciting passive transfer reactions with sera of penicillin-sensitive patients. Also, it showed only slightly increased absorbance of ultraviolet light at 282 $m\mu$ after reaction with mercuric chloride.

Stroma was then subjected to part of the procedure described by Philipson and colleagues¹⁶ for purifying the receptors for hemagglutinating enteroviruses. Sensitized and unsensitized stroma were prepared and washed in 0.01M acetate buffer according to Method I outlined previously. Then equal amounts of stroma, 2.5M pH 6.7 CaCl_2 , and n-butanol were shaken at room temperature, and the aqueous layer was recovered following centrifugation. This solution was dialyzed against pH 7.5 tris buffer containing 0.1mM EDTA and 0.001M MgCl_2 . Then one-fiftieth volume 0.5 per cent heparin solution and one-fiftieth volume 0.5M MnCl_2 were added to the sac contents in an ice bath, and after 2 hours the small amount of precipitate was removed by centrifugation in the cold. These preparations then were dialyzed against saline and sterilized by Millipore filtration. Their solid and N content were given in Table III. The sensitized material showed increased absorbance at 282 $m\mu$ when reacted with HgCl_2 , while the control stroma did not. Likewise, the former, but not the latter, was active in eliciting passive transfer reactions. However, as shown in Table VI, which indicates the least amount of substance capable of eliciting passive transfer reactions, there was at most only a slight increment in the activity of this partially purified material compared with crude stroma, whereas the purified preparation should have shown a sixtyfold increase in its reactivity with certain enteroviruses. Furthermore, with serum F. B. the purified material actually was less reactive than the crude, sensitized stroma.

*Supplied by Dr. Ralph Kathan, University of Illinois.

Table VI. Results of P-K tests with crude and partly purified solubilized erythrocyte stroma

Antigen	Sera		
	K. H. PPL+ Pcn-	F. B. PPI- Pcn-	K. C. PPL- Pcn+
Crude stroma	6.5*	0.8	6.5
Partly purified enterovirus receptor preparation	3.3	6.6	1.6

*Minimal quantity of skin test eliciting material in micrograms.

Table VII. Results of P-K tests employing modified solubilized erythrocyte stroma

	Purified enterovirus receptor ^{16, 17}	Stroma+ P.P.L.+ Pcn-		Stroma+ P.P.L.- Pcn+		Stroma+ P.P.L. Pcn-	Stroma+ P.P.L.+ Pcn+
		K. H.	G. S.	K. C.	R. H.	F. B.	D. W.
56°	-	-	-	-	-	-	-
100°	+*	+	+	+	+	+	±
Ether extraction	-	-	-	-	-	-	-
Periodate	+	+	+	-	-	+	-
Trypsin	-	-	-	-	-	-	-
Chymotrypsin	+	+	±	-	+	±	+
RNase	-	-	-	-	-	-	-
DNase	-	-	-	-	-	-	-
Papain	+	+	+	-	+	-	-
Sialidase	-	-	-	-	±	-	-

*Indicates receptor inactivation.

Table VII provides further evidence both that the hemagglutinating enterovirus receptors are not the substance binding all the penicillin derivatives and that different receptors are involved in binding various penicillin products. For comparison with the present experiments, the second column indicates the effect of the various listed agents on purified enterovirus receptors, according to the observations of Philipson and colleagues.^{16, 17} A plus indicates receptor inactivation. The other columns show the effect of these agents on the receptors reacting with various penicillin derivatives as ascertained by loss of capacity to elicit passive transfer reactions with sera containing various types of penicillin antibodies, as indicated. Most of these tests were repeated 2 to 6 times on the same recipient. Prior to reaction with penicillin, stroma prepared by Method II was treated under conditions similar to those employed by Philipson and colleagues for receptor inactivation: heating for 15 minutes, ether extraction by shaking for 10 minutes, reaction with 10⁻²M periodate for 30 minutes at 37°, and then incubation with trypsin, chymotrypsin, RNase, DNase, and papain (activated with the use of 0.02M cysteine) in final concentrations* of 20 to 40 µg per milliliter for 60 minutes at 37° C. and pH 7.0 to 7.1. Sialidase was employed in a concentration of 12.5 µg per milliliter in a potassium acetate buffer, pH 4.5,

*Higher enzyme concentrations yielded preparations which produced irritant skin reactions.

containing 0.03 per cent of the erythrocyte donor's serum. Ether and periodate was removed by dialysis, and the enzymes were inactivated by heating at 50° C. for 15 minutes. Following this the preparations were dialyzed against pH 8.0, 0.15M phosphate buffer and then treated with penicillin, dialysis, centrifugation, dilution, and filtration as described for Method II.

As indicated in Table VII, heating at 100°, but not 56°, uniformly inactivated the receptors, while ether extraction, trypsin, RNase, DNase, and sialidase had no effect. Treatment with periodate, chymotrypsin, and papain produced variable results which in part related to the type of antibodies present in the sera being studied. This suggests that different penicillin allergens may react with different red cell receptors.

DISCUSSION

The data presented indicate both that soluble material biologically active in eliciting skin test reactions can be recovered from the stroma of penicillin-treated red cells and that these active substances are complex and heterogeneous in their composition. Their biological activity suggests that they contain more than one penicillin hapten per molecule of receptor.¹⁸ However, the fact that the penicillin-stroma complexes used in these experiments could elicit passive transfer reactions at serum dilutions approximately equivalent to the highest dilutions reacting to PPL does not necessarily imply a similar degree of substitution by penicillin derivatives, since it has been shown that conjugates containing a large number of benzyl penicilloyl determinants per molecule of polylysine do not have a greater capacity to elicit wheal-and-erythema reactions than more lightly conjugated complexes.¹⁹ On the other hand, the fact that PPL could be diluted much more than the sensitized stroma before losing its capacity to elicit passive transfer reactions at sites sensitized by relatively concentrated serum suggests that the former contains a much higher molar concentration of active material than the latter.

Several data suggest a heterogeneity in the penicillin derivatives attached to the stroma. Elicitation of passive transfer reactions both by PPL and sensitized stroma suggests that penicilloyl groups may be present in the stroma preparations, and there are data obtained from hemagglutination-inhibition experiments which also suggest attachment of penicilloyl groups to the red cell membranes.^{1, 2} This supposition also is supported by the observations on ultraviolet absorption after reacting the sensitized stroma with HgCl₂. On the other hand, the possibility must be considered that the reactive material in our "sensitized stroma" preparations is the penicilloylated protein contaminants recently found to be present in many penicillin solutions.^{20, 21} However, it would be expected that any such contaminants would be removed during the 3 washings of the sensitized stroma in acetate buffer employed in Method I. Furthermore, in order to exclude this possibility more completely, sensitized stroma was prepared by Method I with penicillin which presumably had been freed of any penicilloylated protein contaminants by dialysis against water and recovery of the penicillin from the dialysate by lyophilization. This "purified" penicillin proved to be equally as active as commercial penicillin in preparing sensitized stroma.

As shown on Table IV, however, the fact that sensitized stroma could elicit passive transfer reactions with a number of sera unreactive to PPL suggests the presence of an additional allergen on the stroma. One such substance may be present in the penicillin solutions used for sensitizing the stroma and be responsible for eliciting passive transfer reactions with sera responding to sensitized stroma and penicillin but not to PPL. Results obtained in passive transfer neutralization experiments employing serum K. C. (Table V) would be in accord with such a hypothesis. The presence of a third penicillin allergen on the sensitized stroma is suggested by the 2 sera which repeatedly reacted to the sensitized stroma but to neither penicillin nor PPL. One of these subjects, F. B., was tested directly with his own sensitized stroma, penicillin, and PPL. He reacted only to the sensitized stroma. This unidentified penicillin product or even a fourth derivative might be responsible for the results reported with sera K. H. and O. W. in Table V. In view of the fact that much more penicillin is required to neutralize these sera to challenge with sensitized stroma than to a biologically equivalent challenge with PPL again suggests that the stroma contains groups other than penicilloyl which react with these particular sera, but the penicillin derivative involved appears to be different from that which elicits passive transfer tests challenged with penicillin solution, since these were negative with these sera. The large amounts of penicillin required for passive transfer neutralization of the sensitized stroma also suggest that the unknown penicillin product involved is present in penicillin solution in lower concentration than penicilloyl groups or that it is formed in the skin to a lesser extent.

Another significant question concerns whether intact B-lactam rings are present in the sensitized stroma. It is believed that the antibiotic activity of penicillin requires the presence of this configuration,¹² but its presence in sensitized stroma would imply a linkage other than through amino groups. Failure to detect intact B-lactam rings in our sensitized stroma by infrared spectrophotometry may have been due to insufficient sensitivity to the method. The same may be said of our test for antibiotic activity of the solubilized, sensitized stroma. Ley²² reported that penicillin bound to red cells is not bactericidal. It may be noted that penicillinase causes rupture of the B-lactam ring, and Ley and colleagues²² reported that this enzyme prevents sensitization of erythrocytes if reacted with penicillin *before* adding red cells but does not cause loss of sensitization of cells previously reacted with penicillin.²²

The most cogent of additional possibilities concerning the nature of allergens producing these skin test reactions with sensitized stroma is that antibodies partially reactive with the stroma carrier are revealed by skin testing with sensitized stroma. Budd and colleagues²³ mentioned a patient reacting by skin test to penicilloyl-human serum albumin but not to PPL, and several investigators have noted that haptenes which include the alkyl chain of lysine and the amide linkage inhibit penicilloyl-specific immunologic reactions at lower concentrations than penicilloic acid.²⁴ Furthermore, there are reports^{23, 25, 26} of penicillin-related human agglutinins which could not be inhibited by a variety of penicillin haptenes. This suggests that some antibodies might be specific for a structure of the red cell formed or exposed during sensitization with penicillin.

Heterogeneity of the stroma portion of the drug-stroma complexes also is

suggested by this investigation. As shown in Table VI, partial purification of the stroma yielded a small increment in reactivity with 2 sera but a substantial diminution with a third serum having different immunologic characteristics. This variability may be due to various penicillin derivatives reacting with different stroma receptors. Some of the data in Table VII support this possibility: in some instances sera containing different types of penicillin antibodies show differing patterns of response to altered, sensitized stroma. While one would expect penicilloyl determinants to combine with receptors having free amino groups, DeWeek⁴ showed that penicillin could sensitize erythrocytes the amino groups of which had presumably been blocked by preliminary treatment with formaldehyde. More recently Josephson and Kaplan²⁷ showed that amino groups in solution failed to inhibit the sensitization of red cells by penicillin and that blocking amino and sulfhydryl groups by reacting the cells with 1, 3 difluoro-4, 6 dinitrobenzene also failed to prevent sensitization by penicillin (but did inhibit sensitization by penicillenic acid). These data were interpreted to indicate that penicillin may react with a receptor other than or in addition to amino groups on red cell surfaces, though this has not been unequivocally demonstrated. While it is known that penicilloyl groups bind to serum proteins,²⁸ it is noteworthy that Levine and Price²⁹ found evidence that human serum albumin and gamma globulin do not act as the carriers in penicillin allergy. There is the possibility that relatively unique structures on red cells may serve as important carriers. Delayed skin test reactions could not be used as a guide to the nature of the haptene carrier in these studies, since almost all tests involved challenge of sites passively sensitized with serum.

Observations on the interaction of penicillin with bacteria may be germane. Based on the capacity of various enzymes, alone or in combination, to release penicillin from fractions of bacteria, Duerksen³⁰ concluded that the receptor may be a lipoprotein associated with the cytoplasmic membrane. Tipper and Strominger³¹ also proposed that penicillin binds to the protein component of a lipoprotein complex located at the outside of bacteria cell membranes. Whether related structures might be present on red cell membranes is conjectural.

Erythrocyte receptors for myxoviruses¹⁵ and hemagglutinating enteroviruses¹⁶ have been studied quite extensively. However, our data suggest that the former are not significant receptors for penicillin derivatives, and the latter also do not appear to be the sole or unique receptors. Our initial attempts to fractionate the crude stroma preparations on DEAE and Sephadex columns have not yielded fractions having greatly enhanced receptor activity for different types of penicillin allergens, but further work of this type appears desirable. On the other hand, this approach will not be fruitful if different penicillin derivatives react with one or more radicals which are common to a variety of biological materials. The presumably covalent bonding of various penicillin derivatives with carrier substances under consideration here must be distinguished from the reversible binding of penicillin with serum albumin.

The possible *in vivo* importance of the interaction of penicillin derivatives with red cell membranes is uncertain. It is possible that the erythrocyte membrane receptor serves as a carrier in the induction of penicillin allergy. How-

ever, it has been stated that detectable amounts of penicillin do not affix to erythrocytes *in vivo* except when large amounts of the drug are given intravenously.³³ Perhaps this may in part be due to binding by serum components, and this may explain why hemolytic anemia from penicillin is not more common. Shelley and Florence³⁴ have suggested that persistence of penicillin antigens on red cells may account for the prolonged allergic symptoms which some patients experience from this drug. In view of this and the fact that possibly a number of other potential allergens firmly adhere to unmodified erythrocyte membranes (see further on), we raised the question of whether unknown allergens might be present on the red cells of patients with chronic urticaria of indeterminate etiology. Accordingly, solubilized stroma preparations were made from their own erythrocytes by Method I described above but *without* reacting the stroma with penicillin. None of 6 patients gave positive skin test reactions which could be passively transferred.

Besides penicillin, it has been claimed that streptomycin, erythromycin, chloramphenicol, and some of the tetracyclines, or their derivatives, also adhere firmly to red cell membranes.^{35, 36} If these reports can be confirmed, the possibility arises that techniques similar to those described herein might be useful in studying additional drug allergens, particularly since other drug conjugates analogous to PPL are not currently available. However, certain risks are entailed which tend to limit this approach to a status of being primarily an investigational procedure. The major concern relates to the possibility of sensitizing individuals either to drugs, erythrocyte antigens, or altered red cell antigens through injecting solubilized drug-stroma conjugates. The immunogenicity of these complexes has not been studied in experimental animals, but there is a definite possibility that they could induce an immune response to drugs,³⁷ a risk that would be related in part to the molecular weight of the receptors. Sensitization to erythrocyte antigens can be avoided by preparing the drug-stroma conjugates from the red cells of the individual to be injected, but this entails either having to prepare conjugates (and control material) from each patient being tested or else doing the studies by passive transfer of patients' sera to a normal recipient who is injected with a stock preparation of drug conjugated to his own red cell stroma. The possible hazards of passive transfer tests are well known.

Finally, consideration perhaps should be given to the hypothetical possibility that by injecting autologous erythrocyte antigens which have drug groups substituted upon them, there might develop loss of tolerance to the normal red cell antigens with subsequent autoimmune hemolytic anemia.³⁸ This would appear to be a relatively remote danger, however, in view of the minute quantities of sensitized stroma injected in the course of skin testing. In the present work risks to patients were avoided by performing all tests (except 2 sets) by the passive transfer method on one investigator. It is noteworthy that following more than 2,100 passive transfer tests performed over a period of 4 years this recipient has shown no evidence of sensitization by penicillinized autologous stroma as indicated by persistently negative control tests where these complexes, penicillin, or PPL are injected into normal skin, and his Coombs test remains negative.

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