for certain substances, at least, it is now possible to obtain at least a lower limit to the cohesive energy density of pharmaceutically important compounds by very simple calculations. These calculations can be simplified further by noting that the ionization potentials in Tables II and III are not far from 10 ev. generally, so this value can be used in place of specific ionization potentials.

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Comparative Stabilities of Ampicillin and Hetacillin in Aqueous Solution

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Abstract □ Ampicillin and hetacillin in aqueous solution showed distinctive chemical alterations within 1 day. Ampicillin formed polymers which were separated according to molecular size by gel filtration on columns of an acrylamide gel. Hetacillin exhibited a rapid generation of a substance with a characteristic absorbance at 317 nm. This material was probably a penicillenic acid. A mechanism for these reactions is suggested.

Keyphrases □ Ampicillin—stability in aqueous solution, compared to hetacillin, mechanisms proposed for chemical alterations □ Hetacillin—stability in aqueous solution, compared to ampicillin, mechanisms proposed for chemical alterations □ Antibiotics, stability in aqueous solution—comparison of ampicillin and hetacillin, mechanisms proposed for chemical alterations

In investigations of the biochemical properties of some semisynthetic penicillins, it was noted that stock solutions of the antibiotics showed colorations, became viscous, and developed precipitates when left to stand at room temperature for longer than a few days. Since the allergic reactions to penicillins have been variously attributed to reactions of penicillins with tissue proteins, endogenous polymer formation, and contaminations with protein impurities among other possibilities, it was felt necessary to investigate more definitively the stability of several semisynthetic penicillins in aqueous solution. This report is specifically concerned with ampicillin [D(−)-α-aminobenzylpenicillin] and hetacillin [6-(2,2-dimethyl-5-oxo-4-phenyl-1-imidazolidinyl)-penicillanic acid] (1). Preliminary studies were reported earlier (2).

EXPERIMENTAL[1]

Ampicillin trihydrate, potassium ampicillin, hetacillin, and potassium hetacillin were obtained from a commercial source[2]. Solutions of the penicillins were prepared in water at 10% w/v concentration and stored in ground-glass-stoppered flasks in darkness at room temperature. At intervals, the pH's of the stock solutions were measured, and 250-μl. aliquots were subjected to gel filtration on columns (43 × 1.8 cm.) of an acrylamide gel[3] with a stated exclusion limit of approximately 1800 daltons. The columns were equilibrated and eluted with 0.05 *M* K^+–PO_4^{-3} buffer, pH 7.4, or 0.05 *M* KBr. Elution patterns with either solvent were identical as detected by the absorbance at 270 nm. of eluant fractions. The latter solvent was necessary for those gel filtration runs whose fractions were examined by IR.

An estimation of polymer size was derived from a correlation of the ratio of absorbances at 1600 and 1765 $cm.^{-1}$ and the position in the elution pattern. These wavelengths (1, 3) correspond to the IR absorbances of the ionized carboxyl group and β-lactam structure, respectively. This estimation is based upon the assumption of a linear structure for the polymer with a terminal unit containing an intact β-lactam.

Assays for antimicrobial activity against *Escherichia coli* K12, a Gram-negative organism, were performed in broth cultures employ-

[1] IR spectral analyses were performed on KBr pellets of lyophilized material using the Perkin-Elmer 21. UV spectra were obtained with the Cary 14.

[2] Bristol-Myers Co., International Division.

[3] Bio-Gel P-2.

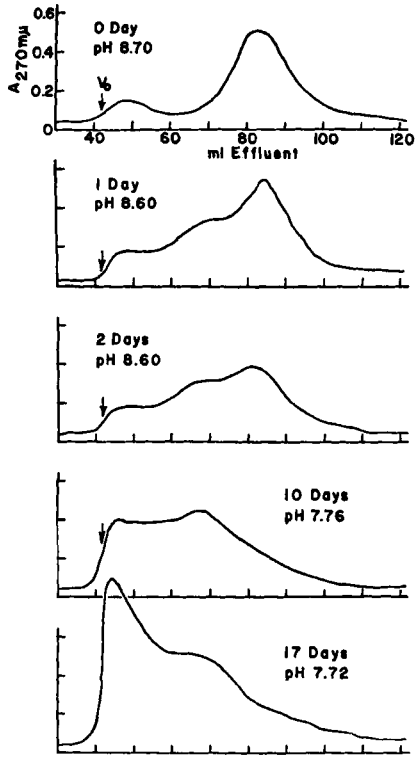

Figure 1—*Gel filtration of aged potassium ampicillin solutions. Ten-percent aqueous potassium ampicillin was passed through columns of acrylamide gel and eluted with 0.05 M K^+–PO_4^{-3}, pH 7.4; 3-ml. fractions were collected. Absorbances of fractions were measured with a spectrophotometer (Beckman DU).*

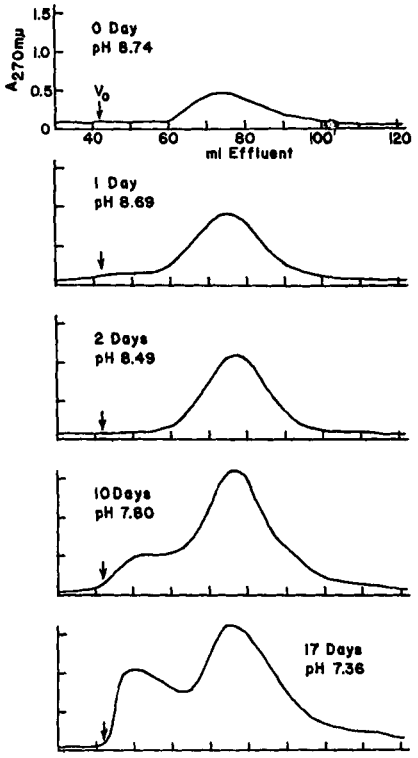

Figure 3—*Gel filtration of aged potassium hetacillin solutions. Ten-percent aqueous potassium hetacillin was passed through columns of acrylamide gel and eluted with 0.05 M K^+–PO_4^{-3}, pH 7.4; 3-ml. fractions were collected. Absorbances of fractions were measured with a spectrophotometer (Beckman DU).*

ing serial dilutions of selected fractions compared with the appropriate standards. Growth was measured turbidimetrically, and antimicrobial activity is expressed as a relative minimal inhibitory concentration compared to a potassium ampicillin standard.

RESULTS

Solutions of potassium ampicillin showed a rapid generation of higher molecular weight material (Fig. 1). Within 24 hr., UV-absorbing substances of a larger size appeared and continued to increase in size and concentration with time, at the expense of the initial potassium ampicillin. After 17 days, the location of the major absorbance was at the exclusion volume of the acrylamide gel column. This process was accompanied by a gradual decrease in pH of the stock solution. After aging for approximately 7 days, precipitates appeared and an increased viscosity was evident.

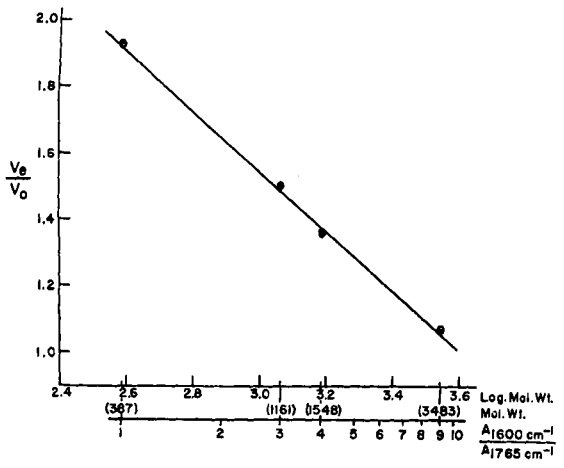

Figure 2—*Relationship of the relative elution position and the logarithm of the molecular weight of fractions from gel filtration runs of aged potassium ampicillin solutions. V_e/V_0 is the ratio of the elution volume and the exclusion volume. The logarithm of the molecular weight is derived from the ratio of the ionized carboxyl to the β-lactam absorbances in the IR (see text for the rationale).*

Variable amounts of heavier material were found in the fresh solutions of several lots of the potassium salt (Fig. 1). Removal of the leading peak material was accomplished in the case of the lot referred to in Fig. 1 by isoelectric precipitation of ampicillin at pH 4.6, filtration of the solid, and resolution with an equivalent of KOH to form the potassium salt. This material gave only one peak when passed through the acrylamide gel column. However, upon aging, it gave a peak distribution pattern identical to the untreated material. A commercial source of "purified" sodium ampicillin[4] (4) also showed only a single peak upon initial solution. With aging, it too showed a peak distribution very similar to that already described. This ampicillin had been passed through a column of Sephadex G-25 to remove high molecular weight materials.

IR analyses were run (with KBr) on selected fractions of potassium ampicillin eluted from the acrylamide gel columns. A decrease at 1765 $cm.^{-1}$ (β-lactam) was found as the molecular weight of the fraction contents increased. If the assumption is made that the material of increasing size is due to a linear polymerization of ampicillin units with the opening of individual β-lactam bonds in all but the terminal unit, then a regular increase in the ratio of the ionized carboxyl to the β-lactam absorbances would be expected. Figure 2 is a plot of the relative elution position and the ratio of ionized carboxyl to β-lactam absorbances. In the case of the monomer, this ratio is close to 1. Thus, a fraction showing a carboxyl to β-lactam absorbance ratio of 2 would be the dimer, a ratio of 4, the tetramer, *etc.* This assignment gives an estimate of the average molecular weight of the material in the fraction. A linear relationship was found (Fig. 2) between the relative elution position and the logarithm of the molecular weight. This finding supports the initial assumption of polymer formation of ampicillin units.

[4] Penbritin.

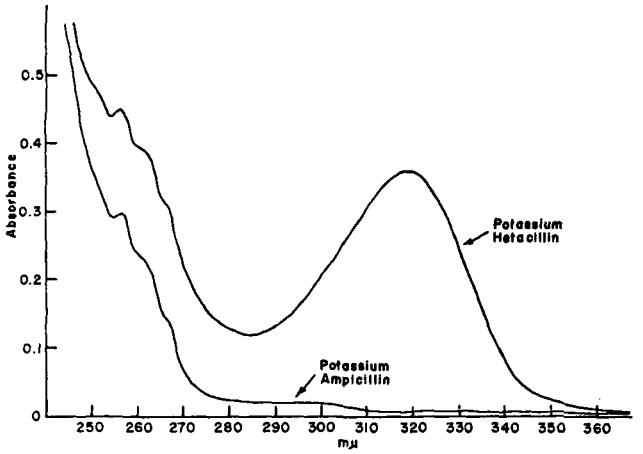

Figure 4—*UV absorption spectra of 2-day-old solutions of 10% potassium hetacillin and potassium ampicillin.*

Antimicrobial activity measurements of column fractions showed monomeric ampicillin to have the same activity per unit of 270 nm. absorbance as the ampicillin standard. This value of full activity was maintained through 2 days of aging with less than 20% loss of activity on the 3rd day. The higher molecular weight fractions corresponding to tetramers or larger showed less than 2% of the specific antimicrobial activity as compared to the ampicillin standard.

In Fig. 3, the comparable aging process exhibited by solutions of potassium hetacillin is illustrated. As was seen with the ampicillin, the pH of the stock solution gradually decreased. Unlike the results with potassium ampicillin, larger size materials made their appearance only after more than a week. After 17 days, most of the UV absorbance remained at the initial elution position. These observations suggest that potassium hetacillin does not form polymeric compounds as readily as does potassium ampicillin under the same conditions[5]. However, although potassium hetacillin retained its original elution position, the absorbance of the single peak was found to increase with time.

Figure 4 gives the UV spectra of 2-day-old stock solutions of potassium hetacillin and, for comparison, potassium ampicillin. The increased absorbance at 270 nm. previously noted can be accounted for by these results. Fresh solutions of both compounds gave a spectrum identical to that shown here for the 2-day-old potassium ampicillin. Aging of potassium hetacillin led to a distinctive absorbance at 317 nm. within 2 days, while none of the ampicillin fractions gave this peak even after 17 days. In fact, the aging process does not alter the ampicillin spectrum except for a small general increase in absorbance in the 280–290-nm. region.

DISCUSSION

The alkali salts of ampicillin and hetacillin in aqueous solutions exhibit pronounced alterations within 1 day. The appearance of higher molecular weight compounds separable by gel filtration indicates that ampicillin readily forms polymers. IR analyses strongly suggest that β-lactam opening is involved in the polymerization process. Polymers with up to nine ampicillin residues have been demonstrated early in the aging process. It is a likely suggestion that the polymers are linear combinations between the opened β-lactam carboxyl group and the free primary amino group of the phenylglycine moiety of another molecule. Similar polymeric forms have been shown for the potassium salt of 6-aminopenicillanic acid at neutral pH (6).

On the other hand, potassium hetacillin shows a rapid generation of a UV-absorbing material, with a characteristic extinction at 317 nm. Materials with similar UV spectra have been noted for various members of the penicillin family (7, 8) and have been identified as the penicillenic acids. It appears likely that hetacillin gives rise initially to its corresponding penicillenic acid. Polymer formation is inhibited because the primary amino group is blocked through its incorporation into the imidazolidinone structure. Formation of the penicillenic acid from hetacillin might occur by a mechanism similar to that ascribed to the formation of the penicillenic acid derived from ampicillin, *i.e.*, *via* a blocked amino group. The latter reaction has been suggested as an analytical tool for the quantitative determination of ampicillin (9, 10). In the case of hetacillin, the imidazolidinone ring might rearrange to free the amide nitrogen for oxazolone formation and retain the primary amine in a blocked state as a Schiff base.

It appears clear that the salts of ampicillin and hetacillin have different stability characteristics in aqueous solution. The solutions give signs of rapid chemical changes which implicate polymer formation in the case of ampicillin and rearrangement to a penicillenic acid in the case of hetacillin. Additional investigations are required to elucidate the significance of these reactions as they may occur in biological systems.

[5] It was recently reported (5) that salts of ampicillin gave more than twice the amount of polymeric substances than those of hetacillin after 14 days.

SUMMARY

Ten-percent solutions of potassium ampicillin in water showed the production of higher molecular weight materials within 24 hr. Polymers of up to at least nine ampicillin residues were demonstrated by IR and gel filtration analyses. These polymers are most likely formed by the reaction of a primary amino group of one molecule with the β-lactam carboxyl of another molecule. Monomeric ampicillin retained full antibacterial activity after having been passed through an acrylamide gel column. Polymers separated from aged ampicillin solutions by gel filtration were found to be devoid of antibacterial activity.

Potassium hetacillin treated in the same manner as potassium ampicillin did not show the rapid production of polymers but instead showed the production of material having an absorption peak at 317 nm. This substance is probably the penicillenic acid.

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