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Immobilization of a Primary Amine-Containing Drug, Adriamycin

Coupling to Crosslinked Polyvinyl Alcohol and Mechanistic Comparison of Hydrolytic Stability

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ABSTRACT

Literature reports have described the covalent coupling of the primary amine-containing anticancer drug, adriamycin, to polymeric supports through the amine group on the drug. These reports also have described drug mechanism studies with the immobilized adriamycin, where the release of the drug would undermine the validity of the conclusions. In the present paper, detailed experimental conditions are given for preparation of nonwater-soluble particles of polyvinyl alcohol by crosslinking water-soluble polyvinyl alcohol with 1,4-benzenedicarboxaldehyde, and for activation with cyanuric chloride and covalent attachment of adriamycin. The expected stability of this drug-support linkage against hydrolytic cleavage is compared mechanistically to that expected for less stable coupling through a carbamate linkage or for less stable coupling via an azomethine link.

Index Entries: Adriamycin; polyvinyl alcohol; immobilization; carbamate; cyanuric chloride.

INTRODUCTION

The immobilization or attachment of drugs to solid supports has found several areas of application. These include the study of drug mechanisms

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of action (*1–3*), the preparation of affinity columns, particularly for the isolation of cell membrane receptors (*4–6*), and the development of sustained release or drug targeting preparations (*7,8*). In the drug mechanism studies, it is important that the linkage between the drug and the support be very stable so that the observed results can be attributed too immobilized, and not released, drug. In the sustained release and targeting preparations, the linkage between the drug and the suport must be labile under certain conditions, in order to allow further distribution of the drug to the site of action.

One class of drugs particularly amenable to mechanistic studies with the compounds immobilized on solid supports is the anthracycline antibiotics used in the treatment of a variety of tumors (*9*). Adriamycin is the member of this class of drugs most widely used clinically. The mechanism of action of adriamycin is not clear. The drug binds to DNA by intercalation; but actions on the cell membrane (*10,11*), radical formation (*12*), and the production of DNA breaks (*13*) as mediated by topoisomerase II activity (*14*) constitute other mechanistic possibilities. In order to study the cell membrane action of adriamycin further, we prepared crosslinked polyvinyl alcohol as the support and attached adriamycin via a cyanuric chloride spacer (*15*) to give a very stable linkage through the adriamycin amino function. Other research groups have coupled adriamycin, again through the amino sugar, to polyglutaraldehyde (*16*) or to 1,1′-carbonyldiimidazole-derivatized agarose (*17*) to form less stable predominantly azomethine (*16*) and *N*-alkyl carbamate (*17*) linkages, respectively. Adriamycin also has been attached to polyglutaraldehyde for drug delivery applications (*18–20*), where the stability of the linkage has not been the primary consideration. Other preparations in which adriamycin has been coupled to different solid supports or antibodies are not covered here, since the preparations have not been used for drug mechanism studies.

Two aspects of the previous work were omitted from the earlier paper (*15*) at the request of the reviewers. These aspects include the detailed procedure for the preparation of crosslinked polyvinyl alcohol (CL-PVA), adapted from the method of Manecke and Vogt (*21*), and mechanistic comparison of the bond stabilities to hydrolysis for CL-PVA-cyanuric chloride coupling of adriamycin with those based on *N*-alkyl carbamate or azomethine linkages. Subsequent inquiries by other laboratories on both of the omitted topics showed that further detailed description of the method and discussion of the relative bond stabilities is needed. Both of these topics are covered in the present paper.

The reaction sequence for the preparation of crosslinked polyvinyl alcohol and subsequent attachment of cyanuric chloride is given in Fig. 1.

MATERIALS

Medium molecular weight (1,700 degree of polymerization) polyvinyl alcohol from 99.5% hydrolysis of polyvinyl acetate was obtained from E.I. duPont de Nemours Co. as Elvanol® 71-30. Solutions of up to about 25%

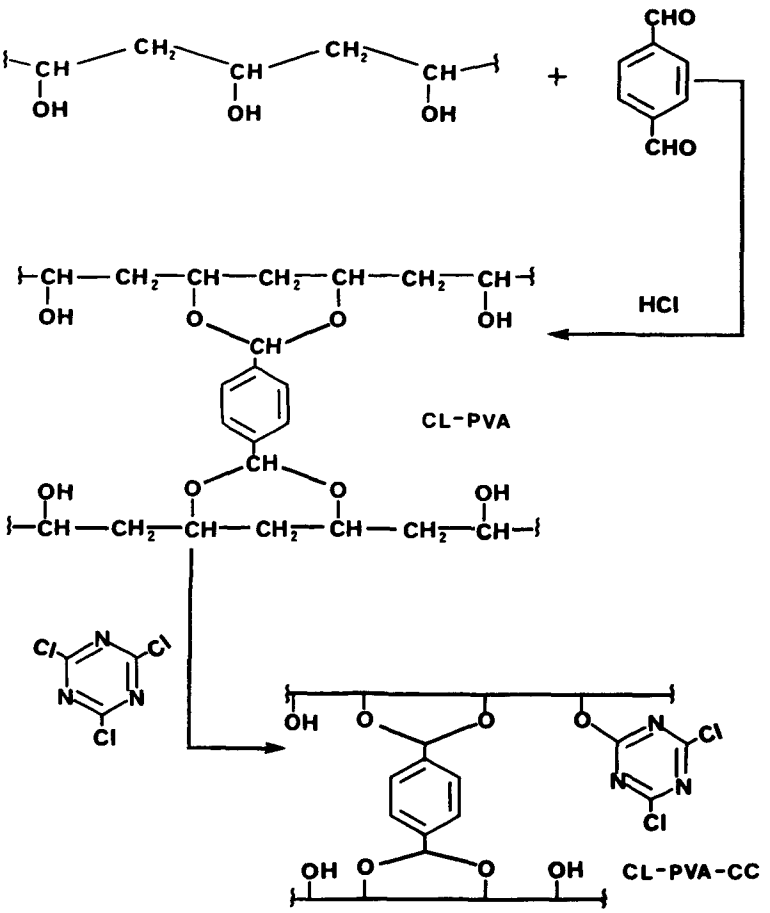

Fig. 1. Reaction sequence for preparation of CL-PVA and conversion to CL-PVA-CC.

by weight of polyvinyl alcohol in water can be prepared at 90°C. The crosslinking agent, 1,4-benzenedicarboxaldehyde (MW 134.13) (Aldrich), was dissolved in 20% methanol/80% water (v/v) at 65°C, treated with decolorizing carbon, cooled on ice, filtered, and dried to give white crystals (m.p. 113.0–114.5°C). The activator, cyanuric chloride (MW 184.4; 100 g) (Aldrich) was dissolved in 700 mL benzene (dried over 4 Å molecular sieves) at 43°C and crystallized after filtration and vacuum rotary evaporation of two-thirds of the solvent. This was followed by recrystallization from acetone (dried over 4 Å molecular sieves). The material was vacuum dried at room temperature and stored in a dessicator at −10°C (m.p. 144.2–146.0°C).

The adriamycin (MW 543.5) was obtained in the free amine form from the National Cancer Institute. HPLC assay with both fluorescence and UV detection (*15*) showed only a single peak that was adriamycin and not the aglycone. ^{14}C-Adriamycin HCl (MW 580) was obtained through the National Cancer Institute from M. Leaffler of Stanford Research Institute.

The radiolabel was on sidechain position 14. Two batches, having specific activities of 12.2 and 7.5 mCi/mmole or 21.0 and 12.9 μCi/mg, respectively, were used. The radiolabel purity showed only one peak by HPLC analysis.

EXPERIMENTAL PROCEDURES

Preparation of CL-PVA

Polyvinyl alcohoil (77.0 g) was dissolved in 3,500 mL deionized water and heated to 80–90°C with stirring. While hot, the solution was filtered through a preheated Buchner filter containing No. 1 Whatman paper. The filtrate was cooled to room temperature, and 11.72 g recrystallized finely divided 1,4-benzenedicarboxaldehyde was added with stirring. Concentrated hydrochloric acid (35 mL) was added with vigorous stirring (using an overhead, mechanical, 3-bladed propeller). When a stiff gel formed (about 30 min), the agitation was stopped, and the mixture was allowed to set overnight. Partial separation of solid and liquid was noted. The wet solid was ground in a mortar to approximately 1–2 mm diameter particles. The solids and liquids were recombined, and 100 mL of methanol was added and stirred for 2 h to cap off unreacted aldehyde groups. The liquid was decanted off, and the solid material washed with water (8 × 1000 mL). The solids were vacuum filtered after each wash, using No. 1 Whatman paper. The material was soaked overnight in phosphate buffered normal saline, pH 7, and then filtered and washed 4 more times with water. Washing of the solids with water was continued until the filtrate was free of chloride ions, as determined by silver nitrate testing. The total washing time usually required several days. The CL-PVA was kept under vacuum at 45–50°C until dry. The particles were sized with a set of sieves having stainless steel screening. The large particles were ground with a mortar and pestle and resized. The total dry weight was 73 g, with the particle sizes between 90–500 μm. Particles between 90–106 μm, obtained with USA Standard Testing Sieves No. 140 (106 μm opening) and No. 170 (90 μm opening), were used for subsequent experiments.

Coupling of Cyanuric Chloride to Produce CL-PVA-CC

Two procedures, one anhydrous and one aqueous, were tested for attachment of cyanuric chloride to the CL-PVA. The two procedures were adapted from published methods (*21–24*).

For the anhydrous procedure, 20 g recrystallized cyanuric chloride was dissolved in 300 mL dry benzene plus 100 mL dry acetone and mixed with 5 g anhydrous solid sodium carbonate of particle size 90 μm or smaller (ground and sieved to get desired particle size) and 5.5 g dry CL-PVA (particle size dry between 90–106 μm, as determined by sieving). The slurry was shaken overnight at about 20°C, filtered, washed 5× with dry petroleum ether followed by 5–10× with UV grade heptane until no cyanuric chloride could be seen in the wash by UV at 240 nm. The solid sodium

carbonate was removed by passage through a 90 μm opening screen. The CL-PVA-CC was vacuum dried at room temperature and used immediately for coupling of adriamycin. Cyanuric chloride loading was estimated by release of chloride from attached cyanuric chloride with carbonate/bicarbonate buffer, pH 10, on an aliquot of the CL-PVA-CC. Overnight shaking of the mixture at room temperature released about 0.04 mmole chloride (determined by AgCl precipitation, with nitric acid washing to destroy any silver carbonate), indicating a loading of roughly 0.02 mmol cyanuric chloride/g dry CL-PVA.

In the aqueous procedure, hot NaOH was used to activate the CL-PVA prior to addition of the cyanuric chloride. CL-PVA (3.5 g) was stirred for 3 h in 60 mL 10% aqueous NaOH at 80–90°C. The liquid was removed by vacuum filtration to minimize subsequent hydrolysis of the active chlorides on cyanuric chloride; and the solids were cooled to room temperature. A solution of 10 g recrystallized cyanuric chloride in 90 g dry acetone was added to the CL-PVA; and the slurry was shaken 10 min at room temperature. Acetic acid (40 mL 20%) was added quickly with stirring to terminate the reaction. The solution was filtered, and the solids were washed with acetone about 3× followed by water 2× and used immediately for coupling of adriamycin. Overnight hydrolysis of an aliquot of the CL-PVA-CC at pH 10, followed by silver chloride precipitation and weighing to determine the released chloride, indicated a loading of at least 8.9 mmol cyanuric chloride/g dry CL-PVA.

Coupling of Adriamycin to Prepare CL-PVA-CC-ADR

Adriamycin (30–40 mg) was dissolved in 5–10 mL distilled water. The addition of 1–2 drops of 0.01 *N* HCl was used to convert the adriamycin to the hydrochloride for rapid dissolution. Next, sufficient concentrated carbonate/bicarbonate buffer was added to give a final solution of 25–30 mL 0.2 *N* buffer, pH 8.9. Immediately, 6–7 g wet or 2–2.5 g dry CL-PVA-CC was added, and the slurry was shaken for 4 h at room temperature with the solution protected from light. The liquid was removed by filtration, and the polymer was washed sequentially with 0.2 *M* phosphate buffer (pH 7.0), methanol, 50% aqueous methanol, and, finally, water. The washing extended over several months time, with each solvent system being employed until no adriamycin was detected in the washes by HPLC analysis. Most of the methanol and water washing was done with the CL-PVA-CC-ADR placed in a 1 cm diameter by 10 cm long Econo column (Bio-Rad) and the wash solution dripped through the column at a rate of a few mL/h. For the methanol washes, about 1,000 mL of wash was collected over a 5-d period, vacuum evaporated to 100 μL; and about 50 μL was applied to the HPLC column and adriamycin detected by fluorescence. This concentrative-HPLC analysis procedure was continued until no adriamycin could be detected, before switching to the 50% aqueous methanol wash material. Where ^{14}C-adriamycin was involved, the washes were assayed for adriamycin by liquid scintillation counting. The coupling of ^{14}C-labeled adriamycin to CL-PVA-CC was carried out by a similar

procedure, as described above, but with slightly different ratios of CL-PVA to CC and CC to ADR. For the nonradiolabeled work, the mole ratios were 0.79 for CC/CL-PVA and 1.2 for ADR/CC, whereas for the ^{14}C-ADR work, the mole ratios were 0.36 for CC/CL-PVA and 0.23 for ADR/CC. Thus, a lower loading was expected for the ^{14}C-case. The loadings, after extensive washing to remove free drug and a 6–7 h incubation at 37°C followed by more washings, were 0.89 mg ADR/100 g support for the nonradiolabeled case and 0.22 mg ADR/100 g support for the ^{14}C-ADR case (*17*). The loading figures were obtained by acid hydrolysis to release the aglycone, followed by HPLC with fluorescence detection, for the nonradiolabeled sample and burning of the CL-PVA-CC-ADR to $^{14}CO_2$, followed by absorption and liquid scintillation counting, for the radiolabeled sample (*17*).

RESULTS AND DISCUSSION

A detailed discussion of the expected relative linkage stabilities, based on mechanistic considerations, for attachment of a primary amino compound, such as adriamycin, to polymeric supports via cyanuric chloride activation, a carbamate linkage, or an azomethine (polyglutaraldehyde) linkage has not appeared in the literature. Such a comparison is important in selecting a procedure for the immobilization of adriamycin or any other compound for drug mechanism studies, where clear differentiation is needed between results caused by immobilized drug and those caused by released drug.

The structure of adriamycin and the three types of linkages being compared are shown in Fig. 2. Any of the following factors can be the basis for hydrolytic cleavage of these linkages: the presence of a good leaving group, whether or not a bimolecular substitution reaction can proceed, or if an addition–elimination reaction is likely, especially under acidic or basic catalysis conditions. Any one of these factors is sufficient to result in cleavage.

The mode of attachment used in the CL-PVA-CC case is particularly stable because of the strong triazinyl linkages. This is evident from consideration of the pathways available for hydrolytic cleavage. Adriamycin or an adriamycin-triazine adduct can be released from the CL-PVA support by possible cleavage at sites "b" and "a," respectively (*see* Fig. 2). Site "a" consists of a strong aromatic ether type linkage. Hydrolysis at this site is very unlikely because of the following mechanistic reasons: a bimolecular substitution reaction at the trigonal carbon is not favored, the oxygen atom at this site is not readily activated to become a good leaving group for release from the triazine, and the addition–elimination sequence, shown in Fig. 3, would not be favored because of the loss of aromaticity in the intermediate that would necessitate summounting a large transition state energy barrier. Therefore, there is no reason to expect a significant degree of cleavage at site "a."

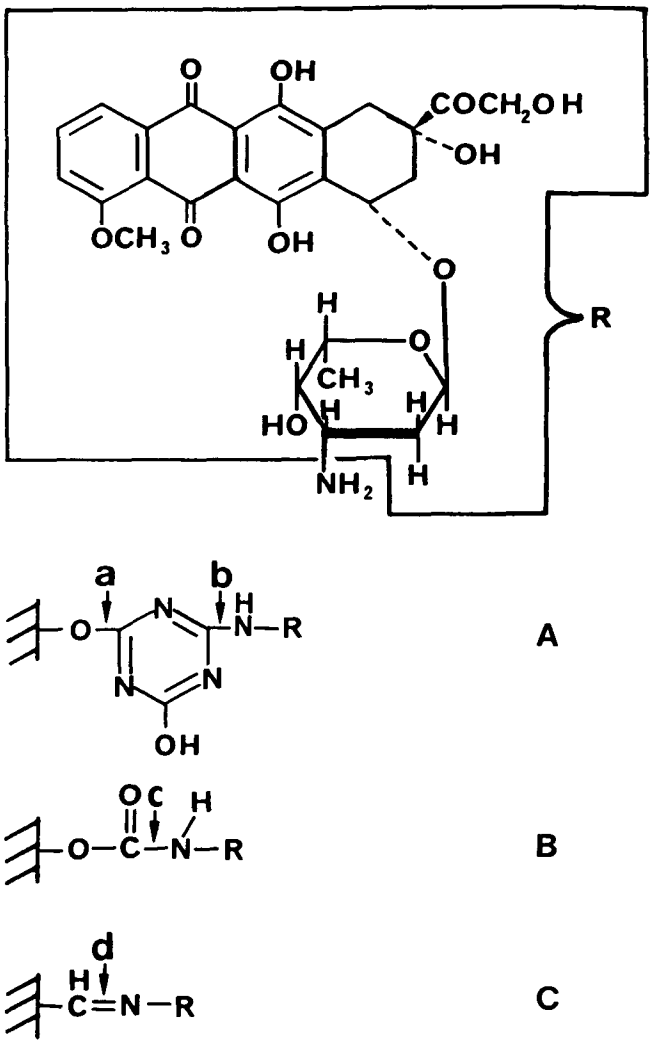

Fig. 2. Structure of adriamycin. Linkage of ADR: A, via cyanuric chloride to CL-PVA (this paper and (*15*)); B, to 1,1'-carbonyldiimidazole activated agarose (*17*); and C, to polyglutaraldehyde (*16*). Sites of potential hydrolytic cleavage are shown as a, b, c, and d. The triazine ring of cyanuric chloride is shown with the third chloride having undergone hydrolysis to leave an OH group. R represents the nonamino part of adriamycin.

Similar considerations also apply to cleavage at site "b" to give release of adriamycin from the triazine. Here again, the release by hydrolytic mechanisms will be strongly resisted, except under quite acidic conditions. At low pH values the adriamycin amino group should undergo protonation. This might facilitate cleavage by making the amine function a better leaving group. However, an addition–elimination step also would need to occur, as shown in Fig. 3; so that cleavage at site "b" would not be expected at a significant extent, particularly in the pH range of 6–8. Plans to

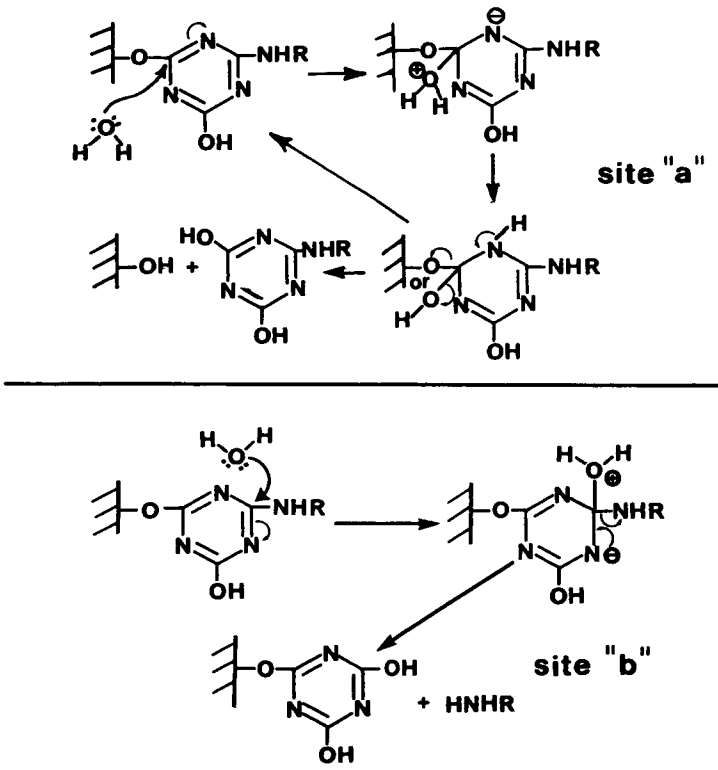

Fig. 3. Expected attack by water on the CL-PVA-CC-ADR preparation at the PVA-CC linkage (site "a") or the CC-ADR linkage (site "b"). R represents the nonamino part of adriamycin.

test experimentally for greater release of adriamycin at strongly acidic pH values (pH 1.0–1.5) were complicated by the fact that the glycosidic sugar linkage in adriamycin undergoes hydrolysis at acidic pH values. Adriamycin has its maximum stability at pH 4 (*25*); but the position 7 sugar linkage is the site of initial hydrolysis at pH values below 4 (*25,26*); and acid hydrolysis of adriamycin is the usual method for preparation of the aglycone. In the case of immobilized adriamycin, acid hydrolysis from the support could not be differentiated from hydrolysis of the glycosidic sugar link since either the ^{14}C-radioactivity or the fluorescence would reside on the released moiety in both cases.

In contrast to the high stability expected by the CL-PVA-CC-ADR preparation, the azomethine linkage (Fig. 2, site "d") that many authors feel is the main coupling formed from the reaction of a primary amine with the aldehyde function of polyglutaraldehyde (*27*) is a reactive bond. This azomethine linkage cannot be expected to have considerable hydrolytic stability even when existing in a conjugated form, especially at lower pH. When a small molecule, such as adriamycin, is immobilized on polyglutaraldehyde, only single point attachment is achieved through the aldehyde–amino functions. However, proteins can have multipoint attachment when coupled to polyglutaraldehyde and hence might display addi-

tional stability. No information is available on the effect of pH on the stability of the adriamycin–polyglutaraldehyde linkage. In drug delivery applications, the release of adriamycin from the polyglutaraldehyde matrix may even be beneficial.

It is possible to stabilize the azomethine (i.e., Schiff-base) type linkage by reduction with borohydride after attachment of the amine. The unstable azomethine linkage (site "d;" -CH=N-) would be reduced to a stable >CH-NH- form. In the earlier study (*16*), in which adriamycin was coupled to polyglutaraldehyde and used in adriamycin membrane studies, the borohydride reduction was not carried out on the attached adriamycin preparation; so that coupling used in the mechanism studies (*16*) may have been subject to hydrolytic cleavage. It is not clear at this time whether borohydride reduction of the adriamycin–polyglutaraldehyde preparation would leave the adriamycin chemically unchanged or not. Mention was made in the referenced work (*16*) that borohydride reduction of the plain polyglutaraldehyde particles, prior to incubation with adriamycin, resulted in loss of ability of the polymer particles to react with adriamycin. This would be expected since the aldehyde groups would have been reduced to alcohols by the borohydride treatment and thus no longer able to couple without a catalyst to a primary amine.

The carbamate linkage (Fig. 2, site "c") also is susceptible to cleavage by hydrolytic attack under both acidic and basic conditions. This is shown schematically in Fig. 4. The degree of stability attributed to a particular type of linkage depends to some extent on the application. For example, the carbamate linkage can be classified as having good stability when used for the immobilization of many enzymes or affinity ligands (*28*), but only fair stability when used for drug mechanism studies.

The attachment of adriamycin to the CL-PVA via triazinyl linkages is quite strong, but the stability of the hemi-acetal bonds involved in the crosslinking of the PVA is less well understood. Hemi-acetal bonds in theory are subject to hydrolytic cleavage under acidic conditions. In the case of CL-PVA-CC-ADR, the hydrolysis of a small percentage of the hemi-acetal bonds should not release adriamycin from the polymer particles since entire polyvinyl alcohol chains would need to be released; and such a high degree of hydrolytic cleavage is not expected at pH values in the 6–8 range. Since cleavage of the hemi-acetal bond does not involve scission of the polymer chain, the likelihood of adriamycin release caused by hemi-acetal bond cleavage is very low.

Comparison of the experimentally determined rates of release of adriamycin for the CL-PVA-CC-ADR and the carbamate linkage preparations showed that the release rate was markedly less for the CL-PVA-CC-ADR preparation. The results at room temperature showed 0.7 ng ADR released/h/g CL-PVA support and 21 ng ADR released/h/g agarose support (*17*), as determined over a 24-h period following several months of washing, 6–7 h incubation at 37°C, and further washing until the 24-h release rate became constant.

The activation of CL-PVA through covalent attachment of cyanuric chloride was carried out by both an anhydrous and an aqueous route. Both

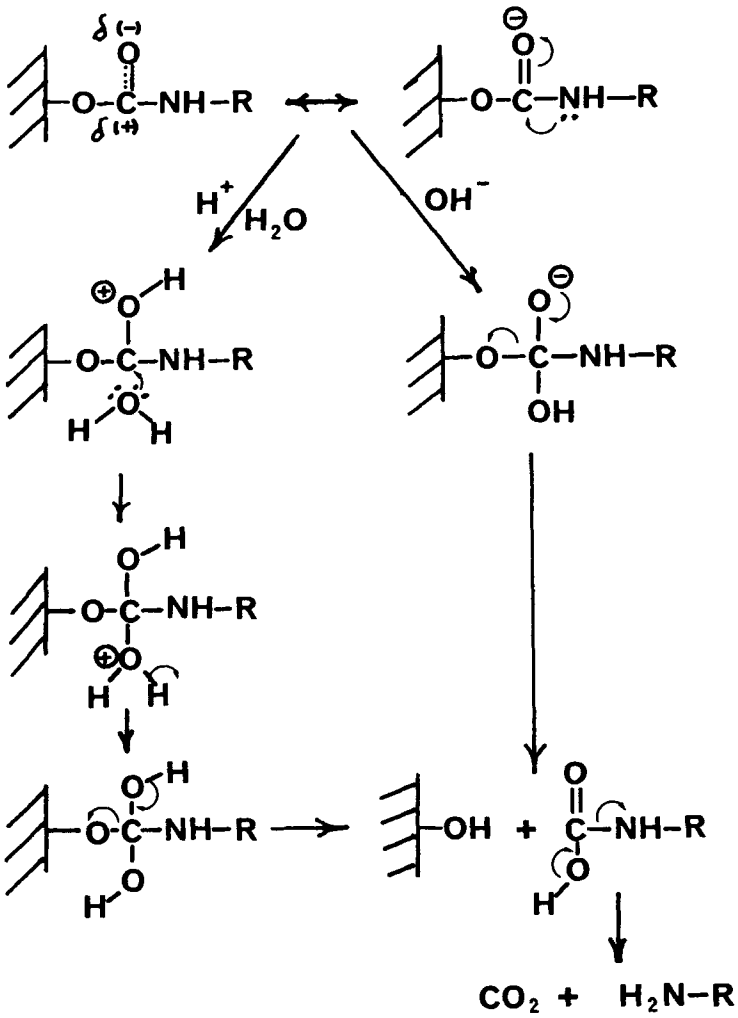

Fig. 4. Expected hydrolytic attack on carbamate linkage under either acidic or basic conditions. R represents the nonamino part of adriamycin.

procedures are described here for reference purposes. The three chlorides all are reactive, particularly with primary amines, with the first chloride reacting at 4°C, the second at 25°C, and the third at 80°C (*29*). By attaching the cyanuric chloride under anhydrous conditions, the reaction of the most reactive chloride with water is eliminated. However, the CL-PVA-CC-ADR preparation that was activated via the aqueous cyanuric chloride attachment route had more attached cyanuric chloride (8.9 vs 0.02 mmol CC/g dry support). In addition, use of the aqueous route gave by far more attached adriamycin (after washing away loose adriamycin) than did the preparation made via the anhydrous cyanuric chloride attachment procedure, as noted by the bright red and washed out pink colors of the two preparations, respectively. Adriamycin is bright red when dry or in solution at pH 6–8.

We conclude that the triazinyl linkages would be expected from a mechanistic standpoint to be much more stable than either the carbamate

or the azomethine couplings. This conclusion is substantiated experimentally so far for the triazinyl and carbamate adriamycin preparations by this lab.

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