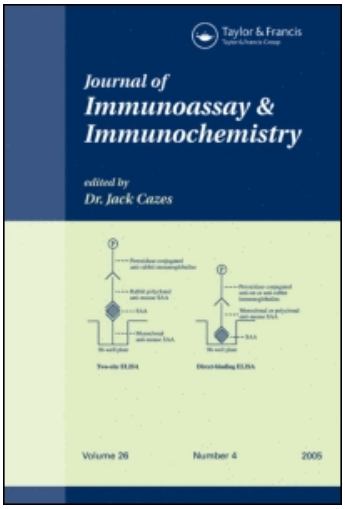

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METHODOLOGICAL CONSIDERATIONS FOR PENICILLIN RADIOIMMUNOASSAY

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ABSTRACT

The objective of this study was to identify and define factors that compromise the utility of radioimmunoassay for quantitation of β-lactam antibiotics in biological aqueous fluids. Serum containing antibodies to benzylpenicillin coupled as a hapten to keyhole limpet hemocyanin was used as the assay primary antibody. Two significant factors that limited the utility of the assay were the tendency for penicillin (1) to hydrolyze in aqueous solutions, resulting in a mixture of immunorecognizable forms; and (2) in the hydrolyzed state, to bind covalently with matrix proteins. Assay sensitivity, crossreactivity, and assay binding parameters varied with the state of hydrolysis of penicillin in tracer, standards, and unknowns and with the composition of the assay buffer. Hydrolysis of the β-lactam ring of penicillin immediately before assay by addition of 0.1 N NaOH or penicillinase resulted in improvements in assay repeatability and uniformity by forming predominantly the penicilloate form of the compound, which was immunologically well recognized by the antibody. Nonspecific binding of penicillin (and derivatives) to proteins in biological fluids such as milk or assay buffers was shown to be a possible cause of error in the immunoassay of penicillins.

(KEY WORDS: Penicillin, Radioimmunoassay, Antibiotics)

INTRODUCTION

The use of hapten-derivatized protein for production of antibody to low molecular weight poorly antigenic compounds is

an established procedure in radioimmunoassay (RIA) (1). The alkaline conjugation procedure described by Parker (2), which was used to attach β-lactam antibiotics such as benzylpenicillin (BP) to protein, is a standard method used to obtain penicillin-based immunogen capable of eliciting the production of antibodies for analytical as well as clinical pathoimmunological (allergy) investigation. However, the necessary incubation of penicillin with protein at pH 10.5 for 30 hours has severe effects on peptide bonds as well as on the integrity of the β-lactam ring structure (3,4). We used rabbit serum containing antibodies to a keyhole limpet hemocyanin (KLH)- conjugated BP, prepared according to Parker (2), in an RIA to measure penicillin in biological fluids.

Because at any given time some β-lactam is present in the hydrolyzed form in standards, tracer, and in "unknown" samples, it may be necessary to prehydrolyze all penicillin to a uniform state before assay. In addition, since conjugation of penicillin to protein in neutral conditions occurs to a limited extent, the effect of storage of biological fluids such as milk on the assay properties and loss of immunoassayable penicillin through irreversible binding to protein was investigated.

MATERIALS AND METHODS

Assay Components and Reagents

Two buffers were compared for effects on the assays. The first buffer consisted of 0.05 M phosphate, 0.01 M disodium ethylenediaminetetraacetic acid, and 0.135 M NaCl with 100 mg/L thimerosal (EDTA-PBS, pH 7.5) and was used for assays that

characterized antibody binding, crossreactivity, and various aspects of time and temperature dependence of binding. The second buffer was buffered milk whey (BMW). BMW was initially prepared by precipitation of milk proteins through addition of rennin or acidification with 1 M HCl to a final pH of 2.2. Following centrifugation, the resulting supernatant was titrated to pH 6.5 with NaOH and thimerosal (100 mg/L) added for stability. BMW was used primarily as the assay matrix for studies dealing with measurement of penicillin in milk and whey.

Antibody directed against BP hapten was donated by Humphreys et al. (5). The particular immunogen was prepared according to Parker (2) by coupling BP to KLH and injecting the resulting emulsified conjugate into rabbits (6). The resulting serum (No. 202) was diluted with EDTA-PBS containing 1.0% normal rabbit serum. For the assay, serum was diluted to achieve a final tube dilution (FTD) of 1:400 or 1:1000, depending on the requirement for sensitivity and practical levels of binding.

Tritiated- and ^{14}C-BP with activities of 144 μCi/mg and 42 mCi/mg, respectively, were obtained from Amersham and used as tracers. Upon receipt, the tracer was diluted with distilled deionized water, distributed into 100 μL aliquots, frozen, and lyophilized. For use in an assay, a vial containing tracer was reconstituted with EDTA-PBS and diluted to 4500 CPM/100 μL (^{14}C) or 4800 CPM/100 μL (^{3}H) corresponding to the addition of a 10 ng or 100 pg mass of tracer, respectively, to each assay tube. In studies where hydrolysis of the β-lactam ring of the tracer was

required, a vial was treated with either penicillinase (7) or 100 µL 1.0 M NaOH (8) for 30 min followed by 100 µL HCl (1 M) and 800 µL EDTA-PBS. Except for the studies of binding versus antibody dilution and general crossreactivity with other antibiotics, only ^{3}H-penicillin tracer was used in the RIA.

Penicillins and other antibiotics used for standards or in crossreactivity studies were obtained from Sigma Biologicals or the U. S. Pharmacopeia as highly purified preparations. Solutions of each test drug were prepared fresh daily as needed with either EDTA-PBS or BMW. The test drugs were added to the assay in appropriate amounts in 100 µL volumes. Hydrolysis of β-lactam in various penicillins was achieved, where necessary, with penicillinase or NaOH with equal efficiency.

General Procedure

Assays were performed in 12 x 75 mm borosilicate glass tubes at room temperature unless otherwise noted. In order, properly diluted BP tracer was added to all tubes. In turn, assay quality control components, standards (U. S. Pharmacopeia), and unknowns in triplicate were added to the tubes. Antibody was added last. The tube contents were mixed on a Vortex mixer and allowed to incubate for the prescribed amount of time pertinent to each phase of the study. Typically, a standard assay would incubate for 2 hours. Bound and free tracer was separated using a double antibody technique, which gave lower variance in triplicate sample tubes than ammonium sulfate preparations. The second antibody (donated by Dr. D. J. Bolt, U. S. Department of Agriculture, Beltsville,

MD), an anti-rabbit γ-globulin, was diluted 1:16 with EDTA-PBS and 200 µL was added to each tube. Assay tubes were incubated at 4°C for 30 min. Chilled polyethylene glycol (8000 MW, 800 µL of a 6% solution) in EDTA-PBS (pH adjusted to 7.5) was added. Tubes were centrifuged at 1850 x g for 30 min, decanted, and swabbed dry. The pellet was dissolved in quaternary ammonium organic base (tissue solubilizer) and added to scintillation vials. Counting fluid was then added. Radioactivity in the vials was quantitated in a Beckman LS-9000 scintillation counter for 5 min or to a σ value of 2.0%.

Data reduction was accomplished using standard log-logit transformation with unweighted linear regression. Comparisons of the effect of different assay components and matrices or crossreactivities were performed in the same assay to alleviate inter-assay variability. Intra-assay variability averaged <10%.

Initial Characteristics of Antisera

To determine the dilution of antiserum that would allow a balance between binding, sensitivity, and reproducibility, several initial dilutions of serum preparation No. 202 were made. Dilutions ranged from 1:10 to 1:1000 and corresponded to final tube dilutions of 1:40 through 1:4000. For this characterization, assay tubes were incubated 24 hours at 4°C before adding the second antibody and counting. Specific binding was defined as the difference between the total tracer bound (counts per minute) and the remaining counts bound in the presence of 10 µg unlabeled BP. With this excess mass of nonlabeled BP, there was no difference in the

total displacing capability of intact or hydrolyzed forms of penicillin.

Effects of temperature and time on tracer binding were further evaluated through a timed incubation of tracer and antibody at 4, 22, or 37°C. Nonhydrolyzed BP was incubated with antibody for 1, 3, 6, or 24 hours before termination of the reaction with a second antibody.

β-Lactam Hydrolysis Effects

The effect of hydrolysis of the β-lactam ring on characteristics of the immunoassay was examined by incubating nonhydrolyzed or hydrolyzed BP tracer with nonhydrolyzed or hydrolyzed BP standards for 1 hour at room temperature. A standard curve ranging from 1 to 1000 ng penicillin per tube was used to determine slope, correlation coefficient, 50% displacement mass (EC 50), and minimal detectable quantity (MDQ, 10% displacement).

Additional information on the degradation of penicillin in aqueous medium was obtained using a fluorometric method developed by Tsugi et al. (9) to measure penicillins and penicilloic acids and modified by Munns et al. (10) to allow reversed phase high performance liquid chromatographic (HPLC) separation and quantitation of fluorescent dansyl BP derivatives. Briefly, quantitation of total penicillins typically involved an initial base hydrolysis of the β-lactam moiety. Acidification and addition of mercuric chloride would yield a terminal aldehyde group to react with dansyl hydrazine. The fluorescent side chains, benzyl in the

case of BP, can be chromatographically separated on a C-18 HPLC column, using an 85% acetonitrile/water mobile phase under isocratic conditions, and quantitated. In the present study, we used this method, omitting the initial base hydrolysis. Therefore, progressive hydrolysis of the β-lactam in aqueous media could be quantitated directly by a relative measurement of the amount of derivative formed as a function of time since only the β-lactam that was hydrolyzed was reactive in the derivatization (4). Spontaneous hydrolysis of the β-lactam ring of BP was estimated by monitoring the generation of penicilloate resulting from the addition of 100 µg sodium BP (Sigma) to 20 mL of either phosphosaline assay buffer (pH 7.5) or BMW (pH 6.5, as in milk). These solutions were incubated at room temperature and 1.0 mL aliquots were withdrawn and reacted for the HPLC assay at 0, 2, 4, 8, 16, and 32 hours. The value at 0 time was used to correct for any hydrolysis product in the penicillin stock before dissolution by subtracting the value from the subsequent time sample values.

Crossreactivity

Percent crossreactivity was defined as EC 50 (penicillin) divided by EC 50 (unknown drug) x 100. Procaine penicillin, amoxicillin, penicilloic acid, penicillenic acid, ampicillin, sulfamethazine, neomycin sulfate, cephalothin, tylosin, streptomycin, bacitracin, tetracycline, oxytetracycline, and erythromycin (U. S. Pharmacopeia) were used to estimate, by regression analysis, the crossreactivity of the antibody with other drugs. With ^{3}H-BP used as tracer, five penicillins chosen from the

acidic, basic, and neutral classes of penicillins were further compared for crossreactivity in both the intact and hydrolyzed states. Crossreactivity assays were conducted with a 1 hour incubation at room temperature using a 100 pg mass intact tracer, 1:1000 FTD antibody, an intact penicillin standard curve, and double antibody precipitation. Additional crossreactivity studies of the other classes of β-lactam antibiotics were further refined to include the effects of hydrolyzed tracer and standards.

RESULTS AND DISCUSSION

As is shown in Figure 1, total binding at an FTD of antiserum No. 202 at 1:400 was adequate and was chosen to examine time- and temperature-dependent aspects of the assay.

The effects of time and temperature on antiserum binding of intact tracer are illustrated in Figure 2. Specific binding (B_0) varied slightly at each time point among the three temperatures at which the assay was incubated. However, instead of increasing linearly as a function of time, binding actually decreased during the initial 6 hours of the assay. Binding at 1 and 24 hours' incubation was approximately equal at 30-35%. However, binding as low as 17-20% was measured at the 6-hour assay incubation time point. Lower binding was more evident with the 37°C incubation at 3 and 6 hours.

Characterization of antiserum No. 202 revealed that the preparation was relatively low in titer in comparison, for example, to antisera for hormone analysis commonly used at dilutions in excess of 1:20,000. However, as judged by the slope of the

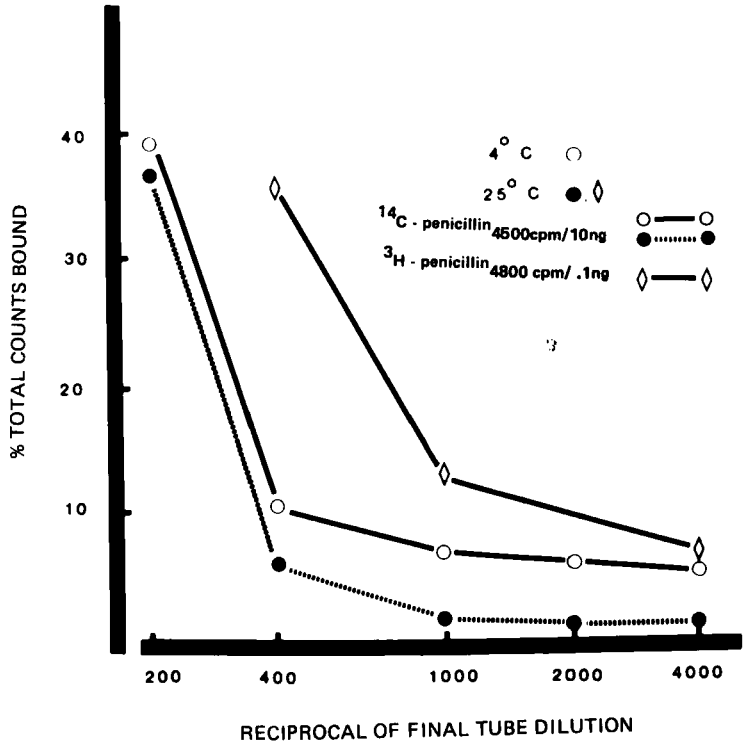

FIGURE 1. Binding of two penicillin tracer preparations with different specific activities to antipenicillin serum at various dilutions.

regression line for both the assay standard curve and a Scatchard analysis (data not shown) the antiserum displayed high affinity characteristics. An additional attempt to further improve the quality of the antiserum was made by affinity purification of antipenicillin fractions. Using BP coupled to bovine serum albumin and immobilizing this conjugate to cyanogen bromide-activated Sepharose 4B, we applied a portion of a similar antiserum, No. 201, to the affinity column and eluted with an increasing salt gradient.

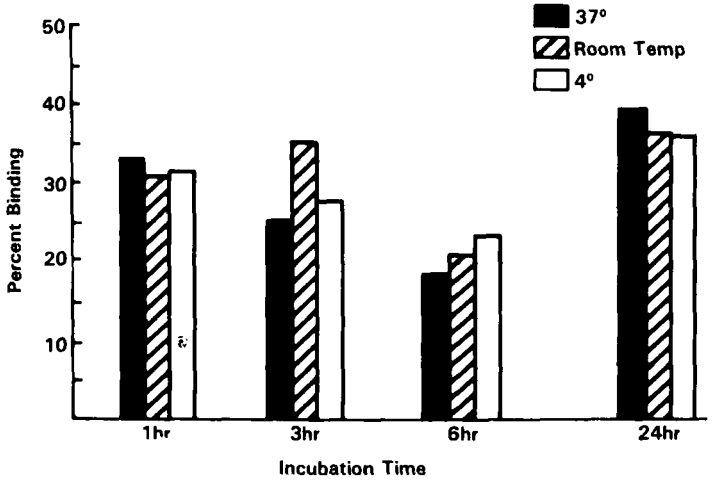

FIGURE 2. Effects of time and temperature during incubation on the B_0 binding of ^{3}H-BP to penicillin antibody.

An estimation of the attached penicillin residues to the conjugate protein was obtained by spectroscopic analysis of penamldate at 285 nm (8). Eluted protein peaks were detected by absorbance and were further characterized for their ability to bind BP tracer. Specific antibody was obtained but the yield was low and the quantitative aspects of the assay were no better or worse than those realized using the diluted native serum No. 202.

We questioned whether some change in the intactness of the β-lactam ring of the tracer during the 24-hour incubation period was influencing the kinetics of the assay. An experiment was performed to determine if the state of hydrolysis of the β-lactam ring would influence assay characteristics. The results are shown in Table 1. Binding varied widely among the group

TABLE 1

Assay Binding Parameters as Influenced by Hydrolysis of Penicillin

Incubation[a]	B_0 Binding (%)	Nonspecific Binding (%)	Slope	EC 50[b] (ng)	MDQ[c] (ng)
Intact Tracer Intact standard	16.7	6.4	-1.76	20.9	1.0
Intact Tracer Hydrolyzed standard	18.0	3.9	-1.00	118.4	0.15
Hydrolyzed Tracer Intact standard	26.8	2.4	-1.25	267.0	0.44
Hydrolyzed Tracer Hydrolyzed standard	25.0	3.2	-1.54	34.4	0.38

[a]When penicillin tracer or standards was hydrolyzed, hydrolysis was effected with a 20-min preincubation with 0.1 N NaOH and subsequent titration to neutrality with HCl.
[b]EC 50 is the mass of standard penicillin that would displace 50% of the B_0 bound counts.
[c]Minimal detectable quantity calculated by regression, using 10% displacement as the assay upper limit cut-off.

treatments. The highest binding occurred when the BP tracer was hydrolyzed before the assay; when the tracer was intact, binding was approximately 10% lower. Nonspecific binding was not measurably different among the test situations. Regarding the general sensitivity of the assay, assays with a mixture of intact and hydrolyzed β-lactam ring were one-half to one order of

magnitude less sensitive than those with a homogenous β-lactam structure (see EC 50). Apparent potency of the standards likewise was dependent upon the intactness of the penicillin molecule. Steepness of the slopes of the standard curves, as projected with linear regression, varied depending on whether the standards and tracer were intact or in a mixed hydrolyzed form. The shallowest (least negative) slopes occurred when there was a heterologous mixture of intact and hydrolyzed forms of the β-lactam ring structure.

Data in Table 2 illustrate that the crossreactivity of this antibody with antibiotics other than the β-lactam class was minimal. However, the antibody crossreacted with two primary metabolites of penicillin, penicilloic and penicillenic acids. The higher potency of penicilloic acid, a naturally occurring alkaline hydrolysis product, possibly reflects the higher affinity of hydrolyzed forms of BP for the antibody. Presumably, this did in fact reflect a short-term, reversible binding as opposed to a covalent-type attachment since the slopes of the displacement curves of penicilloate and hydrolyzed BP were similar. Binding, as estimated by precipitated counts in assay tubes, does not, however, rule out the possibility that subsequent irreversible events could occur. Spontaneous covalent attachment of penicillins to proteins and amino acids has been well documented (2,3). This point must be considered when the utility of RIA is assessed because the kinetics of immunoassays are dependent upon the reversible nature of the binding as exists with respect to equilibrium. In addition, one

TABLE 2
Crossreactivity[a,b] of Several Antibiotics and Metabolites with Penicillin Antibody (No. 202)

Compound	Crossreactivity (%)
Benzylpenicillin (BP)	100
Procaine penicillin	80
Penicillenic acid	31
Penicilloic acid	120
Sulfamethazine	1.2
Neomycin sulfate	0.1
Cephalothin	0.4
Tylosin	0.1
Streptomycin	0.1
Bacitracin	0.1
Tetracycline	0.1
Oxytetracycline	0.1
Erythromycin	0.1

[a]Crossreactivity corrected on a molar basis.
[b]Nonhydrolyzed ^{14}C-BP tracer.

should consider the nonspecific precipitation of tracer counts bound to proteins other than immunoglobulins that might occur when polyethylene glycol is used as a precipitation adjunct in the second antibody reaction.

Data on the crossreactivity of the antibody with other antibiotics of the β-lactam class are shown in Table 3. Crossreactivity or crossreaction potency usually increased when the antibiotic was hydrolyzed, as was especially true with phenoxymethylpenicillin. With other penicillins, whose crossreactivities increased only marginally, no changes in the slope of the dose curve were apparent and the curves lacked any parallel orientation to the standard curve of hydrolyzed BP. The hydrolysis of phenoxymethylpenicillin and methicillin resulted in

TABLE 3
Crossreactivity[a] of Various Penicillins with Antibody No. 202

Drug	State	Crossreactivity (%)
Benzylpenicillin	Hydrolyzed (Std)	100
Ampicillin	Hydrolyzed	3.4
	Intact	0.8
Amoxicillin	Hydrolyzed	0.1
	Intact	0.1
Phenoxymethylpenicillin	Hydrolyzed	43.5
	Intact	4.5
Methicillin	Hydrolyzed	10.9
	Intact	0.1
Cloxacillin	Hydrolyzed	1.7
	Intact	0.6

[a]Comparisons made using hydrolyzed BP tracer.

dose displacement curves for these antibiotics steeper than those observed for their intact form and nearly parallel to the BP standard curve. Hydrolysis gave these drugs an apparent greater ability to displace the BP tracer. Structurally, these compounds are very similar to the native BP. These results imply that the specificity of the antibody was directed slightly more toward the benzyl side chain than the lactam portion of the antibiotic, but that the intactness of the lactam ring was also a key constituent.

The instability of the β-lactam in aqueous solution is well documented. Brodersen (11,12) and others (13) reported that the stability is dependent upon both pH and temperature and that different penicillins and their ionized forms interact with hydrogen ions at different rates. Data also indicate that the pH at which minimum degradation occurs for some β-lactam antibiotics

is 6.5 (3,14). At this pH, the half-life (marked by biological activity) of unstabilized BP at 35°C is less than 1 hour (11).

The utility of the assay to measure penicillin in milk was examined in a comparison of standard curves constructed using phosphate buffer (EDTA-PBS) or BMW as diluent. When penicillin-spiked whole milk was added directly to the phosphate buffer system, no usable information was obtained because of excessive nonspecific binding ($>>B_0$), technical difficulties in the separation of the bound and free forms, and apparent quenching problems associated with the amount of tissue solubilizer needed to dissolve the extra mass of the precipitate (data not shown). Fat-extracted milk presented similar problems, indicating that the fat content of milk was not the major problem hindering the assay. However, when milk was treated with rennin or acid to precipitate the casein, extracted with methylene chloride, and degassed to remove residual volatile organics, serial dilutions of the resulting whey spiked with penicillin yielded a straight line (slope = -1.90, correlation coefficient = 0.98). This line was not parallel to the buffer standard curve line for the same assay generated in the phosphate buffer (slope = -1.55) (Figure 3). Constructing the standard curve in BMW resulted in a standard curve (slope = -1.88) useful for the measurement of penicillin in milk since standards and unknowns were in a similar matrix. Although the EC 50 and MDQ values of the whey assay were somewhat higher than those measured with the phosphosaline buffer assay, the slight loss of sensitivity when BMW was used could be sacrificed for the

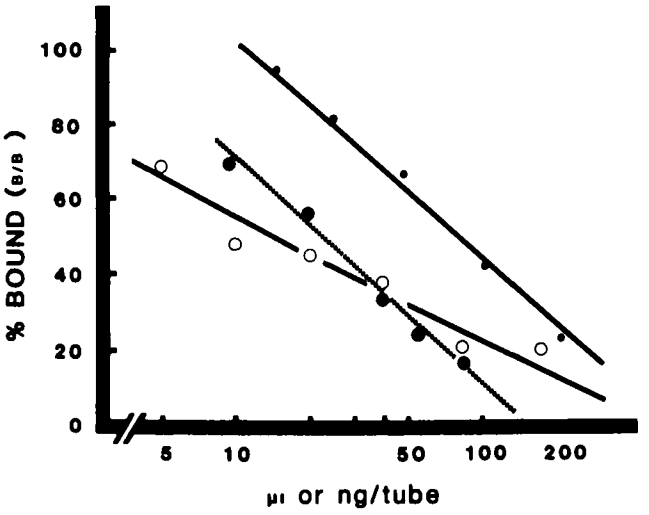

FIGURE 3. Displacement standard curves for hydrolyzed penicillin in PBS (o–o–o) or BMW (●-●-●-) and displacement of tracer by increasing volumes of penicillin-spiked whey to the BMW standard curve (●–●–●).

repeatability of the results and reduced cross interference with whey-associated proteins. Recovery of penicillin from whole milk or whey spiked with hydrolyzed BP was quantitated. The recovery of added penicillin was highly variable between samples (55-80) and considerably lower than that which was calculated to have been present.

The constituents of buffers have significant effects not only on the performance characteristics of immunoassays, but also on the stability of penicillins in solution in general. The effects of buffer components on penicillin stability (stability referring to the intact character of the lactam ring) was first demonstrated by Finholt et al. (15). Dibasic phosphate, dihydrogen citrate, and

borate catalyzed degradation while monobasic phosphate, acetate, and boric acid were noncatalytic. Hou and Poole (7) further investigated buffer salts, ionic strength, and pH effects on β-lactam degradation and found rates that followed pseudo-first order kinetics. Degradations were influenced by general acid-base catalysis, particularly that affected in the presence of base, being 1400 times as rapid as that occurring in acid conditions. The effects of various buffer salts might therefore be related to the pK of the dissociated moiety.

Data showed that a small percentage (up to 9.0% in 6 hours) of labeled penicillin was bound to milk proteins during both 4°C and frozen storage of samples (Figure 4). This penicillin would be unavailable for RIA. Therefore, the immediate removal of casein or milk proteins before sample storage might facilitate the preservation of β-lactam for subsequent analysis. This, plus the problem of hydrolysis, may account for the variability in estimated recovery of unlabeled penicillin from whole milk.

Additional data using HPLC to quantitate penicillin hydrolysis in phosphate and whey buffers are shown in Figure 5. Based on the measurable quantity of "penicillin" in the timed aliquots, up to 6% of the stock penicillin was already hydrolyzed before dissolution in the BMW or PBS. When this value was subtracted from other time values, hydrolysis apparently occurred rapidly in both buffers. Hydrolysis occurred to a greater extent in phosphosaline than in BMW buffer, reaching a measurable peak of 12% at 4 hours in BMW and 31% in phosphosaline at 8 hours. Values lower than this at time

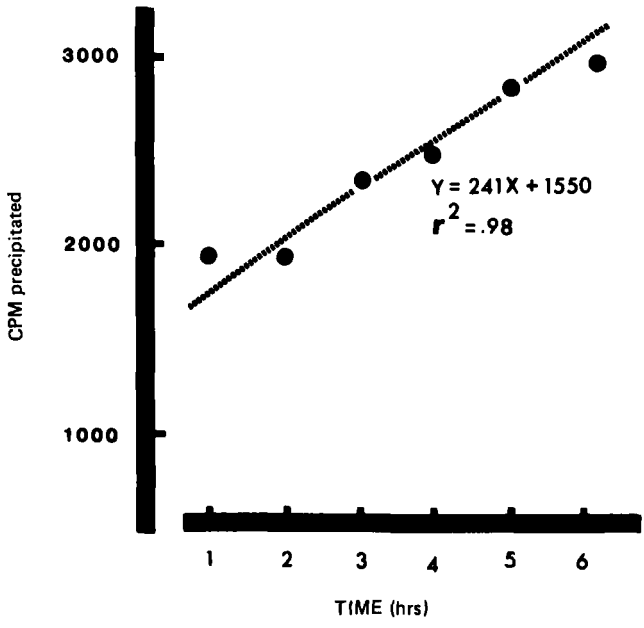

FIGURE 4. Linear increase with time of nonspecific binding of penicillin to acid-precipitable milk proteins. Results were obtained by incubating 32,000 CPM ^{3}H-BP with 0.5 mL whole milk at 37°C for various times, precipitating protein by adding 0.5 mL 1 N HCl, followed by centrifugation, and solubilization of the pellet.

points beyond 8 hours reflect an apparent breakdown of the penicilloate so that the fluorescent derivatization reaction was not obtained. The pH differences in the two buffers may have influenced the rate of spontaneous hydrolysis.

The concept of chemically tuning an RIA, that is, of increasing the specificity for a particular molecular form of a drug, has been previously demonstrated by Mason et al. (16) in assays comparing specificity of different amphetamine preparations

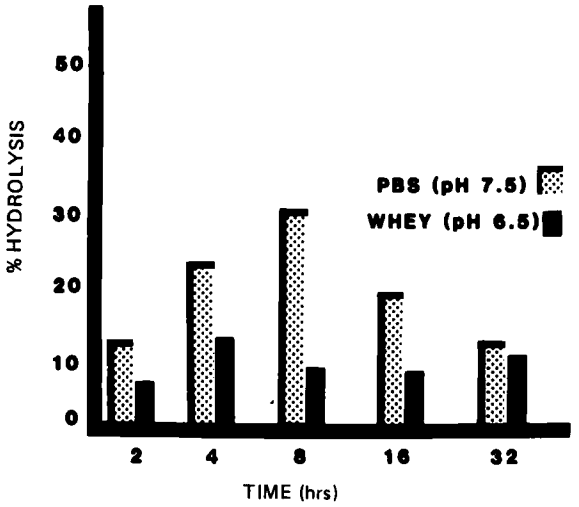

FIGURE 5. Increased hydrolysis of penicillin in phosphate buffer (pH 7.5) or BMW (pH 6.5) during incubation at 37°C as quantitated by HPLC.

for a single antibody. A portion of the antibody formed in response to immunization with an antigen prepared by the Parker conjugation method would preferentially recognize the hydrolyzed form of the ß-lactam ring because the incubation conditions favor the formation of a penicilloyl derivative (2) with the hydrolyzed lactam ring. However, the antibody might not have the highest affinity for free penicilloic acids or penicillins. For example, Atsumi et al. (17) demonstrated that when penicillin was conjugated to polylysine, in some cases it had greater affinity for an antibody than it had when it was not conjugated to polylysine. Data presented here suggest that a penicillin RIA could be improved by including a hydrolysis step.

Atsumi et al. (17) fractionated an antiserum generated against BP-conjugated rabbit γ-globulin, using specific penicillin derivative immunoadsorbants. Their data suggested that there were at least 5 distinct antibody types present, which differed in specificity and affinity for various penicillin antigens. The different reaction products which arise during the alkaline conjugation procedure probably yield several possible orientations of the penicillin as a hapten. The stereochemistry of hapten-protein conjugation can be important (18, 19); the side chain groups of the various penicillins may determine the specificity of the generated antibody. Niswender (18) and England et al. (19) suggested that the greatest probability for antibody production was related to the hapten moiety most distant from the point of protein attachment. Thus, the present demonstration of crossreactivity and dependence on β-lactam structure is possibly related to the presence of numerous types of antibody in antiserum No. 202 with key determinants being the benzyl side chain and hydrolyzed β-lactam of the drug.

Although performed in a nonquantitative fashion, our assay system demonstrated the ability of penicillin-conjugated bovine serum albumin to displace labeled penicillin, although with a slope significantly more shallow than that obtained with the free molecule. This raises yet another possible problem in penicillin by RIA, namely, a mixed set of kinetic reactions occurring between free and protein-bound penicillin for the limited antibody.

It is assumed in RIA that the affinity of the tracer, standards, and measured compounds in "unknown" samples for the

antibody is the same (20). The validity of this assumption is often questioned in protein hormone RIA, where fragmentation of labeled ligand during the iodination reaction or the insertion of radioiodine in a critical recognition site might change the affinity of the antigen for the antibody. This is usually avoided when tracers are ^{3}H- or ^{14}C-labeled. If the assumption of similar affinity of all forms of penicillin ligand for the antibody is valid, then the slopes of the standard curves generated with intact, mixed, and hydrolyzed penicillins should be the same. Since this was not observed here, we concluded that the hydrolysis of penicillin influences the affinity of penicillin for the antibody. However, with the potential for degradation or spontaneous hydrolysis of the β-lactam ring, it would be advisable to convert penicillin (in tracer, standards, and unknowns) to the hydrolyzed form before assay to an approximately homogenous population of ligands with similar structure and affinity for the antibody. This concept is consistent with that of Cooper et al. (21), who alluded to the problems associated with degradation of penicillin tracers.

The use of BMW as an assay matrix for the RIA of penicillin (as well as other substances) in milk may alleviate several problems. Neither B_0 binding nor the slope of the standard curves for phosphosaline, and BMW or whey, respectively, was similar. The relative affinity of the ligand for the antibody in a particular assay is affected by the concentrations of low molecular weight components of the buffer system such as salts and divalent cations

(22). For determination of penicillins in milk or whey, a buffer similar to BMW might be useful as a means of reducing nonspecific matrix interference.

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