

Macromolecular contaminants in penicillin and cephalosporin antibiotics

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High-molecular weight materials in penicillin G, methicillin, ampicillin, and cephalothin were isolated by ultrafiltration utilizing Diaflo UM-10 membranes and dialysis in Visking casing. The quantities of macromolecules recovered from 10 Gm. of the antibiotics varied from as little as 0.7 mg. from penicillin G to as much as 72 mg. from cephalothin. Infra-red spectroscopy revealed that these retentates differed from the original crystalline antibiotics and from one another in their spectra. Amino acid analysis of retentates from methicillin and cephalothin showed amino acids not present in similar analyses of parent antibiotics. However, only a small portion, 1 to 3 per cent, of the weight of retentates was recovered as amino acids, and in control experiments with distilled water comparable quantities of amino acids were recovered during the dialysis step of our isolation procedure. The remainder of the retentates was not chemically characterized but may represent polymers formed from the antibiotics. These results indicate that there are considerable differences in the quantities of macromolecules derived from these antibiotics and that these are largely composed of substances other than protein.

The antigens involved in allergic reactions to penicillin and cephalosporin antibiotics have been extensively investigated,¹⁻⁶ and the results of many studies suggest that these drugs, or metabolites derived from them, combine covalently with serum proteins forming hapten-protein complexes able to provoke allergic reactions. However, Batchelor and associates⁷ and other (8-11) have presented data suggesting that macromolecular proteinaceous contaminants are present in penicillin and cephalosporin antibiotics and are important in reactions to these drugs. Others have not found evidence for such proteinaceous contaminants.^{5, 12} In more recent studies the importance of polymers of penicillin and cephalosporin antibiotics has been emphasized as contaminants in these drugs¹⁹ and as reagents able to elicit allergic reactions in experimental animals.^{7, 13} In this study we analyzed penicillin G, methicillin, ampicillin, and cephalothin for the presence of macromolecular contaminants by membrane ultrafiltration. The results indicate that there are considerable differences in the quantities of macromolecules in these antibiotics and that these macromolecules are largely composed of substances other than protein.

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Supported in part by Grant AI-7187 from the National Institute of Allergy and Infectious Diseases and from the Eli Lilly Research Laboratories.

Received for publication Sept. 4, 1970.

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MATERIALS AND METHODS

Sodium citrate was purchased from Fisher Scientific Company. Glycyl-L-tyrosine, L-lysyl-L-tyrosyl-alpha-L-lysine, and bacitracin were purchased from Mann Research Laboratories. Ribonuclease was purchased from Worthington Biochemical Corporation. Commercial samples of penicillin G, methicillin, ampicillin, and cephalothin were purchased from a local hospital pharmacy. Sodium cephalothin, Lot No. 317-722AD-213-1, and sodium penicillin, Lot No. 315-769AD-276X were supplied by Eli Lilly and Company, Indianapolis, Indiana. Sodium methicillin, Lot No. 69F3788 (PS1162), and sodium ampicillin, Lot No. 69F3787 (G7147), were supplied by Bristol Laboratories, Syracuse, New York.

Ultrafiltration of antibiotic solutions was performed with the use of Diaflo UM-10 membranes, 76 mm diameter (Amicon Corporation, Lexington, Massachusetts). In a typical experiment 10 Gm. of antibiotic was dissolved in 100 ml. of pH 6.8 0.01M K_2HPO_4 - KH_2PO_4 , 0.05M NaCl (PBS), placed in a 400 ml. ultrafiltration cell in the cold room at 4° C., and a pressure of 100 pounds per square inch (p.s.i.) was applied to the cell with nitrogen gas. A diagrammatic representation of this apparatus is shown in Fig. 1. Filtration was continued until only a thin layer of fluid, approximately 2 to 5 ml., covered the membrane. The cell was then refilled with 100 ml. of PBS and pressure applied until the buffer had passed through the membrane. Each such filtration including the initial one was termed a filtration cycle. This process was continued until the final filtrate contained less than one per cent of the original sample, as measured by its absorbance of ultraviolet (UV) light. The material retained by the membrane (retentate) was removed from the chamber by washing with buffer. Retentates were dialyzed against distilled water at 4° C., three changes of 4 L. each, over a 24 hour period to remove salts and any low-molecular weight material remaining from the antibiotic. After dialysis retentates were lyophilized and weighed.

UV spectra of antibiotics and retentates were obtained using a Bausch and Lomb Spectronic 505 recording spectrophotometer. In preliminary experiments the UV absorption spectra of each of the antibiotics was determined, and subsequently the quantity of antibiotic in solution was estimated by its absorbance at 205 nm. for penicillin G, methicillin, and ampicillin and 238 nm. for cephalothin. With all antibiotics absorbance was linearly related to concentration below absorbances of approximately 1.5, using a Beckman DU-2 spectrophotometer. Infra-red spectra of antibiotics and retentates were obtained using a Beckman I-R 18 infra-red spectrophotometer. Lyophilized retentates were mixed with potassium bromide and compressed in a pellet (one per cent sample in potassium bromide).

Analysis of crystalline antibiotics and retentates for amino acids was performed by high-voltage paper electrophoresis and by ion-exchange column chromatography. Samples were hydrolyzed in vacuo at 110° C. for 22 hours with 6N HCl. Hydrolysates were dried over P_2O_5 and NaOH, dissolved in 0.01M citrate buffer, pH 2.2, and analyzed by paper chromatography (Gilson Model D High Voltage Electrophorator) using pH 1.3 formic acid-acetic acid buffer. Paper strips were stained with ninhydrin cadmium acetate. Ion-exchange chromatography was performed using the gradient elution method of Piez and Morris²² and a Phoenix Precision Amino Acid Analyzer, Model 6800.

RESULTS

The Diaflo membranes are molecular filters which separate molecules of differing molecular radius.¹⁴⁻¹⁷ The Diaflo UM-10 was used in this study because it allows the rapid passage of molecules with molecular weights similar to that of penicillin and retains molecules above this size.^{16, 17} The results of experiments with markers of known molecular weight are shown in Fig. 2 and Table I. As shown in Fig. 2, essentially all of lysyl-tyrosyl-lysine passed through the membrane in two cycles, while none of ribonuclease passed through the membrane in four cycles. Bacitracin, of intermediate molecular size, was partially retained by the membrane. These results are shown in Table I.

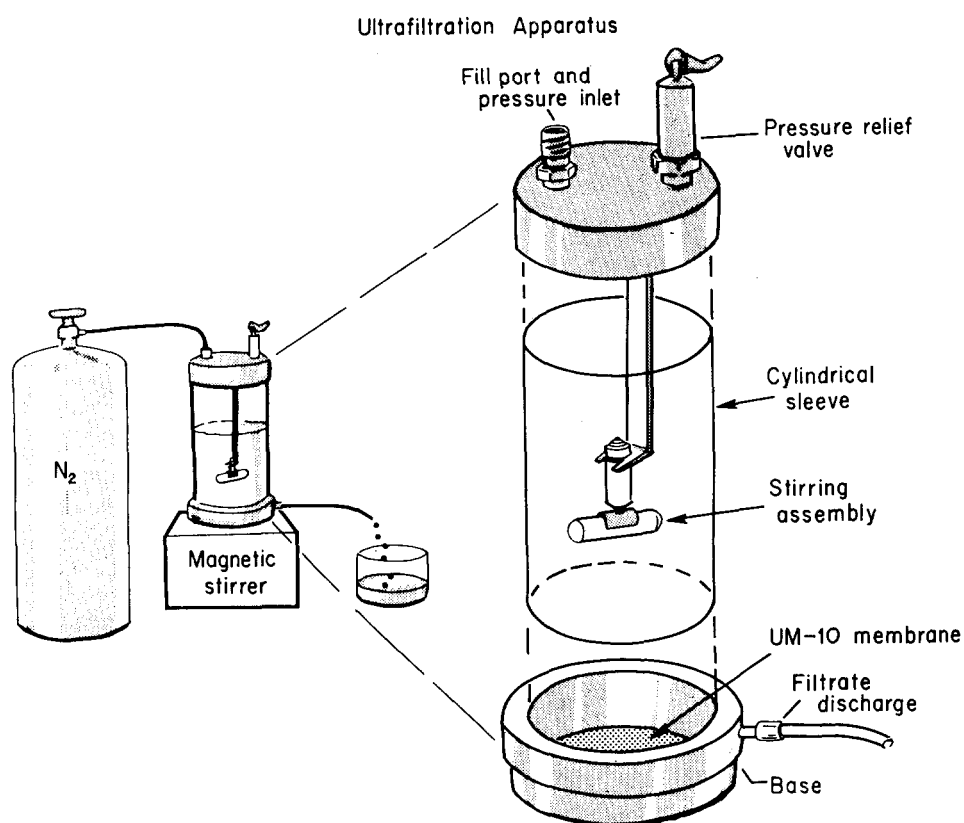


FIG. 1. Diagrammatic representation of ultrafiltration apparatus used to obtain high-molecular weight materials from antibiotics.

TABLE I. Filtration through the UM-10 membrane of materials of known molecular weight

Sample	Quantity	Molecular weight	Number of cycles	Retained by UM-10 (%)*
Citric acid	1.5 Gm.	192	4	0
			4	0
			4	0
			4	0
Glycyl-L-tyrosine	35 mg.	238	4	0
			4	0
Lysyl-tyrosyl-lysine	250 mg.	437	4	0
Bacitracin	42 mg.	1,411	4	36
			3	62
			3	41
Ribonuclease	20 mg.	13,899	6	98
			4	100

*The amount of material in filtrates and retentates was measured by absorbance in ultraviolet light with a Beckman DU-2 spectrophotometer. Citrate was analyzed at 210 nm., glycyl-L-tyrosine at 210 nm., lysyl-tyrosyl-lysine at 223 nm., bacitracin at 210 nm., and ribonuclease at 201 nm.

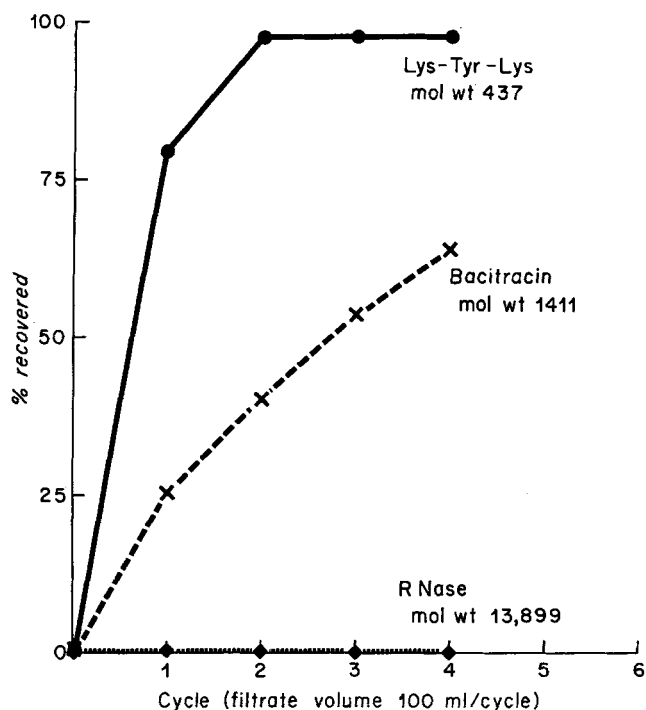


FIG. 2. Recovery of markers of known molecular weight after ultrafiltration.

In Fig. 3 the results of filtration of 10 Gm. of methicillin are shown. Although ultraviolet absorbing material was detected in all filtrates, the bulk of the original material (95 per cent) was recovered by the second filtration cycle. Filtration of penicillin G, methicillin, and ampicillin required about 24 hours, while cephalothin required about 48 hours. In Table II the quantities of retentates recovered as amorphous powders after ultrafiltration, dialysis, and lyophilization are listed. Only trace quantities of material were recovered from penicillin G, while slightly larger amounts were found with methicillin and ampicillin. Cephalothin was remarkable in that rather large quantities of material, approaching 1 per cent of the weight of starting antibiotic, were recovered in the retentate. The average per cent recovery of antibiotics in these experiments was penicillin 95.6, methicillin 100, ampicillin 95.7, and cephalothin 96.5. After filtration of the antibiotic samples, we observed a variable degree of yellow discoloration of the UM-10 membrane. Discoloration was noted even if antibiotic samples were filtered through Millipore 0.45μ filters before testing with the Diaflo membrane. This discoloration, presumably due to binding of antibiotic or its derivatives to the membrane, could not be removed by repeated washings in neutral buffers containing NaCl in concentrations up to 1M, acid, and alkaline solutions, or organic solvents, such as methanol and chloroform.

The retentates from each of the antibiotics were examined and compared to the crystalline antibiotics by infra-red spectroscopy. Each antibiotic had a

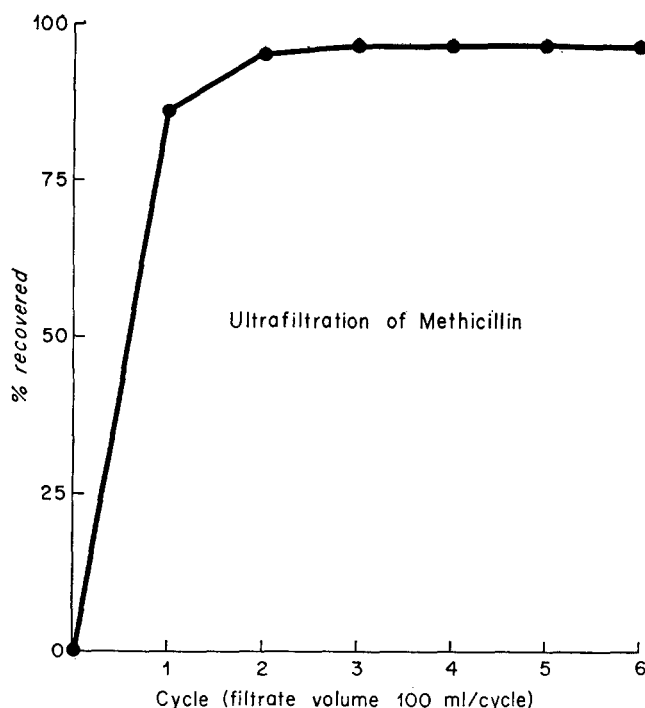


FIG. 3. Recovery of methicillin after ultrafiltration.

unique spectrum; while retentates from a given antibiotic were identical, retentates from the four antibiotics differed from each other and from the parent drug in their spectra. These findings suggested that filtration had resulted in the isolation of materials that differed from the parent antibiotics and that retentates from a given antibiotic differed from those obtained from the other antibiotics. While the parent antibiotics had a strong absorption peak at 5.62μ , indicative of an intact β -lactam structure, retentates either lacked this peak or it was markedly diminished in intensity and broadened.

To determine if the high-molecular weight material retained by the membranes was protein in nature, retentates were hydrolyzed and examined by high-voltage electrophoresis for the presence of amino acids. Retentates of penicillin G were of such small quantity that we were unable to examine them. Retentates of ampicillin, methicillin, and cephalothin were analyzed and compared to parent antibiotics examined in a similar fashion. Analysis by high-voltage electrophoresis indicated that the retentates contained ninhydrin-positive material not present in the parent antibiotics. However, the hydrolysates of the antibiotic frequently produced diffuse zones of staining that made interpretation of the results difficult. A consistent difference between antibiotics and retentates was the presence of staining in the area of lysine and ornithine in all retentates.

Retentates of methicillin and cephalothin were studied further by ion-ex-

TABLE II. Retentates from antibiotics

Antibiotic	Weight of sample (Gm.)	Filtration cycles	Weight of retentates (mg.)	Original Sample retained (%)
Potassium penicillin G*	10	9	1.1	0.011
	10	5	0.7	0.007
Sodium methicillin†	9.9	6	2.6	0.026
	9.9	6	5.1	0.052
Sodium ampicillin‡	10	17	3.0	0.030
	10	6	7.5	0.075
Sodium cephalothin§	10	6	37.5	0.375
	10	5	20.1	0.201
	10	6	72.8	0.728

All of the drugs were purchased from a hospital pharmacy and are representative of the materials in day-to-day clinical use.

*Lot Nos. Squibb 8F728 and Upjohn W242407, respectively.

†Lot Nos. Squibb 7M534 and Bristol C8628, respectively.

‡Lot Nos. Wyeth 2681921 and 2692571, respectively.

§Lot Nos. Lilly, 2JA90A, 2JA90A, and 2NU93A, respectively.

change chromatography, and the results are listed in Table III. The chromatograms of hydrolysates of the antibiotics and retentates revealed the presence of substances that had retention times and reacted with ninhydrin like known amino acids and were present in significant amounts. Although the quantities of amino acids in antibiotics and retentates were not greatly different, numerous amino acids, up to 16, were found in retentates, while only a few amino acids were recovered from crystalline antibiotics. We were concerned that the amino acids in retentates might have been introduced during our isolation procedure. If the material isolated by filtration and dialysis was a high-molecular weight proteinaceous contaminant present in the original antibiotic, the filtrate of the antibiotic should be free of it or it should be markedly reduced in concentration. To test this hypothesis, filtrates of methicillin and cephalothin obtained after passage through the UM-10 membrane were refiltered. After dialysis and lyophilization the retentates were weighed and results are shown in Tables III and IV. These experiments demonstrated that even after preliminary ultrafiltration of methicillin and cephalothin, high-molecular weight materials were still present. Ion-exchange chromatography following hydrolysis of these retentates revealed the same pattern of amino acids and in approximately the same quantities as in the original retentate (see Table III under "Retentate from initial filtrate"). Thus, preliminary ultrafiltration had not removed the macromolecular contaminants.

The presence of amino acids in retentates might have resulted from contaminants in the water used in these experiments. In our laboratory, deionized water supplied by the institution is passed through a deionizing column (De-Ionizer, Illico-Way Research Model, Belleo Glass, Vineland, New Jersey) and distilled in a Loughborough glass still. Ultrafiltration of 600 ml. of distilled water through the UM-10 membrane and subsequent lyophilization did not reveal any visible material. Pervaporation of 4 L. of distilled water followed by hydrolysis and

TABLE III. Amino acid analysis of antibiotics and retentates

Amino acid	Amino acids recovered (μ g)				
	Methicillin			Cephalothin	
	Crystalline drug	Initial* retentate	Retentate from* initial filtrate	Crystalline drug	Retentate
Quantity hydrolyzed (μ g)					
	2,200	1,800	1,900	6,380	6,300
Aspartic acid		2	2		4
Threonine		1	1		1
Serine		5	5	0.7	3
Glutamic acid	0.4	4	7		4
Proline			1		
α -Aminoadipic		2	1	0.6	10
Glycine	10	20	17	75	48
Alanine	12	2	3		2
Valine		1	1		1
Methionine	1	2	1	1.3	1
Isoleucine		2	0.3		
Leucine		0.1	1.2	12.2	
Tyrosine		2			1
Phenylalanine		6	3		1
Histidine		1.1	1		1
Ornithine		0.2	2		1
Lysine		1	7		2
Total	23.4	51.5	53.5	89.8	80.0
Quantity hydrolyzed (%)					
	1.06	2.86	2.82	1.41	1.30

*Here "Initial retentate" refers to the quantity of material present after filtration of freshly dissolved drug while "Retentate from initial filtrate" refers to the material recovered after subsequent refiltration of the drug which passed through the UM-10 membrane and was freed from any pre-existing macromolecular contaminants.

TABLE IV. Refiltration of methicillin and cephalothin

Sample	Weight of sample (Gm.)*	Weight of retentate (mg.)	Sample Retained (%)
Methicillin filtrate	6.6	2.4	0.036
Cephalothin filtrate	3.9	3.7	0.09

*Estimated on the basis of absorbance of filtered antibiotics.

ion-exchange chromatography yielded 16 μ g of amino acids. This could not account for the amino acids found in the retentates as this volume of solvent is seven times that used in processing the antibiotics, and therefore only 16/7 or approximately 2 μ g could be contributed by the distilled water.

Additional experiments were carried out to determine if amino acids were introduced during dialysis. A 5 foot length of Visking dialysis casing (Size No. 20, Union Carbide Co., Chicago) was rinsed, filled with 100 ml. of distilled water, and dialyzed against six changes (4 L. per change) of distilled water over a period of 3 days. The fluid inside of the casing was lyophilized and the resulting gum was dissolved in 6N HCl and hydrolyzed. Ion-exchange chromatography revealed the same pattern of amino acids noted in the retentates, and 98 μ g

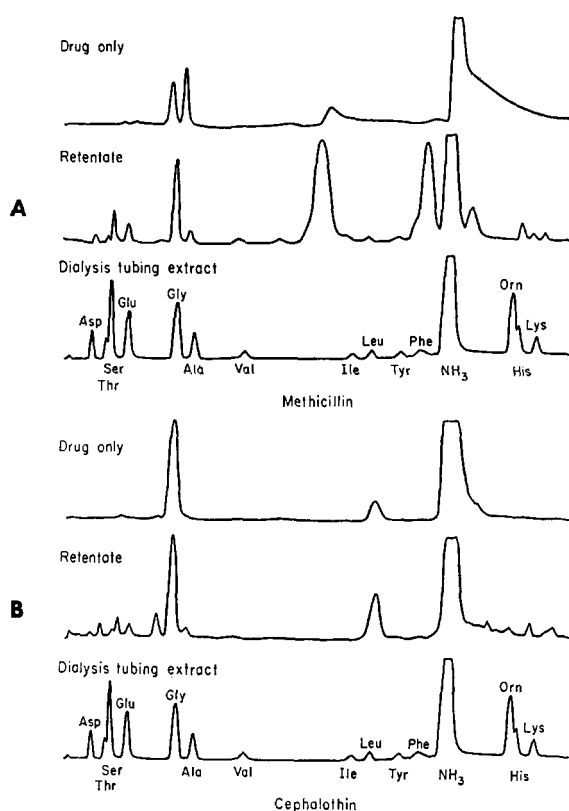


FIG. 4. Ion-exchange chromatograms to methicillin (A) and cephalothin (B), retentates derived from these, and dialysis tubing extract. The elution position of amino acids is indicated for dialysis casing extract. Peaks not corresponding to the elution position of amino acids were not used in the results shown in Table III. The quantities of amino acids in the dialysis casing extract are greater than those in the retentates presumably because of the greater length of Visking casing used in this experiment.

of amino acids were found. The varieties and quantities of the recovered amino acids were essentially the same as those from the methicillin retentate (Table III, "Initial retentate") except that α -aminoadipic acid was not detected while arginine was detected. Because the length of dialysis casing in this experiment was approximately three times that used in our other experiments, up to 98/3 or 33 μ g may have been introduced into retentates during dialysis. In Fig. 4, A and B, the chromatograms of methicillin and cephalothin crystalline antibiotics and retentates are shown, and these are compared to the chromatogram of the material recovered after the dialysis experiment above.

To document further that the contaminant introduced during dialysis was the source of the amino acids found in the retentates, the following experiment was performed. Distilled water was added to the usual length, approximately 11½ feet, of Visking tubing and dialyzed as described above. The fluid inside the casing was added to methicillin and cephalothin crystalline antibiotics,

lyophilized, hydrolyzed, and examined by high-voltage electrophoresis. Methicillin and cephalothin diluted in distilled water were similarly treated. The antibiotics dissolved in dialysis fluid gave a pattern of amino acids absent in the controls but identical to the pattern found in the retentates.

DISCUSSION

In these experiments high-molecular weight materials present in penicillin and cephalosporin antibiotics were isolated by ultrafiltration and dialysis. In early studies with materials of known molecular weight, the selectivity of the Diaflo UM-10 membranes was examined and their ability to retain molecules with molecular weights between bacitracin (1,411) and ribonuclease (13,899) demonstrated. In the case of the antibiotics, filtration was continued until the bulk of the antibiotic was recovered, and only trace quantities of material absorbing in the ultraviolet continued to pass through the membrane. To ensure that all low-molecular weight materials were removed, the retentates were finally dialyzed. As shown by the results in Table II, only small quantities of material were recovered; with penicillin G these quantities were in the range found by Dürsch¹² using dialysis only.

These experiments confirm in part the studies of others^{7, 9, 11, 18, 19} who also have found macromolecular contaminants in penicillin and cephalosporin antibiotics. However, from our studies it appears that the bulk of these materials are not protein in nature, as only 1 to 3 per cent of the weight of retentates could be accounted for as amino acids. With typical proteins the bulk of the material can be recovered as amino acids, depending, of course, on the content of carbohydrate. Even the small quantity of amino acids found cannot be clearly attributed to protein contaminants in the parent antibiotics because it is evident that during the dialysis step of our purification scheme, contamination with amino acids was introduced. It would seem unlikely that these were derived from our distilled water because control experiments showed that only a small quantity of amino acids were found in the water used in these analyses. Our experiments do not answer the question whether the amino acids were derived from the dialysis casing itself or represented bacterial growth during the dialysis step. In either case it is clear that the dialysis step was responsible for the appearance of amino acids and that one must be aware of this as a source of potential contaminants. In subsequent studies we have found that extraction of Visking dialysis casing with distilled water yields solutions which absorb strongly in the ultraviolet at 225 and 277 nm., and some of these ultraviolet absorbing substances elute from Sephadex G-25 columns with volumes indicative of molecular weights over 1,000.²⁰ The remaining portions of the retentates not containing amino acids appear to be derived from the drugs themselves and not from the dialysis casing because: (1) There was a marked difference in yield of retentates from the different drugs, even though each was analyzed in a similar manner. (2) Retentates differed from each other in their infra-red spectra. If retentates were derived from dialysis casing, one would expect approximately the same quantity of material from all drugs and one would also expect that the infra-red spectra would be identical.

The difference in the quantities of high-molecular weight materials derived from these antibiotics was striking and suggests that cephalothin has a considerably greater propensity to form these contaminants than do the other drugs. The least quantity of retentate was derived from penicillin G. The reasons for these differences are not evident, but the large amount of retentate from cephalothin may in part reflect an inherently greater chemical reactivity of this antibiotic. It has been noted by others that administration of cephalothin to patients may result in a positive direct Coombs test,²¹ an observation suggesting that cephalothin is a highly reactive molecule able to bind serum proteins to erythrocytes even under physiologic conditions of temperature and pH.

Since the study of Batchelor and associates⁷ and Stewart,⁹ numerous reports have appeared in the literature concerning macromolecular contaminants in penicillin and cephalosporin antibiotics.^{11-13, 19} Initially these stressed the importance of protein impurities, some of which presumably had reacted with penicillin to introduce the penicilloyl determinant, although even in the initial report of Batchelor and co-workers⁷ the formation of penicillin polymers was noted. More recent reports have stressed the formation of polymers by the antibiotics themselves, and Stewart¹⁹ has found in an experiment similar to that shown in our Table IV that macromolecules are formed from antibiotics which have been put through a preliminary purification step to remove high-molecular weight materials. Batchelor and associates⁷ claimed that polymers formed from penicillin G were able to react with antisera to the benzylpenicilloyl determinant, and Butcher and Stewart¹³ claimed that macromolecules derived from ampicillin reacted with antisera to the proteinaceous residue from penicillin G. However, the importance of these macromolecules in allergic reactions in patients remains to be determined.

We thank Dr. V. R. Mattox for assistance in interpretation of infra-red spectrograms.

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