Journal of Controlled Release, 2 (1985) 205—213
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

OPTIMIZATION OF MACROMOLECULAR PRODRUGS OF THE ANTITUMOR ANTIBIOTIC ADRIAMYCIN*

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In our earlier work [10] on aminoribosyl-bound prodrugs of adriamycin (ADR) using poly(α-L-glutamic acid) (PGA) grafted in high yield (90—100 mol.%) with various peptide spacers as a plasma-soluble macromolecular carrier we observed rather low cytotoxic activities in L1210 leukemia and B16 melanoma in vitro *assays. These results may be tentatively explained by a decreased susceptibility of the spacer-bound adriamycin moiety to hydrolysis by lysosomal enzymes due to the high spacer load. This hypothesis was tested by the study of two conjugates prepared by a different route. Peptide conjugates of adriamycin (Gly-Gly-Leu—ADR and Gly-Gly-Gly-Leu—ADR) were synthesized using the trityl* N*-protecting group and were coupled to PGA in 4.5 mol.% load according to the method described earlier [11]. However, these conjugates were almost totally devoid of cell growth-inhibiting activity in L1210 and B16* in vitro *tests. The data suggest that either the uptake of the polymeric prodrugs into the cell by pinocytosis is highly dependent on spacer load or molecular weight, or that lysosomal digestion is too slow for efficient release of ADR. Possibly, enzymatic degradation of PGA which is known to occur only between pH 4 and 6 is rate-limiting for release of the drug. Current studies include the enzymatic degradation of PGA—peptide spacer—drug systems using* p*-nitroaniline as a model drug and papain as the enzyme. By variation of the length and load of spacer it can be estimated under which conditions the release of drug (using UV spectrometry) is faster than degradation of the polymer (as determined by viscometry). In addition, the uptake of PGA and derivatives with a fluorescent label into tumor cells is studied using laser flow cytometry and laser microscopy.*

INTRODUCTION

High molecular weight prodrugs of cytostatic agents have been developed to decrease the toxic side effects of the drugs and to achieve a more selective administration of the agents to tumor cells [1–6 and references cited therein]. Ideally the conjugates of the drug and the polymeric carrier should be stable in the circulation and should degrade only after uptake by tumor cells. Degradation of the conjugate takes place in the lysosomal compartments by exposure to a number of

*Paper presented at the Second International Symposium on Recent Advances in Drug Delivery Systems, February 27, 28 and March 1, 1985, Salt Lake City, UT, U.S.A.

0168-3659/85/$03.30

digestive enzymes at an acidic pH (4—5) [7—9] releasing the parent drug *in situ*.

In previous papers [10,11] we have described a series of high molecular weight prodrugs of adriamycin using poly(α-L-glutamic acid), PGA, as a carrier and peptides of different length and composition as spacer units. Depending on the composition of the conjugates different cytotoxic activities against L1210 leukemia cells were observed. However, the cytotoxic activities were always lower as compared to that of the free drug. In principle, this may be due to a slow uptake or no uptake at all of the conjugate by the tumor cell or a to a very low rate of degradation of the conjugate after internalization by the tumor cell.

In this paper we will report on the synthesis of some new conjugates of adriamycin and PGA, the interaction of conjugates with mouse leukemia L1210 cells studied with laser flow cytometry and the enzymatic degradation of the carrier and the conjugates using papain as a model enzyme.

SYNTHESIS AND CYTOCIDAL ACTIVITY OF MACROMOLECULAR PRODRUGS OF ADRIAMYCIN

The synthesis of poly(α-L-glutamic acid), PGA, was described previously [12,13]. The conjugates PGA_2, PGA_7 and PGA_8 had molecular weights ($\bar{M}_w$) of 13×10^4, 7.1×10^4 and 6.3×10^4, respectively, as determined by viscometry using the relation $[\eta] = 3.13 \times 10^{-3} (\bar{M}_w)^{0.965}$ [14]. Direct coupling of adriamycin (ADR) onto PGA was carried out by using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, EEDQ, as a coupling agent (Fig. 1). This procedure allows the selective coupling of the amino group in the daunosamine moiety of adriamycin without the concomitant formation of esters. The conjugates designated as P_x—A (x refers to the batch of PGA) were obtained in yields up to 97% and with 16—22 weight percent of adriamycin. It was noticed that adriamycin readily gives π complexes with the P—A conjugates and complexed adriamycin has to be removed by ion-exchange chromatography [11] before biological testing of the conjugates.

The attachment of adriamycin onto PGA through spacer groups was carried out in two ways, which are depicted schematically in Fig. 2. In an earlier approach [11] we first introduced different peptide spacers onto the PGA via preactivation with saccharin and *N,N'*-carbonyldiimidazole and subsequent reaction of the saccharin imides with *N,N,N',N'*-tetramethylguanidine salts of the oligopeptides. The degrees of substitution usually obtained were in between 90—100% of available carboxylic acid groups of the polymers. Typical spacers used are Gly-Gly-Leu and Gly-Gly-Gly-Leu. After grafting of

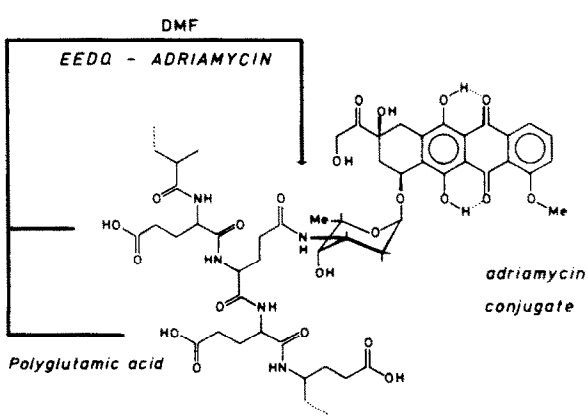

Fig. 1. Chemical coupling of adriamycin with PGA.

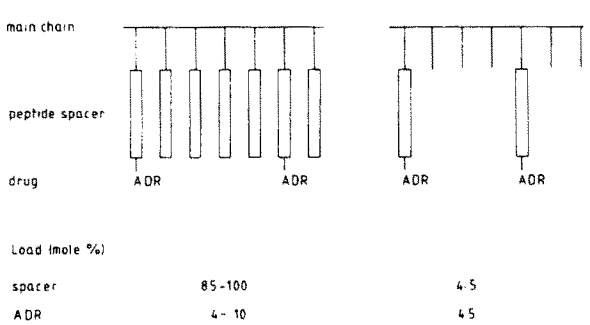

Fig. 2. General structures of two types of macromolecular prodrugs of adriamycin using PGA as the carrier.

PGA with the spacer, adriamycin was again coupled with EEDQ. The resulting conjugates are designated for instance as P_x—GGGL—A, where P = PGA, G = Gly, L = Leu and A = adriamycin, and the index indicates the batch number of PGA. The degree of substitution of adriamycin onto the grafted carriers varies between 3.6 and 10 mol.% of available carboxylic acid groups.

An alternative way to attach adriamycin onto PGA through spacer functions is to first prepare adriamycin-spacer entities which are subsequently coupled onto PGA. The synthetic approach is depicted in Fig. 3. The appropriate *N*-trityl protected spacers were synthesized in overall yields of 75—97%. After activation through the *N*-hydroxysuccinimide ester, adriamycin was coupled giving the protected adriamycin spacer compounds in 80—82%. Removal of the *N*-trityl group by 75% acetic acid gave the unprotected adriamycin spacer, which was isolated as the HCl salt in 75—79%. These residues were then coupled onto PGA using the EEDQ method described previously. After purification and lyophilization polymers with 4.5 mol.% adriamycin residues were obtained. These conjugates are further designated as P_x—[spacer—A].

All conjugates described above were tested for their anti-tumor activity using the L1210 clonogenic and the B16 mouse melanoma liquid assays. The results are given in Table 1.

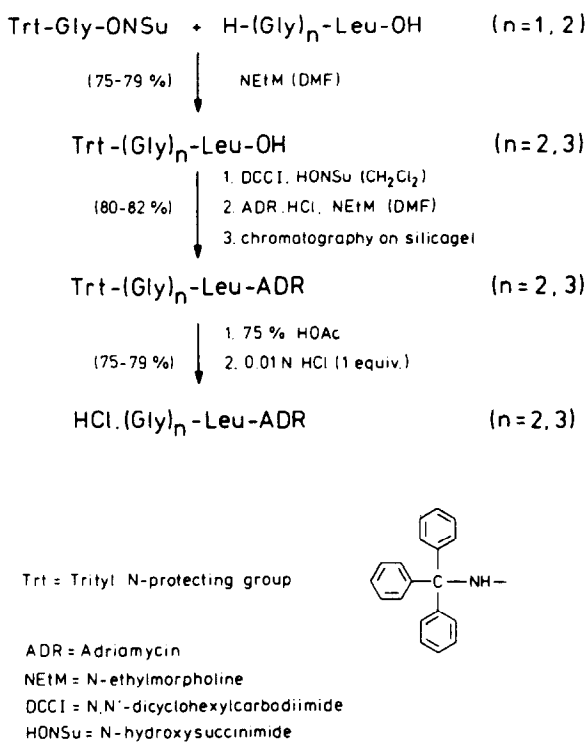

Fig. 3. Synthesis of peptide—adriamycin conjugates.

TABLE 1

ID_{50} values of adriamycin and adriamycin conjugates obtained with the L1210 clonogenic and the B16 liquid assays

Compound	Code[a]	Load[b] (mol.%)	ID_{50} L1210 assay (ng A/ml)	ID_{50} B16 assay (ng A/ml)
Free drug	A	–	21/24	4—5
Conjugate	P_2—A	5	1100/4100	–
Conjugate	P_3—GGL—A	96/4.3	200	70
(spacer)	P_2—G—GGL—A	95/67/10	1100/3300	270
	P_7—GGL—A	89/8	3000	220
	P_7—GGGL—A	84/8	560	200
	P_8—[GGL—A]	4.4/4.4	>65000	⩾650
	P_8—[GGGL—A]	4.5/4.5	>65000	⩾650

[a] Indices indicate batch numbers.
[b] Subsequent load percentages refer to subsequent substitution with spacer entities and adriamycin; A = adriamycin, P = poly(α-L-glutamic acid), G = Gly and L = Leu.

From Table 1 it can be observed that the conjugate P_2—A has a very low activity as compared to the free drug. When the P_3 and P_2 conjugates are compared, conjugates with the GGL spacer provide higher activities as compared to those with the G—GGL spacer. The latter spacer, however, was prepared in a two step procedure with incomplete substitution with G and GGL. This still leaves the possibility that considerable amounts of adriamycin are coupled onto a G spacer as compared to the G—GGL spacer. In the P_7 series conjugates with the GGL spacer give a very low activity. However, the adriamycin load is twice that of the P_3 conjugate, which possibly may influence the cytocidal activity. Increasing the spacer length of the P_7 conjugate with one glycine residue, using a direct coupling of GGGL onto PGA, gives higher activities as compared to the P_7—GGL conjugate. Surprisingly the P_8 conjugates with both a GGL and GGGL spacer show almost no activity, although only 4.4—4.5 mol.% of adriamycin spacer entities were attached.

In order to obtain further information, we have initiated studies on the interaction of conjugates with L1210 cells using laser flow cytometry [15]. We have also studied the enzymatic degradation of some of the conjugates using papain as a model enzyme.

INTERACTION OF CONJUGATES WITH MOUSE LEUKEMIA L1210 CELLS

The conjugates of PGA and adriamycin are designed in such a way that after uptake by the cells, degradation by lysosomal enzymes may take place and adriamycin or low-molecular weight adriamycin-peptide units may be released. After additional conversion of the peptides, free adriamycin enters the nucleus and intercalates with DNA which causes an inhibition of RNA- and DNA-mediated protein synthesis.

Two mechanisms can be distinguished by which the conjugates are taken up by the cells. These are adsorptive and passive (fluid-phase) pinocytosis as reviewed by Pratten et al. [16]. Duncan and Lloyd [8] described the pinocytosis of soluble synthetic polymers as drug carriers, whereas Kooistra [17] and Munniksma et al. [18] studied the pinocytosis of proteins. Parameters affecting the pinocytosis of synthetic polymers include the type of cell [16], the structure of the polymer including the molecular weight distribution [8], the composition of the medium [19] and the temperature [20,21]. When the polymer has affinity for the cell surface, adsorptive pinocytosis takes place and the rate of capture of the polymers by the cells is enhanced [8,22]. Complexes of anthracyclines and DNA [23] and covalently bound conjugates of daunomycin and albumin were studied *in vivo* [5] and *in vitro* [9] with mouse leukemia L1210 cells. The interaction of the former complexes with sarcoma, lymphoma and human leukemia cells *in vitro* was also studied with fluorescence microscopy and compared with that of the free drug [4]. It was shown that the macromolecular anthracycline complexes were internalized by the cells and then accumulated in lysosomes. The interaction of protein-bound methotrexate with L1210 cells was studied by Chu and Whitely [6].

We have used laser flow cytometry using the adriamycin fluorescence [15] to study the interaction of L1210 cells with the polymeric conjugate P_7—A, which presumably has the same very weak cytotoxic action as P_2—A (cf. Table 1) and appears to be quite stable on treatment with papain (see below). L1210 cells (0.5 ml of 1×10^6 cells/ml in culture medium) were exposed to the conjugate P_7—A which has a load of 6.3 mol.% of adriamycin-equivalents (0.125 ml of 1.0 mg/ml in PBS) at 37°C for 3 h. The cells were washed twice with phosphate buffered saline (PBS) centrifugated and resuspended in PBS prior to flow cytometry. The samples were measured in a home-built flow cytometer using a 3 watt argon ion laser tuned to 488 nm. The forward light scattering, orthogonal light scattering and fluorescence emission at

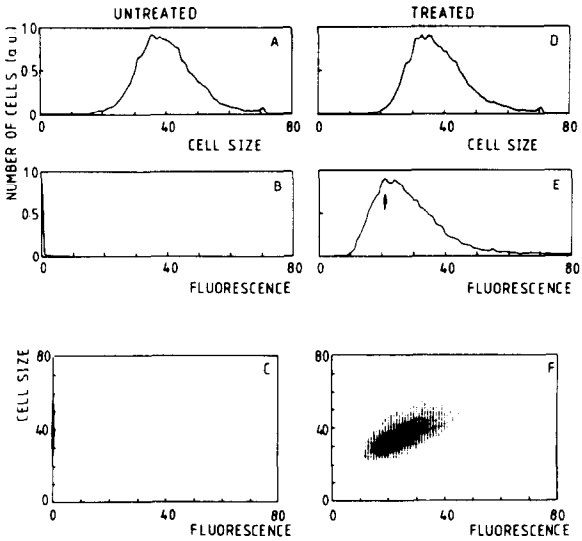

Fig. 4. Flow cytometric analysis of untreated mouse leukemia L1210 cells and of cells which were exposed to the polymeric conjugate P_7—A at 37°C for 3 h. Conditions: see text.

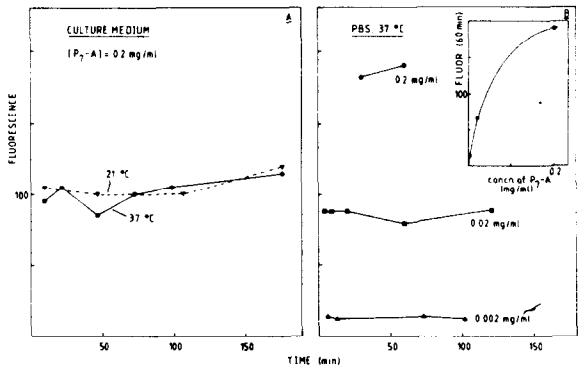

Fig. 5. Interaction of L1210 cells with conjugate P_7—A as determined with flow cytometry (Fig. 4). Dependence of the cell-associated adriamycin fluorescence on incubation time in culture medium at two temperatures (A) and in PBS at 37°C with different concentrations of P_7—A (B) Dependence of cell-associated fluorescence after 60 min on the concentration of P_7—A in PBS (B insert).

90° were analyzed with a home-made multichannel analyzer connected to a LSI 11/23 minicomputer. For each experiment data were collected for 10,000 to 12,000 cells. The forward light scattering gives information about the cell size and the fluorescence is related to the amount of adriamycine associated with the cells.

Figure 4 shows the relative number of cells versus forward light scattering and the relative number of cells versus fluorescence (histogram) as well as two-dimensional dot plots of forward light scattering versus fluorescence (scatter plot) for cells exposed and cells unexposed to the conjugate P_7—A. The significant fluorescence observed with the treated cells indicates that the polymeric conjugate either binds to or is taken up by the cells. The fluorescence intensity at maximum frequency (Fig. 4E, arrow) of cells exposed to P_7—A in culture medium was determined at various incubation times at 21°C and 37°C (Fig. 5A). Very similar results at both temperatures were obtained. The fluorescence increases rapidly (within 10 min) to a plateau value. Cells were also incubated for various periods in PBS at 37°C with different concentrations of P_7—A. After incubation the cells were also washed twice with PBS. Within 10 min plateau values were obtained for each concentration of P_7—A. Higher plateau values were observed when the conjugate concentration in the solution was increased (Fig. 5B). These data strongly suggest that the observed fluorescence is mainly due to adsorption of P_7—A onto L1210 cells as will be discussed below.

Pinocytosis is a process requiring metabolic energy and is strongly dependent on temperature [20,21]. In our experiments the cell fluorescence was independent on the temperature. Furthermore, the rate of uptake of nondegradable polymers by pinocytosis is generally constant while we have observed a rapid initial increase in the amount of cell bound conjugate, which then remains constant for at least 3 h. Pinocytosis of *N*-(2-hydroxypropyl)methacrylamide copolymers by rat visceral yolk sacs occurs with a constant rate for a period of at least six hours [25]. Therefore, we conclude that the measured fluorescence of the cells is mainly caused by adsorbed prodrug.

Due to the apparent adsorption of P_7—A onto the cells, a possible pinocytosis in our

case may be obscured. The binding of P_7—A onto the cells is quite strong, because a substantial amount of adsorbed P_7—A was not removed after two washing steps with PBS. Tritton and Yee [26] reported that adriamycin coupled to an insoluble agarose support does not enter the cell and is still actively cytotoxic for L1210 cells. This was explained by a membrane-mediated cytotoxicity. It has been reported by Murphree et al. [27] that adriamycin induced changes in the surface membranes of sarcoma 180 ascites cells. It cannot be excluded that the adsorption of P_7—A onto L1210 cells is mediated by the adriamycin moiety. Contrary to the results of Tritton et al. [26] the P—A conjugates are practically non-cytotoxic, suggesting that adriamycin bound to PGA interacts in a different way with the cell membrane than the insoluble agarose-bound adriamycin residue.

DEGRADATION OF PGA-DERIVED ADRIAMYCIN PRODRUGS WITH PAPAIN

A prerequisite for an efficient lysosomotropic endocellular release system is that after internalization the system is degraded at a sufficient rate by the lysosomal enzymes. The enzymatic cleavage of drugs from macromolecular carriers has been studied by Trouet et al. [5,9] using daunomycin bound to derivatized proteins and by Kopecek and coworkers [4,25] focusing on the release of *p*-nitroaniline as a model compound from poly(acrylamide)-type matrices. These studies revealed that the length and sequence of the peptide spacers used is crucial for the rate of drug release. Generally, the spacer should consist of at least three amino acids, while the sequence may be optimized for lysosomal (cathepsin mediated) degradation [4].

To study the susceptibility of our conjugates for enzymatic attack, we have performed degradation studies with papain [28] as a model enzyme. Solution of conjugates in citrate buffer (pH 5.0) were incubated with papain. At appropriate times samples were taken, which were mixed with borate buffer (pH 10) and extracted with chloroform/methanol (4:1, v/v). The combined fluorescence intensity of adriamycin and/or neutral peptide derivatives of adriamycin in the organic layer was followed (λ_{exc} = 480 nm and λ_{em} = 560 nm) for different conjugates as a function of time (Fig. 6).

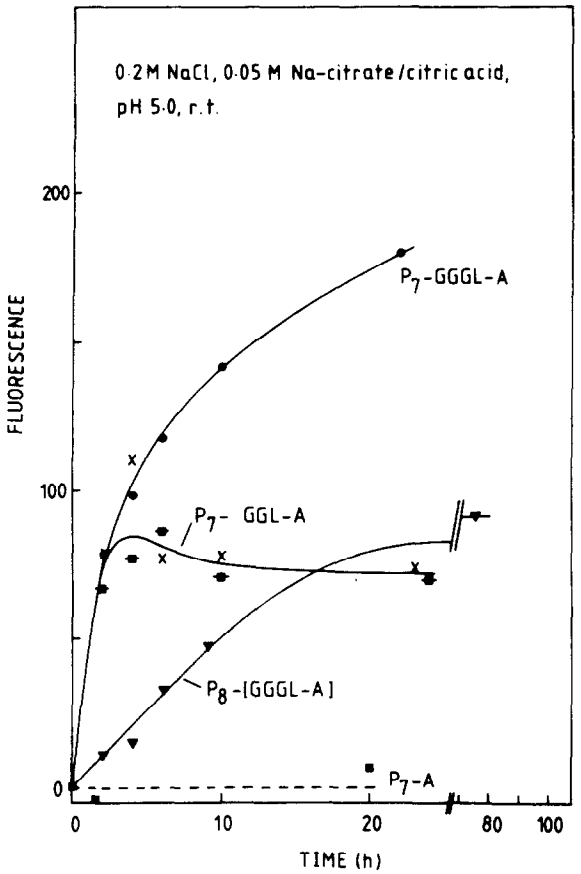

Fig. 6. Time-dependent release of extractable fluorescent products (λ_{exc} = 480 nm, λ_{em} = 560 nm) after enzymatic hydrolysis of various PGA-bound adriamycin prodrugs with papain in buffer, pH 5.0 (0.2 *M* NaCl, 0.05 *M* Na citrate/citric acid, 1 m*M* EDTA and 5 m*M* cysteine). Concentrations: spacer-bound conjugates, 2.0×10^{-3} *M* of ADR equiv., 7×10^{-5} *M* of papain; P_7—A, 2.1×10^{-4} *M* of ADR equiv., 3.2×10^{-5} *M* of papain.

The data presented in Fig. 6 show that the initial release of fluorescent compounds from P_7—GGGL—A and P_7—GGL—A, which are

highly substituted by spacers, is significantly higher than the release from P_8—[GGGL—A] with only one spacer residue per adriamycin molecule. These data are consistent with those on cytotoxic activities presented in Table 1. The release of fluorescent compounds from P_7—GGL—A levels off after a very short period, which cannot be interpreted at the present time. The conjugate with γ-glutamyl-bound adriamycin (P_7—A) does not release fluorescent extractable products to a significant extent, which is consistent with the specificity of papain for cleavage of α-amide bonds.

TLC analysis of release products from conjugates with a tetrapeptide spacer reveals that adriamycin and at least one other compound (possibly Gly-Leu—adriamycin) were present in the organic phase. The tri-spacer conjugate mainly released adriamycin. All data were obtained from degradation experiments carried out at a pH value of 5.0, which is the optimal pH for papain-mediated degradation of PGA [29]. The optimum pH for degradation is related to the helix—coil transition of PGA which has a pH mid value of 5.0.

We have determined the pH dependence of the adriamycin fluorescence intensity in the P_7—A conjugate (Fig. 7). A pH mid value of about 5.5 was found, which indicates that the covalently bound adriamycin moiety only slightly modifies the conformation of the main chain. Therefore, it cannot be excluded that with the P_7—A conjugate also cleavage of the main chain takes place. The pH dependent fluorescence of adriamycin showed a different course as compared to that of the conjugate.

We have applied viscometry using a micro Ubbelohde type instrument for the study of the PGA-backbone degradation by papain. PGA as well as the conjugate P_7—A were incubated with papain in a buffer solution at pH 5.3 and the relative viscosity (η_r) was determined at various times (Fig. 8). The parent polymer, PGA_8, which has a molecular weight comparable to that of PGA_7, was degraded as expected [29]. The viscosity of the adriamycin derivative P_7—A is much lower

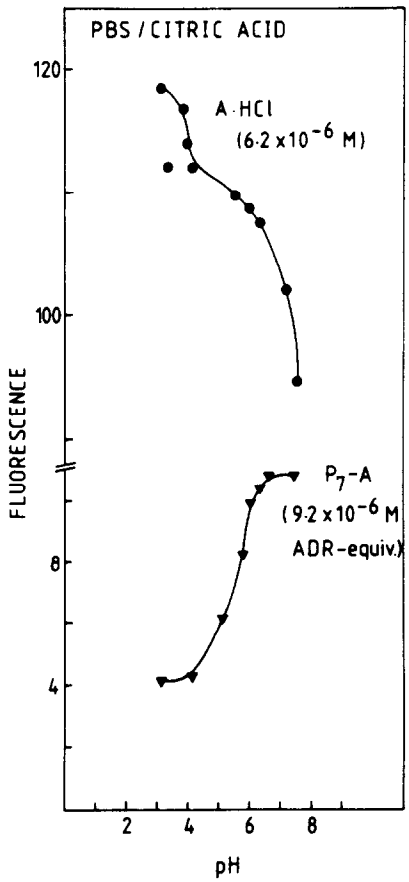

Fig. 7. Dependence of the fluorescence emission (λ_{exc} = 488 nm, λ_{em} = 564 nm) of adriamycin and P_7—A on pH.

than that of the parent polymer and remains constant after a small initial decrease upon treatment with the enzyme. The lower initial viscosity of the conjugate may be explained by some bond cleavage during the coupling procedure or by an effect of the adriamycin moiety on the polymer conformation. The latter possibility is unlikely because a conformational transition of the P_7—A conjugate occurs at almost the same pH as with PGA. The small initial decrease in viscosity observed with P_7—A after papain treatment can be explained by rapid cleavage of some of the amide bonds in the main chain. Possibly oligomers of glutamic acid with adriamycin residues are not susceptible to further enzymatic degradation.

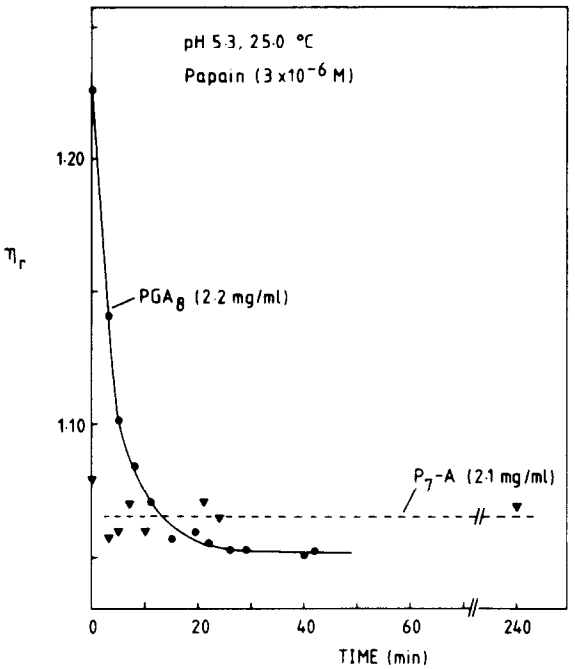

Fig. 8. Enzymatic degradation of PGA_8 and P_7—A with papain in buffer, pH 5.3 (0.2 *M* NaCl, 0.05 *M* Na citrate/citric acid, 1 m*M* EDTA and 5 m*M* cysteine) at 25°C. Dependence of relative viscosity (η_r) on incubation time. Polymer concentrations: PGA_8, 2.2 mg/ml (free acid); P_7—A, 2.1 mg/ml (Na salt).

CONCLUSIONS

Adriamycin and adriamycin—peptide residues were released from PGA conjugates by papain enzyme when tri- or tetrapeptide were used as spacers. This result is consistent with the findings of Trouet et al. [5,9] and Kopecek et al. [4,25].

PGA conjugates of adriamycin with one spacer unit per adriamycin molecule show a substantial lower cytotoxicity as compared to conjugates which were first almost completely substituted with spacer units and then reacted with adriamycin.

The rate of enzymatic degradation of these conjugates is in accordance with their cytotoxicity.

When conjugates of adriamycin and PGA without a spacer were contacted with L1210 cells a rapid concentration dependent adsorption was observed.

ACKNOWLEDGMENTS

Dr. W.J.F. van der Vijgh, Mr. J. v.d. Poort, Prof. Dr. H.M. Pinedo and Dr. P. Lelieveld are acknowledged for testing the cytotoxicity of the conjugates. Thanks are due to Mrs. G. Nolten for culturing the L1210 cells and to Miss L. Rotman for typing the manuscript.

Parts of this study were supported by a grant from the Dutch Cancer Foundation.

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