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Synthesis of Antigenic Branch-Chain Copolymers of Angiotensin and Poly-L-lysine*

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ABSTRACT: Two branch-chain copolymers comprising backbones of poly-L-lysine and branches of the physiologically active octapeptide angiotensin II were synthesized. In one of these, the amino-terminal end of angiotensin was coupled to poly-L-lysine via *m*-xylylene diisocyanate. In the other instance, carbodiimide condensation was performed between the carboxyl-terminal end of angiotensin and the ε-amino groups of poly-L-lysine.

Both polymers elicited in rabbit the production of antibody specific for angiotensin. Binding of isotopically labeled angiotensin to antibody was demonstrated by gel filtration. One of these branch-chain polymers is active as a pressor, while the other is not. The antibody does not appear to compete with angiotensin for its physiologic binding site.

Polypeptides of low molecular weight are usually poor antigens. Angiotensin, an octapeptide with molecular weight 1031, appears to be nonantigenic (Deodhar, 1960). In an effort to make antibodies directed against angiotensin, the molecule was coupled covalently to poly-L-lysine, itself nonantigenic, and the resultant complex was tested for antigenicity in rabbits. By appropriate techniques, angiotensin could be attached to the polyamino acid backbone via either the carboxyl- or amino-terminal end. Antibodies binding isotopically labeled angiotensin were demonstrated. The techniques involved in making antibodies against angiotensin have wide application in immunoassay and in studies directed toward the mechanism of action and biological properties of this and other low molecular weight polypeptide hormones.

Materials and Methods

Materials. Synthetic asparagine[1] valine[5] angiotensin II (Hypertensin Ciba, lot A-7930) was provided free of excipients.[1] This material was demonstrated to be homogeneous by amino acid analysis, by high-voltage electrophoresis in formic–acetic acid buffer, *p*H 1.9, at 98 v/cm, and by gel filtration on Sephadex G-25. Infrared spectroscopy showed no evidence of residual blocking groups. Poly-L-lysine·HCl was obtained from the Mann Chemical Co. (lot J-2094). The molecular weight of this material was 17,000 as determined by a short-column equilibrium ultracentrifugation (Richards and Schachman, 1959) using a partial specific volume of 0.79 (Friedman *et al.*, 1961). *m*-Xylylene diisocyanate was obtained from the Mann Chemical

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Co. Dicyclohexylcarbodiimide was obtained from Sigma Chemical Co. Leucine aminopeptidase and diisopropylfluorophosphate-treated carboxypeptidase were obtained from the Worthington Biochemical Corp.

Synthesis of Angiotensin Coupled to Poly-L-lysine via Its Carboxyl-Terminal Phenylalanine [A-(Phe)-PL].[2] A carbodiimide condensation was carried out according to the method of Sheehan and Hess (1955). In a typical experiment 153 mg of angiotensin and 46 mg of poly-L-lysine·HCl were dissolved in 11.6 ml of distilled water, and the solution was added to 23 ml of a solution containing 463 mg of dicyclohexylcarbodiimide in tetrahydrofuran (final *p*H 6.4). The mixture was stirred at 20–25° for 18 hours. At the end of this time the precipitated dicyclohexylurea was removed by centrifugation. The supernatant was then brought to *p*H 2.0 by the addition of formic acid and dialyzed against 0.5 M formic acid for 48 hours. If the acidification step was omitted, the solution assumed a reddish color with absorption maximum at 340 mμ, possibly owing to the formation of a lysyl carbodiimide compound.

Synthesis of Angiotensin Coupled to Poly-L-lysine via the Amino-Terminal Asparagine [PL-(Asn)-A]. A method similar to that described by Schick and Singer (1961) was used. In a typical experiment 150 mg of angiotensin was dissolved in 6 ml potassium phosphate buffer at *p*H 7.0. *m*-Xylylene diisocyanate (30 μl) in 12 ml dioxane was added with the immediate formation of a precipitate. Poly-L-lysine·HCl (50 mg) was then added and the *p*H was raised to 9.5 by the dropwise addition of 0.1 M NaOH. After several minutes of incubation the precipitate dissolved. The mixture was stirred at 20–25° for 4 hours and then was dialyzed against 0.1 M acetic acid.

Characterization of Synthetic Products. Amino acid analyses were performed according to the method of Spackman *et al.* (1958). End-group analyses were done by the dinitrophenol method, employing thin-layer chromatography to separate the products (Walz *et al.*, 1963). Leucine aminopeptidase amino-terminal analyses and carboxypeptidase carboxyl-terminal analyses were done according to methods described by Potts *et al.* (1962). For qualitative analysis of the products of enzymatic hydrolysis, the high-voltage electrophoretic method of Dreyer (1960) was used.

Immunization of Animals. The two poly-L-lysine angiotensin copolymers were prepared in an emulsion with complete Freund's adjuvant. One mg of antigen suspended in the emulsion was injected into rabbits intramuscularly, intraperitoneally, and into the toe pads. In the instance of PL-(Asn)-A an additional intravenous injection of 1 mg of the compound was made 3 months after immunization. Bleedings were performed 14 weeks after initial immunization.

Synthesis of Isotopically Labeled Angiotensin Derivatives. [^{131}I]Angiotensin was prepared (Hunter and Greenwood, 1962) employing carrier-free iodine obtained from Amersham, Ltd. In a typical experiment, 5 μg of angiotensin was treated with 2.0 mc of ^{131}I which had previously been oxidized with chloramine T. To the reaction mixture was added 2 ml of serum from rabbits immunized against PL-(Asn)-A and the mixture was incubated at 0° for 10 minutes. The mixture was then applied to a Sephadex G-25 column (1 × 40 cm) and developed with 0.1 M Tris-acetate buffer, *p*H 7.5, containing lysozyme. The first radioactive peak (void volume) was collected and acidified to *p*H 2.0 with formic acid. This was reapplied to the same column and developed with 0.5 M formic acid. The first portion of the included volume contained labeled angiotensin capable of being bound quantitatively by the same rabbit antiserum. This product was used directly in the antibody binding studies.

Acetyl [^{14}C]angiotensin was prepared by the reaction of angiotensin with a 4-fold molar excess of [^{14}C]-acetic anhydride (10 mc/mmole) obtained from New England Nuclear Corp. The product was purified and separated from unreacted angiotensin by successive chromatography on Dowex 50-X4 and CM-cellulose. A spectrophotometric titration of the tyrosyl hydroxyl of acetylated angiotensin demonstrated no change from native angiotensin, indicating that acetylation occurred predominantly at the amino-terminal nitrogen. The titration was performed by increasing the *p*H of a solution of acetyl angiotensin in increments of 0.5 *p*H unit over the range *p*H 8.0–12.0, and observing the ultraviolet absorption spectra. A quantity of acetyl [^{12}C]angiotensin was prepared in the same manner.

Assay of Antibody Activity. Either acetyl [^{14}C]-angiotensin or [^{131}I]angiotensin was incubated for 15 minutes with 50 μl of serum from normal or immunized rabbits at 0° in 1.0 ml of 0.1 M Tris-acetate buffer at *p*H 7.5, containing 1 mg/ml lysozyme.

The mixture was applied to a 0.9 × 29-cm column of Sephadex G-25 and developed with the same buffer at 2°. The addition of lysozyme was found necessary to prevent nonspecific binding of the radioactive peptides to glass and gel. The column effluent from the ^{14}C-labeled mixture was collected in 0.6-ml fractions and counted on a low-background end-window counter. The column effluent of the ^{131}I-labeled mixture was passed directly through a Teflon coil in a 51-mm sodium iodide crystal well counter with pulse-height analyzer. Constant flow rates of 1 ml/minute were maintained by a metering pump. The time for a column run was 70 minutes. The counts were integrated directly at 1-minute intervals by a scaler, and total counts in the void volume and included volumes were determined.

Counts within the first peak or void volume, if present, were taken to represent isotopically labeled angiotensin bound to antibody. Counts within the second peak or included volume represent unbound angiotensin. Specific displacement of isotopically labeled angiotensin from antibody was demonstrated by incubating the serum for 30 minutes with increments of native angiotensin and a constant quantity of the isotopically labeled compound. Nonspecific binding of

[2] Abbreviations used in this work: A-(Phe)-PL, angiotensin coupled to poly-L-lysine via its carboxyl-terminal phenylalanine; PL-(Asn)-A, angiotensin coupled to poly-L-lysine via the amino-terminal asparagine.

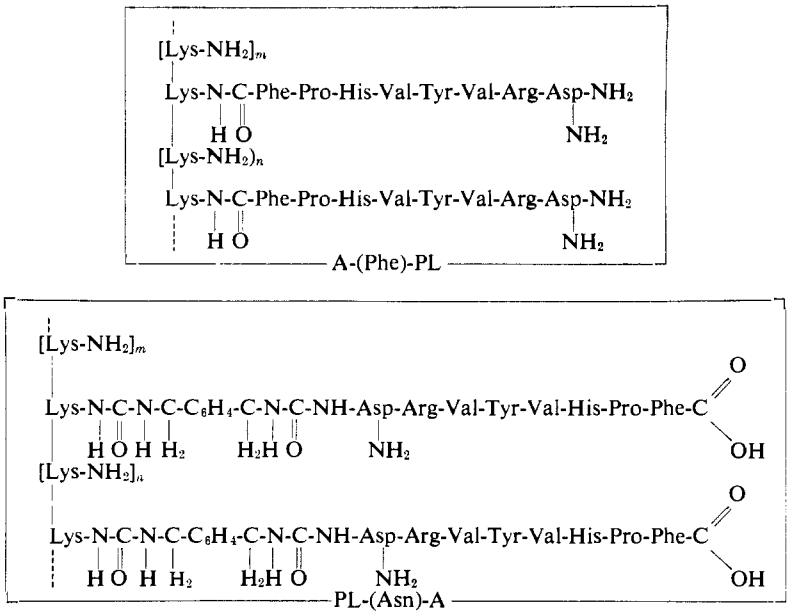

FIGURE 1: Schematic representation of angiotensin poly-L-lysine copolymers.

isotopically labeled angiotensin to serum was tested by experiments employing serum from nonimmunized rabbits. Nonspecific displacement of isotopically labeled angiotensin from antiserum was tested with vasopressin, bradykinin, and a high concentration (1 mg/ml) of a peptic digest of lysozyme.

Biological Assay. To determine the biological activity of the angiotensin derivatives, a modification of the rabbit aortic strip assay of Helmer (1961) was designed. A 2 × 45-mm spirally cut strip of rabbit aorta was suspended in a 12-ml chamber, bathed in oxygenated Krebs-Ringer solution at 37°, and attached to a Sanborn Model 7 DCDT-100 force transducer (full-scale, 1 g). The output of the transducer was recorded graphically using a Sanborn Model 311 amplifier-indicator and a Photovolt recorder Model 43. The force of isometric contraction elicited by angiotensin and its derivative was compared. Standard solutions of the compounds to be tested were introduced into the chamber in amounts varying from 0.1 to 10 μg.

Results

Characterization of Compounds. The amino acid analyses of A-(Phe)-PL and PL-(Asn)-A are shown in Table I. Molecular weights, molar ratios, and amino- and carboxyl-terminal analyses are shown in Table II. End-group analysis data indicate that the carboxyl-terminal end of poly-L-lysine does not participate significantly in the carbodiimide condensation. It appears that angiotensin branch chains are coupled only via their carboxyl-terminal end to the poly-L-lysine backbone in A-(Phe)-PL and only via the amino-terminal end in PL-(Asn)-A (Figure 1). The absence of carboxyl-terminal phenylalanine in the product of the carbodiimide reaction indicates that self-condensation to form polyangiotensins did not occur or that such products were of sufficiently low molecular weight to pass through dialysis tubing.

TABLE 1: Amino Acid Analyses[a] of Angiotensin Poly-L-lysine Copolymers.

Amino Acid	PL-(Asn)-A[b]	A-(Phe)-PL
Asp	0.2	1.1
Arg	1.0	1.0
Val	2.0	2.0
Tyr	0.5	0.9
His	1.0	1.1
Pro	1.1	1.1
Phe	1.0	0.8
Lys	10.0	33.3

[a] Expressed as molar ratios in relation to valine, which is taken to equal 2.0. [b] The low value of aspartic acid in PL-(Asn)-A is probably related to the stability of the xylylyl aspartic hydantoin formed from the xylyl carbamyl amino acid on exposure to 6 N HCl (Stark and Smyth, 1963). The lower than expected value for tyrosine in this compound is explained by oxidation during hydrolysis.

Demonstration of Specific Antibody Activity. Sera from five of six rabbits immunized with PL-(Asn)-A and from two of five rabbits immunized with A-(Phe)-PL were found to bind acetylated [^{14}C]angiotensin and

[^{131}I]angiotensin. Typical experiments are illustrated in Figures 2 and 3. When present in excess, the antisera proved capable of binding all of the [^{131}I]angiotensin (Figure 2A). In experiments utilizing the sera of nonimmunized rabbits, no [^{131}I]angiotensin could be demonstrated in the void volume, indicating the complete absence of nonspecific binding. The addition of increments of unlabeled angiotensin to the reaction mixture resulted in increasing displacement of [^{131}I]angiotensin from antibody (Figure 3). In sufficient excess, virtually complete displacement was effected (Figure 2B). The specificity of this displacement is supported by the finding that a 50-fold excess of vasopressin and bradykinin and a 10^5 excess of a mixture of peptides obtained from a peptic digest of lysozyme failed to effect any displacement of [^{131}I]angiotensin from antibody. [^{131}I]-Angiotensin and acetyl [^{14}C]angiotensin were equally well bound by antisera produced in response to PL-(Asn)-A and A-(Phe)-PL.

Biologic Activity. Rabbit aortic strip preparations varied considerably in relative responsiveness to standard preparations of the test substances. A-(Phe)-PL elicited contractions from one-tenth to one-third as great as equivalent quantities of angiotensin. PL-(Asn)-A failed to produce any contraction. The acetyl derivative of angiotensin was one-third to one-half as potent as angiotensin, whereas poly-L-lysine was inactive.

In an attempt to demonstrate inhibition of biologic activity by specific antibody, excesses of the two types of antisera were incubated for 18 hours at 3° with varying amounts of angiotensin. When these mixtures were applied to the aortic strip, the magnitude of the contractile force was identical to that of control preparations without antibody.

Discussion

It has been demonstrated that relatively small peptides comprised of amino acids in random arrangement can act as specific antigenic determinants when attached to large carrier molecules (Sela *et al.*, 1962).

Deodhar (1960) attempted to evoke antibodies against angiotensin by immunizing with bovine γ-globulin to which angiotensin had been coupled via a diazonium compound. Recently Goodfriend *et al.* (1964) have reported formation of antibody against angiotensin and bradykinin coupled by the carbodiimide reaction to serum albumin. In both these methods the carrier molecule is itself antigenic. In the former, various locations of the diazonium bridge are possible, both with respect to angiotensin and to protein carrier. In the latter method random attachment of the peptide at both its amino- and carboxyl-terminal ends may occur.

The present study demonstrates the synthesis of a class of branch-chain amino acid copolymers employing a backbone of poly-L-lysine, and branches comprised of peptides of specific amino acid sequence attached to the backbone in a specific orientation. These compounds are capable of elicting the production of antibody specific for the small peptides comprising the branches. The binding of the free peptides to antibody is demonstrated and the method may be applied to an immunoassay of this hormone. Poly-L-lysine is a particularly suitable backbone for such branch-chain polymers, because it is not intrinsically antigenic, and the ε-amino groups provide an opportunity for coupling peptides specifically either by their carboxyl- or their amino-terminal ends.

Gel filtration has been employed to separate anti-

TABLE II: Molar Ratios, Molecular Weights, and Amino- and Carboxy-Terminal Analyses of A-(Phe)-PL and PL-(Asn)-A.[a]

Compound	Coupling Agent	Moles Angiotensin per Mole Poly-L-lysine	Moles Angiotensin Bound per Mole Lysine Residue	Molecular Weight	Amino-Terminal Amino Acids	Carboxyl-Terminal Amino Acids
PL-(Asn)-A	Xylylene diisocyanate	11.6	0.10	29,000	Lysine	Phenylalanine lysine
A-(Phe)-PL	Dicyclohexylcarbodiimide	3.5	0.03	20,600	Aspartic acid lysine	Lysine

[a] Molar ratios of angiotensin to poly-L-lysine and molecular weights of the polymers were calculated from amino acid analyses and molecular weight of poly-L-lysine·HCl determined by short-column equilibrium ultracentrifugation. Amino-terminal analyses were done both by the dinitrophenylation method and with leucine aminopeptidase. Carboxyl-terminal analyses were done with carboxypeptidase.

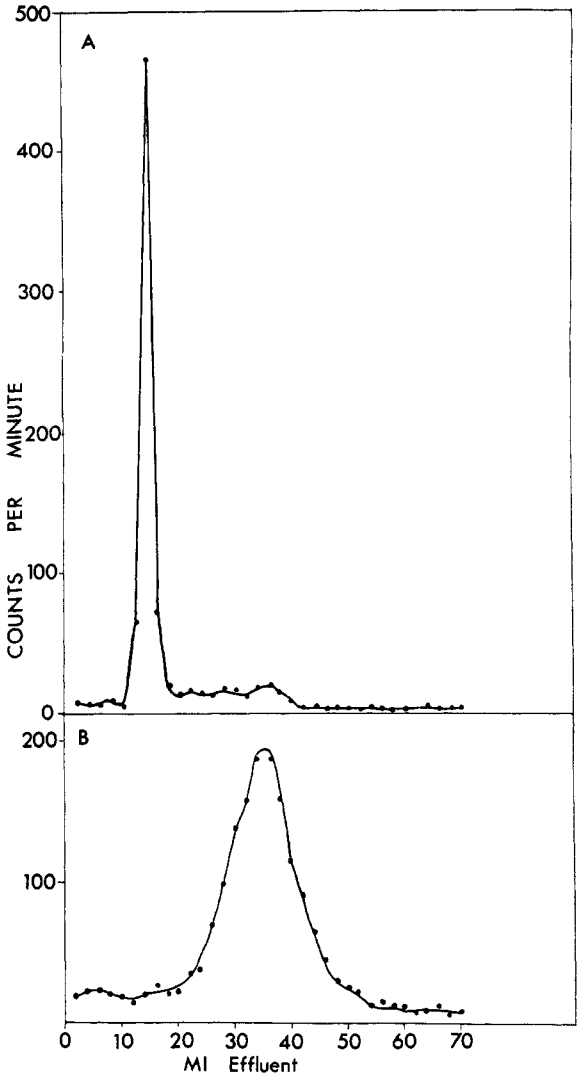

FIGURE 2: Radioactive assay of PL-(Asn)-A and A-(Phe)-PL. (A) PL-(Asn)-A antiserum (50 μl) was incubated 30 minutes with a quantity of [^{131}I]angiotensin in 1 ml of 0.1 M Tris-acetate buffer, *p*H 7.5, containing 1 mg/ml lysozyme. The mixture was separated on a 0.9 × 40-cm column of Sephadex G-25 at 2°. 84% of the radioactivity is within the void volume (first peak). (B) A-(Phe)-PL antiserum (10 μl) was incubated under the same conditions with a quantity of [^{131}I]angiotensin and 100 mμg of unlabeled angiotensin. All the radioactivity is contained within the gel volume (second peak).

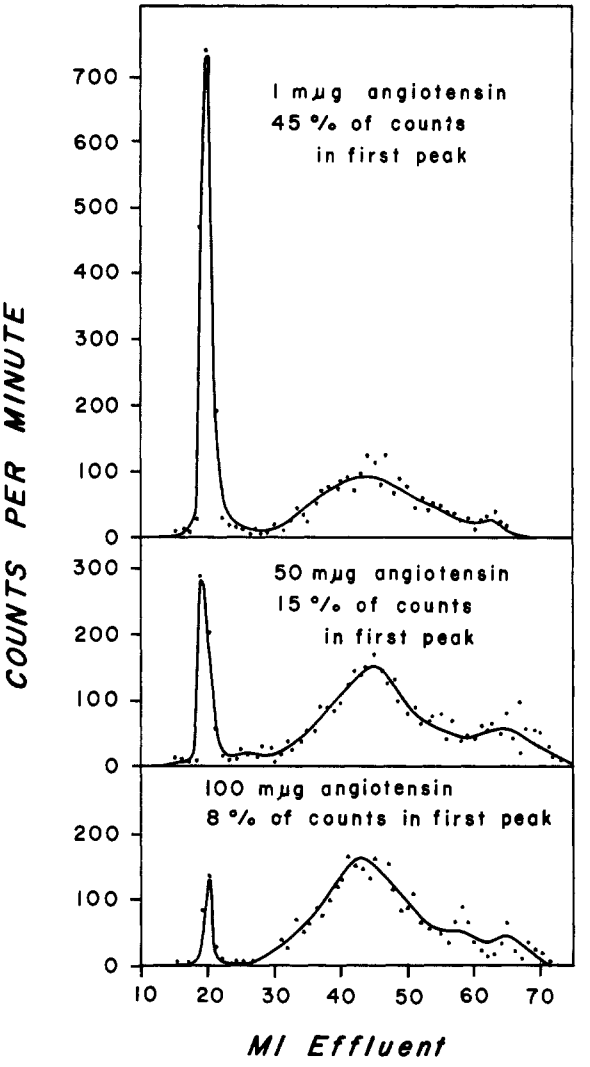

FIGURE 3: Radioactive assay of a mixture of A-(Phe)-PL antiserum, [^{131}I]angiotensin, and unlabeled angiotensin. A-(Phe)-PL antiserum (50 μl), a fixed quantity of [^{131}I]angiotensin, and varying amounts of unlabeled angiotensin were incubated in 1 ml of 0.1 M Tris-acetate buffer, *p*H 7.5, containing 1 mg/ml lysozyme at 0° for 30 minutes. The mixture was separated on a 0.9 × 40-cm column of Sephadex G-25 at 2° and effluent radioactivity was determined directly in a flow-through well counter. Quantities of nonlabeled angiotensin and per cent of total counts in void volume (first peak) are noted in the diagrams.

body-bound angiotensin from the free molecule with a high degree of resolution. There is no discernible change in equilibrium which may decrease resolution in chromatoelectrophoretic separation (Berson *et al.*, 1956).

The high degree of specificity of binding by antibody is indicated by displacement of the isotopically labeled material by minute quantities of unlabeled angiotensin, while excesses of vasopressin, bradykinin, or mixtures of peptides have no effect.

The association of angiotensin with its antibody was employed to fractionate antigenically competent ^{131}I-labeled angiotensin from other products of iodination, the labeled antigen being later disassociated from antibody by acidification. In this manner, a labeled antigen capable of quantitative binding to antibody is obtained.

It is both interesting and surprising that A-(Phe)-PL retains substantial biologic activity, since previous stud-

ies suggest that a free terminal carboxyl is necessary for pressor action of angiotensin (Bumpus *et al.*, 1961).

A failure of the antibody to inhibit the biological effect of angiotensin on the isolated aortic strip suggests as one possibility that the binding affinity between the hormone and its physiologic site of action is considerably greater than that between the hormone and its antibody.

The availability of specific antibody directed against a peptide of small size and definite amino acid sequence, rather than a random copolymer, will facilitate study of the structure of antibody and the precise requirements and relationships of antigenic determinants. This type of copolymer allows for amino acid substitutions, inversions, and other specific modifications. Such flexibility of alteration is not possible in either random copolymers or natural protein antigens.

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U-21,669: A New Lincomycin-related Antibiotic*

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ABSTRACT: U-21,699 is a new antibiotic very closely related to lincomycin. Both U-21,699 and lincomycin are produced by *Streptomyces lincolnensis* var. *lincolnensis*. Isolation of the activities from the fermentation broth has been achieved by carbon adsorption, followed by elution with aqueous acetone. U-21,699 has been separated from lincomycin by countercurrent distribution, followed by Florisil chromatography, and has been isolated in crystalline form as the hydrochloride salt. The structure of U-21,699 has been determined and the work supporting the proposed structure is discussed.

Antibiotic U-21,699 is a new lincomycin-related antibiotic, produced concomitantly with lincomycin[1] in fermentations of *Streptomyces lincolnensis* var. *lincolnensis*. The isolation and chemistry of lincomycin have been reported by Mason *et al.* (1962), Herr and Bergy (1962), and Hoeksema *et al.* (1964). The present paper describes the isolation and chemical properties of U-21,699. Biological properties of this new antibiotic have been described by Mason and Lewis (1964).

Experimental and Results

Isolation of U-21,699. Recovery from Fermentation Broth. A culture broth of lincomycin fermentation (24 kl) was adjusted to *p*H 3.0 with concentrated sulfuric acid, and filtered using filter aid. The filtered beer was adjusted to *p*H 8.0 with 50% aqueous sodium hydroxide solution. The alkaline clear beer was then passed through columns containing Pittsburgh Type CAL, 12–40 mesh granular carbon (109 kg). The carbon columns were

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[1] Lincocin is the trademark of The Upjohn Company for lincomycin hydrochloride.