Chromatography of Penicillins, Penicilloates, and Penicilloylamides on Dextran Gels

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The factors influencing the chromatographic behavior on dextran gels of penicillins and their derivatives were investigated by comparing elution profiles and partition coefficients (K_D and K_{AV}) of penicillins differing in side-chain structure and among penicillin derivatives of identical side-chain but different nuclear structure. Under the conditions of pH and ionic strength employed (pH 7.4, 0.145 M NaCl, 0.05 M PO_4), side-chain adsorptive effects best explained the anomalous behavior of benzylpenicillin and of oxacillin and its chlorine-substituted analogues. Polar side-chain substituents, such as the amino group of ampicillin and the carboxyl group of carbenicillin, and cleavage of the β -lactam ring, exemplified by penicilloates and penicilloylamines, both appeared to interfere with side-chain-directed adsorption. The differential adsorption of penicillins and their derivatives to dextran gels is not only of theoretical interest relative to the mechanism of chromatography but of practical application to analytical and preparative procedures in penicillin chemistry.

Dextran gels have been used by a number of workers to study penicillin polymerization and the products of reacting penicillins with larger molecules.

Stewart (29) and Schneider and de Weck (26) used highly cross-linked gels to separate penicilloylated proteins from unconjugated penicillin, and Schneider and de Weck (24, 25) used the same method to separate unreacted monomer penicillin derivatives from penicilloylated polylysines and penicilloylated carbohydrates. Other workers have employed gel filtration chromatography to demonstrate polymerization of penicillins in aqueous solutions (4, 8, 11, 27, 28, 30, 31). In the course of their studies, some data on chromatography of monomer penicillins was accumulated, but no systematic examination of the factors influencing their chromatographic behavior has been published. Recently, Murakawa et al. (22) have compared the binding of different penicillins to proteins with their gel binding characteristics.

Our own interest in the chromatographic behavior of penicillins arose in the course of studies on the reaction products of penicilloylation and penicillanylation of proteins with radio-labeled penicillins (N. E. Hyslop, Jr., R. J. Milligan, and J. F. Calvert, manuscript in preparation). During the chromatographic sep-

aration of conjugated proteins on Sephadex G-25, we observed that the elution volume, V_e , of penicilloic acid, the major side product of the penicilloylation reaction, differed substantially from its precursor of nearly identical molecular weight. Furthermore, the order of elution of the penicillin precursor, 6-aminopenicillanic acid (6-APA), and of two penicillins of different side-chain structure, benzyl penicillin or penicillin G (PG), and oxacillin (OXA), was in inverse relation to their molecular weights. The penicillins and penicillin derivatives studied are shown in Fig. 1.

In this paper, we describe the chromatographic behavior on Sephadex G-10 and G-25 of dilute solutions of representative penicillins and their penicilloic acid and penicilloylamide derivatives. Chromatography was conducted under conditions of moderate ionic strength, which excluded Donnan salt exclusion effects, and neutral pH. The results suggest that the anomalous K_D values observed for penicillins differing in side-chain structure are primarily due to differences in side-chain adsorptive effects (13, 18). The reduction in K_D values of penicilloylamide and penicilloic acid derivatives of individual penicillins is probably due to inhibition of side-chain adsorbance resulting from opening of the β -lactam ring. The molecu-

FIG. 1. Chemical structures of representative penicillins and cephalosporins. Both families of compounds contain a β -lactam ring. The second ring in the penicillin nucleus is a five-membered thiazolidine ring and in the cephalosporins is a six-membered thiazine ring. Both have side chains attaching to the β -lactam ring (R_1 -substituents). Cephalosporins with biological activity also have R_2 side chains attached to the thiazine ring. Factors influencing the chromatographic behavior of penicillins presumably also apply to cephalosporins.

lar size of penicilloyl amides is further increased by adding an R_2 side chain.

Chromatography of penicillin reaction products on highly cross-linked dextran gels may offer significant quantitative and preparative advantages over standard paper and silica gel analytical techniques. Moreover, in our hands it has proved to be especially useful in analyzing radiolabeled derivatives designated for animal or human studies.

MATERIALS AND METHODS

Radiolabeled [^{35}S]potassium benzyl penicillin ([^{35}S]PG-K, 3.136 $\mu\text{Ci}/\text{mg}$), [^{35}S]labeled sodium oxacillin ([^{35}S]OXA-Na, 2.302 $\mu\text{Ci}/\text{mg}$), and [^{35}S]6-APA (5.322 $\mu\text{Ci}/\text{mg}$) were gifts from Bristol Laboratories, Syracuse, N.Y. Structural formulas are shown in Fig. 1. Carbon-14-potassium benzyl penicillin, [^{14}C]PG-K, of three separate batches (batches 24, 29, and 31, each with 28.6 mCi/mmol) and [^{35}S]H₂SO₄ (50 $\mu\text{Ci}/\text{ml}$) were purchased from Amersham-Searle, Chicago, Ill. Tritiated water (^3H -labeled water, 3.07 $\times 10^6$ dpm/g) was purchased from Packard Instruments, Downers Grove, Ill. Epsilon-amino-*n*-caproic acid (EACA) was

purchased from Calbiochem, San Diego, Calif. Radiolabeled penicillins were stored in the dry state at 5 $^{\circ}\text{C}$; fresh solutions were made for all experiments unless otherwise indicated.

Penicillin derivatives of two types were prepared by standard methods from each of the three penicillins studied.

Radiolabeled penicilloyl amides (Fig. 2) were prepared with EACA in 25% or 10 molar excess by the usual method (20, 26) at pH 11.5 with reaction periods of either 2 or 24 h. All penicilloyl-EACA derivatives were at a final concentration of 10 $\mu\text{mol}/\text{ml}$ after reacting 0.125 mmol of EACA (16.4 mg) in 10 ml of saline buffer, adjusted to pH 11.5 with N NaOH, with 0.10 mmol each of [^{35}S]PG-K (37.2 mg), [^{35}S]OXA-Na (42.3 mg) and [^{35}S]6-APA (21.6 mg). Penicilloyl amides of EACA are abbreviated as PG_o-EACA, etc.

Radiolabeled penicilloic acids (Fig. 2) at final concentrations of 10 $\mu\text{mol}/\text{ml}$ in saline buffer were prepared from precursor penicillins at the same concentration by alkaline hydrolysis at pH 11.5 for 24 h (21). At completion of the reactions, both penicilloic and EACA-penicilloyl derivatives were readjusted to pH 7.4 with N HCl. Penicilloates are abbreviated as PG_o-ate.

Radiolabeled intact penicillins were diluted in saline buffer for chromatography to concentrations of 10 $\mu\text{mol}/\text{ml}$ for ^{35}S isotopes and 20 nmol/ml for ^{14}C isotopes since the latter had substantially greater specific activity.

Unlabeled intact penicillins were diluted in saline buffer at a concentration of 1 mg/ml; 1-ml samples were chromatographed on the same columns as used for radiolabeled penicillins. Effluents were monitored for absorbance at 230 nm.

The eluant buffer, phosphate-buffered saline (PBS) (0.005 M in PO₄, 0.145 M in NaCl, pH 7.4 before any adjustment), was used in solubilizing all penicillins and conducting reactions used to prepare derivatives. At this ionic strength penicillins are

FIG. 2. Alkaline hydrolysis of penicillins. Hydrolysis of the β -lactam ring occurs rapidly at alkaline pH, forming the penicilloic acid derivative. In the presence of available amino groups, a competitive reaction ensues between hydrolysis and formation of the penicilloyl amide derivative. In the experiments reported here, the penicilloylamides were made with ϵ -aminocaproic acid.

prevented from precipitating, and the pH chosen corresponds to the range of greatest stability for the intact molecules.

In column chromatography, Sephadex G-25 Fine and G-12 (A. B. Pharmacia, Uppsala, Sweden), of particle size 20 to 80 and 40 to 120 μ m, respectively, were used as the stationary phase. The dextran was swelled in PBS at 100 C in a boiling water bath. The supernatant was decanted after the bulk of the gel had settled. Decantation was repeated twice to remove fine particles. The gel was packed into a glass column of cross-sectional areas of either 3.14 or 3.80 cm^2 . Gel bed height was usually 35 cm, the exact height being recorded during experiments. Columns were used for periods of up to 6 months. No preservatives were used. All fractionations were conducted at room temperature (23 ± 2 C).

Before sample application, a freshly packed column was eluted with 1 liter of buffer. Sample volumes of 1.0 ml were applied at the top of the gel and were washed in with two 1.0-ml portions of buffer before reconnecting the buffer reservoir (9). Columns were eluted with PBS at the rate of 14.5 ml/h, although rates up to at least 30 ml/h did not alter chromatographic behavior. The effluent was collected in 0.5- or 1.0-ml volumes on an ISCO model A fraction collector (Instrumentation Specialties, Lincoln, Neb.).

Fractions were analyzed for counts per minute (cpm), and results were correlated with elution volumes by identifying the V_e as the fraction containing the greatest cpm. Elution volumes were reproducible on multiple columns poured at different times over a 1-year interval. Counting was performed in a Packard Tri-Carb Liquid Scintillation Counter, model 3320 (Packard Instruments, Downers Grove, Ill.). Column fractions were decanted into glass vials (Packard, no. 6001009), and 10.0 ml of Instagel was added (Packard, no. 6002174). The exact counting efficiencies established were 75% for ^{35}S , 84% for ^{14}C , and 25% for ^3H . Sample recovery averaged 97%.

RESULTS

The results of chromatographic experiments are expressed in representative elution profiles and in terms of the standard distribution coefficients K_D (1, 13, 14) and K_{AV} (19). Calculations of K_D and K_{AV} values for compounds chromatographed on individual columns and gels were based on derivations of the expressions shown in equations 1 and 2:

$$V_e = V_o + K_D V_i \quad (1)$$

$$V_e = V_o + K_{AV} (V_i - V_o) \quad (2)$$

where V_e is the elution volume, V_i is the internal volume, V_o is the void or external volume, and V_t is the total volume (gel phase [V_x] and liquid phase [V_o]).

For each new column, the value of V_i , the volume of unbound solvent internal to the gel phase, was established either directly with tritiated water (1, 14) or indirectly from the V_e for

$[^{35}\text{S}]\text{H}_2\text{SO}_4$, which for each particular gel type bore a constant relationship to the V_e of ^3H -labeled water. The void volume (V_o) consisting of the total volume of liquid external to the gel phase was measured with blue dextran in a 0.2% solution.

Following the model of Laurent and Killander, where the total volume of the gel bed, V_t , is regarded as consisting of two phases, one gel phase with the volume V_x and one liquid phase with the volume V_o (the void volume, measured in the usual way), V_i was calculated from direct measurements of the height of the bed volume and the internal diameter of the column. The calculated value of V_i agreed with actual volumetric determinations when performed (32).

The elution position of $[^{35}\text{S}]\text{H}_2\text{SO}_4$, an interacting solute, is shown in all representative elution profiles. For each column, its position was reproducible and its elution profile symmetrical, due to use of an eluent of physiological ionic strength and pH (23). The V_e for $[^{35}\text{S}]\text{H}_2\text{SO}_4$ serves to mark the following K_D and K_{AV} positions: for G-10, K_D 0.419 and K_{AV} 0.120; for G-25 F, K_D 0.563 and K_{AV} 0.384.

The K_D values obtained by us for seven penicillins and their derivatives are based on "limiting elution volumes" (23) as indicated by the lack of influence of higher eluent salt concentrations on elution position or profile. The profiles were all reasonably symmetrical, and the elution positions highly reproducible. Fractional differences in K_D values would have been improved by employing a larger column to enhance the actual volumetric differences. However, K_D values did not vary greater than ± 0.08 on replicate chromatograms of individual solutes on different columns at different times.

The concentration of solute chosen was deliberately low to avoid possible complex formation or polymerization and to take advantage of radioisotope labeling. The solvent electrolyte concentration and pH chosen were those of biological fluids. The amount of electrolyte in buffers was greater than the minimum necessary to prevent electrostatic interactions between solute and gel, as confirmed by identical K_D values and elution profiles for $[^{35}\text{S}]\text{H}_2\text{SO}_4$ in 0.15 M and 1.0 M NaCl buffers (23).

The pH value chosen was in the pH range of maximal stability for the penicillins, which are especially susceptible to hydrolysis of the β -lactam ring in solution. Alkaline pH values greater than 10 rapidly convert penicillins to their penicilloic acid derivatives. Acid pH values reduce aqueous solubility of some penicillins. With the exception of a study of OXA at pH 6.0, we did not examine the influence of pH on

chromatography of penicillins. K_D values were identical for OXA chromatographed at pH 6.0 and 7.4, indicating that pH difference in this range had no significant influence on chromatography of oxacillin.

Apparent partition coefficients of intact penicillins. Chromatography of radiolabeled and unlabeled intact penicillins was conducted on Sephadex gels G-10 and G-25F. The radiolabeled penicillins, PG and OXA, and the penicillin precursor, 6-APA, were compared with unlabeled penicillins whose structures resembled either PG (ampicillin [AMP] and carbenicillin [CARB]) or OXA (cloxacillin [CLOX] and dicloxacillin [DICLOX]). Representative elution profiles for samples chromatographed on G-10 and G-25 are shown in Fig. 3 and 4, respectively.

Partition coefficients were calculated from elution volumes (V_e). Table 1 presents comparative partition coefficients on BioGel P-2 and acid-treated Sephadex G-10 and also contains averaged K_D values derived from individual experiments with radioisotopes on G-10 and G-25F. Table 2 summarizes the partition coefficients determined for both radiolabeled and unlabeled penicillins on Sephadex G-10.

When ranked by relative order of elution appearance (Table 2), it was apparent that differences in molecular weight were not correlated with elution position. However, side-chain structure was correlated with chromatographic behavior as reflected in two types of behavior relating to side chains: AMP and CARB were the only side-chain-bearing penicillins tested whose K_D values were <1 ; and PG, OXA, CLOX, and DICLOX each had $K_D > 1$ and $K_D - G-10/K_D - G-25 > 1$. Acid treatment of G-10 gel did not alter the partitioning of any of the three penicillins examined. The polyacrylamide gel BioGel P-2 did not partition penicillins as effectively as did dextran gels.

Apparent partition coefficients of penicillin derivatives: penicilloic acids and penicilloylamides. The common nuclear structure of the penicillin precursor, 6-APA, and two representative penicillins differing in side-chain structure, PG and OXA, was altered in order to examine the influence of nuclear structure on the chromatographic behavior of intact penicillins. The penicilloic acid (e.g., PG_o-ate) and penicilloylamide (e.g., PG_o-EACA) derivatives of each of the radiolabeled penicillins were chromatographed, and partition coefficients

FIG. 3. Elution profiles on G-10 of penicillins bearing different side chains. An equimolar mixture ($\square$) of radiolabeled (^{35}S) 6-APA ($\bullet$), PG-K ($\blacksquare$), and OXA-Na ($\blacktriangle$) was fractionated (A) on the same column used to study the elution of the individual penicillins (B). K_D 0.419 is marked by the V_e of [^{35}S]H₂SO₄ ($\times$).

FIG. 4. Elution profiles on G-25F of penicillins with identical side chains and differing nuclear structure: PG, PG_o-ate, and PG_o-EACA. Native radiolabeled (³⁵S) benzylpenicillin, PG (●), and its penicilloic (○) and penicilloylamide derivatives (■) were chromatographed. K_D 0.563 is marked by the V_e of [³⁵S]H₂SO₄ (—). Note that some of the PG has decayed while in solution for several days. The difference between the V_e of PG_o-ate and PG_o-EACA is significant and reproducible but is smaller than on G-10 (see Fig. 5).

TABLE 1. Apparent partition coefficients for ³⁵S radiolabeled penicillins and selected penicillin derivatives on Sephadex G-10^a, G-25F^a, and BioGel P-2^c

Penicillin	Derivative	Average K _D values			Average K _{AV} values			
		G-10	G-10 (acid treated ^b)	G-25	G-10	G-10 (acid treated ^b)	G-25	BioGel P-2 ^c
6-APA	Intact	0.501	0.501	0.965	0.214	0.214	0.655	ND
	Penicilloate	0.233	ND ^d	0.900	0.093	ND	0.621	ND
	Penicilloylamide	0.133	ND	0.791	0.053	ND	0.531	ND
PG-K	Intact	1.58	1.58	1.22	0.652	0.652	0.820	0.230
	Penicilloate	0.267	ND	0.845	0.107	ND	0.580	0.100
	Penicilloylamide	0.168	ND	0.783	0.065	ND	0.531	0.080
OXA-Na	Intact	2.90 ^e	2.90	1.37	1.20 ^e	1.20	0.935	ND
	Penicilloate	0.567 ^e	ND	1.00	0.233 ^e	ND	0.680	ND
	Penicilloylamide	0.467	ND	0.982	0.186	ND	0.667	ND

^a Eluted with 0.145 M NaCl, 0.05 M PO₄, pH 7.4.
^b Acid-treated gel; elution with PBS, pH 7.4.
^c 200 to 400 mesh.
^d ND, Not done.
^e K_D values were identical when oxacillin and its penicilloic acid were eluted with 0.145 M NaCl, 0.05 M PO₄, pH 6.0.

were derived from elution volumes. Representative elution profiles on G-10 and G-25F are shown in Fig. 5 and 6. Average partition coefficients from individual experiments on G-10 and G-25F are presented in Table 1. The relative elution order of these derivatives on G-10 and their molecular weights and structures are compared with intact penicillins in Table 3.

From these data it is apparent that opening the β -lactam ring to form the penicilloic acid

TABLE 2. Experimental K_{AV} and K_D values for several intact penicillins at pH 7.4^a on highly cross-linked dextran gel (Sephadex G-10)

Penicillin	K_D	K_{AV}
CARB ^b	0.400	0.160
6-APA ^c	0.501	0.214
AMP ^b	0.750	0.302
PG ^c	1.58	0.652
OXA ^c	2.90	1.20
CLOX ^b	4.80	1.95
DICLOX ^b	9.30	3.70

^a Eluted with PBS, pH 7.4.

^b V_e determined from monitoring optical density at 230 nm; 1.0 mg/ml concentration of penicillin in PBS, pH 7.4; 1 ml of sample applied.

^c Radioisotopic study.

derivative reduces partition coefficients. Further reduction is observed when a five-carbon R_2 -side chain is added as a result of penicilloylating EACA.

Use of gel chromatography to analyze purity and stability of radiolabeled penicillins.

The stability and purity of commercially available [¹⁴C]PG was compared with custom-made (Bristol) ³⁵S-labeled PG, OXA, and 6-APA. Purity was analyzed by determining the number and K_D values of peaks visualized on gel chromatography with either G-10 or G-25. Sephadex G-10 was more reliable for exact identity of the breakdown compounds on the basis of specific K_D values. The proportions of intact and denatured penicillins were determined by integrating the areas beneath the peaks. Penicillins allowed to stand in aqueous solution at neutral pH and physiological ionic strength developed a second elution peak; the K_D value of this peak was identical to that of alkalized penicillin and therefore was assumed to be penicilloic acid. Although the chromatography of other degradation products (penicillenes, penillics, and penilloics) was not investigated, their different structure would likely result in major differences in partition

TABLE 3. Chemical characteristics of penicillins and relative order of K_D values

Mol wt (as acid)	Ordered by increasing side-chain complexity	Relative elution position on G-10 ^a	K_D Value ^a (G-10)	Chemical characteristics									
				Side chain						Nucleus			
				π Electron-rich substituents		Substitution of benzyl		Ionizable substituent		Ionizable groups			
				Ben-zyl	Meth-oxazol	Halo-genation (chlorine)	Meth-oxyla-tion	—NH ₂	—COOH	—NH ₂	>NH	H O —N—C	—COOH
216	6-APA	7	0.501							1			1
234	6-APA _o -ate	3	0.233							1	1		2
347	6-APA _o -EACA	1	0.133							1	1		2
334	PG	11	1.58	1									1
352	PG _o -ate	4	0.267	1							1		2
465	PG _o -EACA	2	0.168	1							1	1	2
348	AMP	9	0.750	1				1					1
374	CARB	5	0.400	1					1				1
396	METH	10	1.2 ^b	1			2						1
389	OXA	12	2.90	1	1								1
407	OXA _o -ate	8	0.567	1	1					1			2
520	OXA _o -EACA	6	0.467	1	1					1		1	2
423	CLOX	13	4.80	1	1	1							1
457	DICLOX	14	9.30	1	1	2							1

^a Observed in these experiments.

^b Approximate, inferred from relative V_e reported by Murakawa et al. (22).

coefficients when compared to the intact penicillin.

^{35}S radioisotopes had been supplied as powders after crystallization from solution and were of sufficient amounts to allow weighing of dry solute for each experiment.

^{14}C penicillins were supplied as approximately 1 mg of dry powder, which necessitated solution, repackaging, and storage to be available for multiple experiments. Therefore, the effect of storage conditions on stability was analyzed by comparing elution profiles of material stored as dried powder at -20°C after lyophilization or as a dilute aqueous solution frozen at -70°C .

The purity of ^{35}S radioisotopes remained constant at $>99\%$ over a 2-year period. However, as indicated in Table 4, under conditions of either lyophilization or freezing of aqueous solutions, storage of ^{14}C PG was associated with extensive degradation to the penicilloic acid derivative, although initial purity was 95 to 98%. Subsequent experiments suggest that storage at -70°C in the nonpolar solvent dimethylformamide will stabilize dilute solutions of the ^{14}C PG isotope for at least 7 months (Table 4).

Analysis of penicilloylation reaction by gel chromatography of reaction products.

The efficiency of the penicilloylation reaction was studied by chromatographing the products of the reaction and analyzing the number of peaks, their K_D values, and the area beneath the peaks (Fig. 5, 6, and 7). This was the only means for examining the efficiency of the penicilloylation of EACA with 6-APA, as neither 6-APA_o-ate nor 6-APA_o-EACA reacts with *p*-hydroxymercuribenzoate to form the penamaldate, in contrast to comparable derivatives of PG and OXA (26).

When penicilloylamides have substantially different K_D values from the penicilloic acid derivative, this method allows quantitative analysis of reaction efficiency. Figure 7 demonstrates its application to the analysis of penicilloylation of EACA with 6-APA. The identification of the reaction products formed can be improved by chromatographing the reaction products on G-10 instead of G-25, where there is a greater difference in K_D values of the penicilloic acid and the penicilloylamides and by using longer columns to enhance the separation of the derivatives.

TABLE 4. Effect of mode of storage on three commercial preparations of ^{14}C PG-K; analysis by chromatography on G-25 F^a

Preparation	Procedure	Date	Results of chromatography				Calculated purity ^b	
			Peak 1		Peak 2		% Penicillin (PG-K)	% Penicilloic acid (PG _o -ate)
			K_{AV}	K_D	K_{AV}	K_D		
Batch 24	Synthesis	9/71					98	
	Solubilized in water; samples frozen at -20°C ; sample thawed	11/72	0.592	0.855	0.840	1.23	95	5
	Sample thawed	1/73	0.592	0.855	0.840	1.23	93	7
	Sample thawed	6/73	0.592	0.855	0.838	1.17	55	45
Batch 29	Synthesis	12/72					98 ^c	
	Solubilized in water; samples lyophilized	1/73						
	Resolubilized and fractionated	5/73	0.592	0.855	0.841	1.24	65	35
Batch 31	Synthesis	6/73					98 ^c	
	Solubilized in DMF ^d ; samples stored at -70°C	8/73						
	Chromatographed in buffer ^a	8/73	0.592	0.855	0.840	1.23	98	2
		12/73	0.593	0.855	0.840	1.23	94	6
		3/74	0.593	0.855	0.840	1.23	94	6

^a Eluted with 0.145 M NaCl, 0.05 M PO_4 , pH 7.4.

^b Composition calculated by integrating areas of elution profiles.

^c Commercial specifications based on microbiological assay of potency and chromatography.

^d Dimethylformamide.

FIG. 5. Elution profiles on G-10 of penicillins with identical side chains and differing nuclear structure. Individual chromatographs of the same derivatives of PG that were shown chromatographed on G-25F in Fig. 4 are shown here as a composite. ^3H -labeled water (—) marks the $K_D = 1$. The PG-O-EACA derivative (■) was made by alkalizing PG-K (●) in the presence of a 25% molar excess of EACA. PG-O-ate (○) was made by alkaline hydrolysis.

DISCUSSION

Penicillins are especially interesting probes for analyzing the mechanism of dextran gel chromatography of small molecules because in their intact form the penicillins are organic acids of common heterocyclic nucleus and varying side-chain structure. A rich array of side-chain differences is found in the commercially available penicillins, and derivatives can be prepared in which only the nuclear structure is altered, as represented by the penicilloates. As observed in these experiments and those of Murakawa and colleagues (22), the penicillins and their derivatives do not behave identically in dextran gel chromatography. Each of the penicillins examined had a unique partition coefficient, which was not predictable from its molecular weight, and the penicilloic and penicilloylamide derivatives which were examined differed substantially from their precursor in their partition behavior.

Although the behavior of penicillins in dextran gel chromatography may be explained on several grounds, the phenomena observed ap-

pear to have predictive value for the behavior of penicillins in other interactions, notably protein binding (22). Recent studies by Hsu et al. (16) suggest that the interacting site on proteins is hydrophobic in character and that differences in binding constants are related to the hydrophobic or hydrophilic nature of substituent functions on the penicillin side chain.

The partitioning of penicillins in dextran gel chromatography could be based on this same property of relative hydrophobia. This characteristic can be assigned a numerical value by determining partition coefficients of penicillins in octanol-water mixtures, followed by calculation of Hansch constants (15). According to Hansch and Steward (15) the partitioning of penicillins between aqueous and nonaqueous phases may be predicted from these values, which reflect the relative "lipophilic" character of the molecule. Although the Hansch constant is an attractive method for rationalizing the biological activity of penicillins (5), sufficient data is not presently available to determine how well Hansch constants correlate with the partitioning of penicillins in dextran gel chromatog-

raphy. Published data consist only of calculated values for phenoxyphenicillins, derived from substituent constants of model compounds (15). However, summing of substituent constants does not always correctly predict the value for the whole molecule, as group interactions may occur which are not possible in the parts (17). Therefore correlation of behavior of penicillins in dextran gel chromatography with their respective Hansch constants must await their direct measurement for the penicillins, penicilloic acids, and penicilloylamides examined in this study.

The behavior of penicillins, penicilloic acids, and penicilloylamides in liquid chromatography on dextran gels can be explained in terms of concepts derived from chromatographic studies with other organic compounds (2, 3, 6, 10, 33). In these terms the behavior of any solute in liquid chromatography on dextran gels is a combination of molecular sieving and gel-solute interactions.

The behavior of noninteracting or "ideal" solutes is a function of steric hindrance to gel

permeation. The critical factor for the gel is average pore size and for the solute is molecular size, as expressed in terms of Stokes radii (1, 14).

The behavior of interacting or "nonideal" solutes is termed anomalous behavior (13). Nonideal or anomalous behavior is characterized by elution positions and profiles that would not be predicted from considerations of steric hindrance to gel penetration as imposed by solute molecular size and average pore size of a specific gel. Anomalous behavior is therefore recognizable by the occurrence of either premature or delayed appearance of V_e , as compared to a noninteracting standard of identical molecular size. Another manifestation is asymmetry of the elution profile. Both phenomena may occur together. K_D values reflect elution volumes, and symmetry of the elution profile is tested by dividing the area in front (F) of the V_e by the back area (B). When symmetry exists, $F/B = 1$ (23). Elution profiles of the compounds studied were reasonably symmetrical.

By definition the solvent equilibrates com-

FIG. 6. Elution profiles on G-25F of oxacillin derivatives: composite diagram. OXA (●), OXA_o-ate (○), and OXA_o-EACA (■) were chromatographed individually. [³⁵S]H₂SO₄ (—) indicates $K_D = 0.563$. OXA_o-EACA was made in the presence of a 25% molar excess of EACA; a greater difference in elution position between OXA_o-EACA and OXA_o-ate was obtained on G-10 (see Tables 1 and 3).

FIG. 7. Use of chromatography to study penicilloylation of EACA by 6-APA. Penicilloyl and penicilloate derivatives of 6-APA do not form the penamaldate. Therefore, the efficiency of 6-APA penicilloylation was studied by chromatographing ^{35}S -labeled products of the reaction conducted with EACA at 25% molar excess (■) and at 10 molar excess (□). Elution profiles of reaction products were compared with the elution profiles of the penicilloate derivative, penicilloic acid, or 6-APA₀-ate (○), and native 6-APA (●), as shown in this composite figure. Unlike penicilloylation with PG (Fig. 5) or OXA (Fig. 6), a 25% molar excess of EACA was insufficient to prevent formation of significant amounts of penicilloate. The similar K_D values of 6-APA and 6-APA₀-ate on G-25 are evidence of the dominance of gel filtration in their chromatography. Note that in comparison to native PG and OXA, their penicilloates have reduced K_D values due to interference with side-chain adsorption resulting from β -lactam ring cleavage.

pletely with the liquid phase of the gel-liquid system. Therefore the K_D for the solvent is 1, and noninteracting solutes should have a K_D of <1 . When solutes interact with the gel so as to retard elution beyond the solvent elution volume, the K_D of the solute will be >1 . K_D values of >1 therefore indicate anomalous behavior, as was observed for several intact penicillins on both dextran gels but not on polyacrylamide gels.

Gel-solute interactions can also produce premature elution, in which case K_D will be <1 and could be misinterpreted as indicating no interaction. To recognize when $K_D <1$ represents anomalous behavior it is necessary to calibrate the system with an "ideal" or noninteracting standard solute of identical molecular size. By identifying anomalously low K_D values, ion exclusion effects and the presence of complexes and polymers can be recognized (13, 14, 18). In an analogous manner, Dennen (8) and

others have used K_{AV} of monomeric penicillins as a marker in searching for penicillin polymers.

Several types of interactions can occur between gel and solute which cause anomalous behavior.

Electrostatic repulsion may cause anomalously low K_D values and electrostatic attraction anomalously high K_D values. Electrostatic effects are due to ionized groups in the dextran (12), which exert maximal effect on charged solutes in deionized water (13, 18, 23). The K_D values of penicillins in deionized water are substantially reduced over K_D values observed in the presence of NaCl (22), presumably due to electrostatic repulsion attributable to the C₆ carboxyl and other ionizable substituents. Electrostatic effects can be abrogated by saturation of the ion-exchange sites with rather trivial amounts of NaCl incorporated into gel, solute, and eluting buffers, as was done in our experiments.

In the absence of electrostatic effects, it is apparent from our data and that of Murakawa et al. (22) that adsorption of penicillin to gel is the principal determinant governing the behavior of most intact penicillins on highly cross-linked dextran gels. Furthermore, these adsorptive effects are attributable to the side-chain structure, and when the nucleus is intact the side-chain structure can be used to predict relative elution positions and K_D values.

Direct evidence for the preeminence of adsorption as the factor governing the chromatographic behavior of penicillins is severalfold. First, nearly all penicillins tested exhibited anomalous behavior ($K_D > 1$) in the presence of sufficient electrolyte to abolish any electrostatic attraction. Exceptions were AMP and CARB. Second, for penicillins with $K_D > 1$, K_D values were enhanced rather than diminished on G-10 as compared to G-25, consistent with observations of greater adsorption with higher degrees of cross-linking (10). Third, K_D values were also enhanced by very high molarities of salt (22), also a consistent feature of adsorptive behavior (18).

Evidence pointing to the side-chain structure as responsible for the adsorption effects comes from several observations. First, all intact penicillins except AMP, CARB, and methicillin showed increasing K_D values with increasing side-chain complexity and molecular weight. Second, the increase in K_D value with increasing side-chain complexity was limited to penicillins whose additional substituents are either rich in π bonds (e.g., the methoxazol group) or where the substituent directly enhances the intrinsic adsorptivity of the benzyl ring. Thus, the addition of methoxy groups to the phenoxy group (methicillin) had a mild inhibitory effect (22), but the halogenation of the benzyl group markedly enhanced the K_D values of the methoxazol derivatives CLOX and DICLOX over the precursor OXA. These effects are consistent with the observations of Brook and Housley (3) on the resultant K_D values when phenol is substituted with methoxy groups or halogenated with chlorine atoms.

Adsorption is a property associated with highly cross-linked gels and is presumed to arise from interactions between solute and ether groupings in the gel (10). The greater the degree of cross-linking, the greater is the content of ether groupings and the extent of adsorption effects. Hydroxyalkylation of G-25 to produce LH-20 has a similar effect (10). The strength of the adsorptive interaction for substances in aqueous solutions may be enhanced by high

concentration of electrolyte (e.g., 3 M NaCl). Jansen (18) attributes enhancement of gel-solute interaction by electrolyte to a smaller layer of hydration surrounding the solute molecule.

Compounds exhibiting adsorbance characteristically have a rich π -electron structure, an extending system of double bonds, or a planar configuration. Each of these characteristics may be found in penicillins and, to varying degrees, penicilloic acids and penicilloylamides. Aromatic substituents, such as occur in side chains, regularly are adsorptive.

The adsorptive behavior of a molecule is strongly influenced by the presence of ionizable substituents and whether or not they are ionized (2, 6). Ionized substituents interfere with adsorbance contributions of other functions, such as the benzyl ring in derivatives of benzyl penicillin: the reduced K_D values of AMP and CARB, which differ from PG by an $-\text{NH}_2$ and a $-\text{COOH}$ function, respectively, illustrate this principle.

The mechanism by which ionizable substituents interfere with adsorptive phenomena is not completely clear. It is not due to simple ion exclusion since interference occurs in the presence of high concentration of electrolyte. Jansen (18) has suggested that ionization may cause rearrangement of π electrons. Enlargement of molecular size resulting from hydration of the ionized solute may also contribute to hindrance of gel-solute interactions, as shown for inorganic salts by Neddermeyer and Rogers (23) and exemplified by the low K_D of $[\text{S}^{35}]\text{H}_2\text{SO}_4$ in these experiments.

The reduced K_D values of penicilloic acid derivatives compared with their precursor penicillins can be explained as due to steric hindrance of access to adsorption sites in the gel. Two sources of increase in the effective molecular size of a penicillin are possible on hydrolyzing the β -lactam ring to form the penicilloic acid. Opening the β -lactam ring allows some independent free rotation of the sidechain and thiazolidine rings. Also, an ionizable COOH function is substituted for the C_7 , $\text{C}=\text{O}$ and adds its own hydration requirements to those of the other ionizable groups on the molecule. It is not known whether or not this structural change influences the π -electron structure of the side-chain functions to reduce adsorptivity. The further reduction in K_D value of the penicilloylamides PG_0 -EACA and OXO_0 -EACA over PG_0 -ate and OXA_0 -ate suggests that steric hindrance is the predominant factor in opposing adsorption, since the penicilloylamides are only

different in net change from the penicilloates by

virtue of the C_7 $\begin{array}{c} O & H \\ || & | \\ -C & -N- \end{array}$ function but have larger effective molecular size due to the seven-member R_2 side chain.

Additional evidence for steric hindrance of penicilloates comes from comparison of K_D values on G-10 and G-25. Although the intact penicillins that showed adsorptivity on G-25 showed greater adsorptivity on the more highly cross-linked G-10 gel, their penicilloic acid derivatives showed the greatest interference with adsorptivity on the same gel. These results suggest that the smaller pore size of the more highly cross-linked gel resulted in increased steric hindrance of solute access to the adsorptive sites.

An alternative explanation would be that greater cross-linking provides increased numbers of hydrophobic sites in the gel. By increasing the number of ionized groups on the penicillin molecule, the hydrophobic nature of the side chain is opposed by the hydrophilic character of the ionizable groups. This hypothesis would be consistent with the results observed with intact penicillins and their penicilloic acid and penicilloylamide derivatives.

The behavior of 6-APA was of interest because of its relationship to the penicillin nucleus. Differing by the introduction of an $-NH_2$ function at C_6 , any adsorptivity intrinsic to the heterocyclic ring system of 6-APA was subject to inhibition by this extra ionizable substituent. Under the conditions employed, 6-APA gave no evidence of adsorptive behavior and appeared to be governed solely by steric considerations, as indicated by the reduction of K_D values on the more highly cross-linked gel. Progressively smaller K_D values for 6-APA_o-ate and 6-APA_o-EACA were observed, as would be expected by applying either the steric hindrance hypothesis or the hydrophobic hypothesis.

These hypotheses seem to be valid explanations for the chromatographic behavior of penicillins observed in these experiments. It must be emphasized that the stated partition coefficients in the tables are valid only for the conditions under which the data was collected. The influence of pH on the chromatography of solutes bearing ionizable substituents can be profound and is clearly applicable to all penicillins and their derivatives. In their study of the effect of pH on observed K_D values of organic acids and bases, Brook and Housely (2) demonstrated a striking relationship between pH titration curves and observed adsorptive behavior.

They concluded that K_D values of molecules with ionizable substituents were only valid when given for a specified pH value. They turned this observation to inventive use in separating related molecules differing only in pK_a values.

Knowledge of the factors influencing the chromatographic behavior of penicillins has both theoretical and practical application. Gel-solute interactions between penicillins and highly cross-linked dextran gels appear to be an excellent model for predicting protein binding of penicillins (22). Moreover, Crone and Keen (7) suggest that gel-solute interactions also have relevance to transport of solutes across biological membranes.

Several practical applications are apparent besides the established use of gel chromatography to separate penicilloylated and penicillan-ylated macromolecules from reaction systems (25; N. E. Hyslop, Jr., R. J. Milligan, and J. F. Calvert, manuscript in preparation) and penicillin polymers from solution (11, 30). Quantitative analysis of chemical reactions is possible, as shown by our experiments on the penicilloylation of EACA with 6-APA, and chromatographic resolution can undoubtedly be improved with appropriate manipulations of pH and ionic strength. Gel chromatography of penicillins is also useful for preparative separation of penicillins from degradation products and could be adapted to the identification of the latter in biological fluids.

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