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DEGRADATION OF SIDE CHAINS OF N-(2-HYDROXYPROPYL) METHACRYLAMIDE COPOLYMERS BY LYSOSOMAL ENZYMES

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SUMMARY

A series of twenty two N-(2-hydroxypropyl) methacrylamide copolymers, each containing a different, potentially degradable side chain, were incubated with rat liver Tritosomes. Four of the side chains were digestible as judged by the liberation of a terminal 4-nitroaniline residue. The pH optimum for the degradation of the side chain -ε-aminocaproyl-phenylalanyl-4-nitroanilide was in the range 5.75-6.5 over the first hour of incubation and somewhat lower (pH 5.5-6.0) after this time. The degradation of the above side chain had a Km value of 58.3 mg/ml.

INTRODUCTION

The binding of pharmacologically active compounds to macromolecules offers a potential mechanism to enhance the specificity of drug action. If a drug that rapidly penetrates all cells by random diffusion is associated with a larger carrier molecule, endocytosis becomes its only mode of entry, and this can be a highly cell- and substrate-specific mechanism (1,2). Substances captured by endocytosis are transported into the lysosomal compartment of the cell where the drug, if it is to have therapeutic efficacy, would need to be released from its macromolecular carrier and escape into the cytoplasm.

Some biopolymers have been used to this end. DNA has been complexed with daunorubicin, an antibiotic that binds avidly to DNA and interferes with replication (3). DNA-daunorubicin complexes administered intra-

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peritoneally into mice were less toxic than free daunorubicin and somewhat more effective against L 1210 leukaemia. More recently it has been shown (4) that attachment of methotrexate, the dihydrofolate reductase inhibitor, to poly-L-lysine enabled the drug to penetrate and retard the growth of a (normally resistant) Chinese hamster ovary cell line. Synthetic polymers offer certain advantages over naturally occurring ones in that they can be specifically tailored to possess properties appropriate to the biological system in which they are to be used; the theoretical application of such compounds as drug carriers has been extensively discussed (5,6).

For controlled release of drugs within the lysosomal system it is necessary that degradable side chains are incorporated into the polymeric carriers, thus providing a site for drug attachment and release. In order to test the feasibility of such a system, water-soluble copolymers of methacrylamide and N-(2-hydroxypropyl) methacrylamide have been synthesized, in which the methacrylamide residues were extended by side-chains that contained potentially degradable linkages and that terminated in a 4-nitroaniline residue. Here we report a preliminary investigation to assess the susceptibility of these copolymers to hydrolysis by rat liver lysosomal enzymes. Using the most readily digestible copolymer we have also examined the effect of pH and substrate concentration on the rate of release of 4-nitroaniline.

MATERIALS AND METHODS

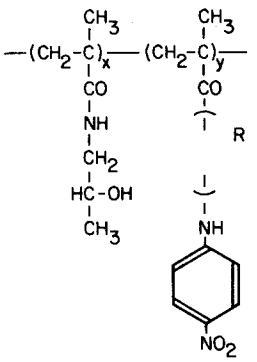
Chemicals. All the copolymers used (see Table 1 for chemical structures) contained approximately 98% N-(2-hydroxypropyl) methacrylamide and approximately 2% degradable side chains. They were prepared as described previously (7,8,9). All other chemicals were from B.D.H. Chemicals Ltd., Poole, Dorset.

Degradation of Synthetic Copolymers. Rat liver 'Tritosomes' in sucrose were prepared according to the method of Trouet (10). Copolymer was weighed out (5 mg, except in the experiment involving a range of substrate concentrations) in a 1 ml cuvette and then dissolved in 0.7 ml of potassium phosphate buffer (0.2M, containing 0.2% Triton X-100) pH 5.5 (except in the experiment involving a pH range). At the beginning of the incubation 0.3 ml of undiluted Tritosomes was added and degradation followed by measuring the liberation of 4-nitroaniline at 410 nm, using a Cecil CE 545 dual beam spectrophotometer, each experimental cuvette

being read against a blank containing 0.7 ml of buffer and 0.3 ml of Tritosomes. During the incubation (time periods up to 5h) the cuvettes were maintained in a 37°C water bath.

To confirm that the copolymers were stable under all the conditions of incubation, but in the absence of Tritosomes, control experiments were performed with each experiment in which copolymer was incubated with appropriate buffer and the absorbance monitored.

Table 1.



x 97.08 - 98.29%

y 1.71 - 2.92%

CODE NO.	SIDE CHAIN R	AMOUNT OF POLYMER (g) CONTAINING 1 mole OF ACTIVE GROUPS	MEAN RATE OF DEGRADATION (n mole/h)	MEAN RELATIVE RATE OF DEGRADATION
1	-Gly-Ala-Phe-NAp	6591	-	-
2	-Gly-Ileu-Phe-NAp	8610	-	-
3	-Gly-Val-Phe-NAp	8065	-	-
4	-Gly-Gly-Phe-NAp	5550	-	-
5	-Gly-Gly-Phe-Phe-NAp	6043	4.3	0.32
6	-Gly-Ala-Tyr-NAp	6448	-	-
7	-Gly-Ileu-Tyr-NAp	8770	-	-
8	-Gly-Val-Tyr-NAp	8293	-	-
9	-Gly-Gly-Tyr-NAp	5683	-	-
10	-Gly-Gly-Phe-Tyr-NAp	6125	8.3	0.75
11	-Acap-Phe-NAp	5627,7447*	15.6	1.0
12	-Acap-Leu-NAp	5193	-	-
13	-Gly-Phe-NAp	5712	-	-
14	-Gly-Gly-Gly-Phe-NAp	4241	insoluble so not determined	
15	-Gly-Leu-Phe-NAp	4773	7.8	0.45
16	-Gly-Phe-Phe-NAp	9253	-	-
17	-Gly-D-Phe-Phe-NAp	7538	-	-
18	-Ala-Gly-Val-Phe-NAp	8014	-	-
19	-Gly-Gly-Val-Phe-NAp	8503	-	-
20	-Gly-Phe-Tyr-NAp	8707	-	-
21	-Gly-βAla-Tyr-NAp	5717	-	-
22	-Gly-Leu-NAp	4420	-	-

The structure of the N-(2-hydroxypropyl)methacrylamide copolymers are shown. The rate of degradation of each sidechain was assessed as described in the "Methods" section and results are expressed as nmole of 4-nitroaniline released per hour. Measurable degradation was only observed with copolymers 5,10,11 and 15. The mean relative rate of degradation is also shown and this value represents the ratio of the rate of degradation measured for a copolymer to the rate measured for copolymer 11 in the same experiment. This value serves to normalize the data which were collected with several different preparations of Tritosomes.

* Two different preparations of this compound were used.

RESULTS

At pH 5.5 only four of the copolymers (numbers 5, 10, 11 and 15) showed any measurable release of 4-nitroaniline over the incubation period (Table 1). The release of 4-nitroaniline from these copolymers was linear with time. The maximum rate of release was 15.6 nmole/h from copolymer 11. All the copolymers tested were completely stable during incubations at 37°C in the absence of Tritosomes, and those that were hydrolysed in the presence of Tritosomes released less than 10% of their chromophore over the incubation period.

Further experiments were carried out with copolymer 11 to determine the pH optimum for degradation and also the effect of substrate concentration. At pH values above 5.5 the rate of release of 4-nitroaniline was not linear with time over a 5h incubation period. A high initial linear release of nitroaniline over approximately the first hour was followed by a slower release which was maintained throughout the rest of the incubation. Fig. 1 shows the rates of degradation during the initial and later phases

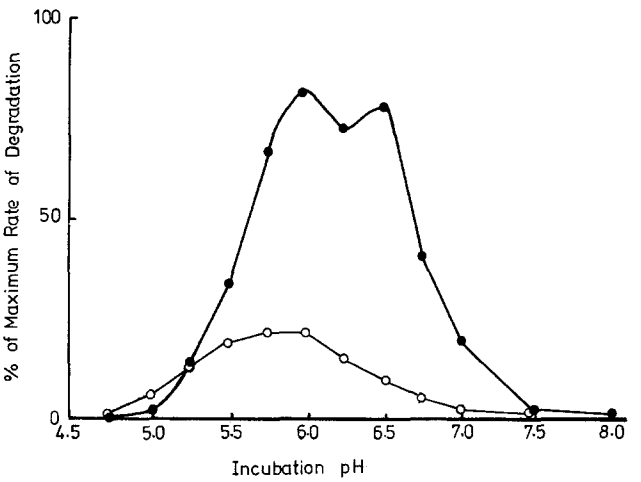


Fig. 1. Effect of incubation pH on the rate of release of 4-nitroaniline from copolymer 11 by rat liver Tritosomes, during the initial (0-60 min) (●——●) and second (1-5h) (○——○) phases of incubation. Since each experiment used a different preparation of Tritosomes, it was necessary to normalize rates observed in any given experiment by expressing them as a percentage of the maximum rate observed at any pH in that experiment. Each value shown on the Figure is a mean derived from four separate experiments.

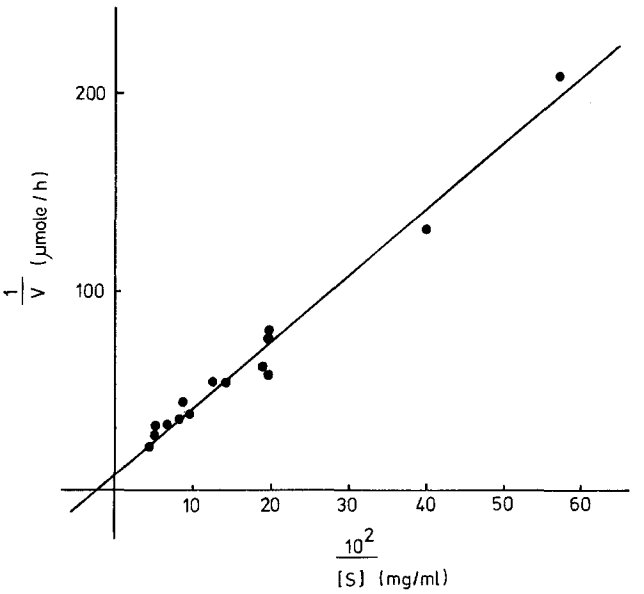


Fig. 2. The Linewear -Burk Plot shows the relationship between concentration of copolymer 11 (side chain -Acap-Phe-NAp) and its rate of degradation.

of incubation, for a number of pH values. It can be seen that the initial phase has a broad pH optimum in the range 5.75-6.5, whereas the slower rate of release over the later phase of the incubation has a pH optimum in the range 5.5-6.0.

The rate of release of 4-nitroaniline from copolymer 11 was measured over 95 min at substrate concentrations from 1.75 to 20 mg per ml. Fig. 2 shows a Lineweaver-Burk plot of the data, from which a Km value of 56.3 mg/ml can be calculated. In no case were more than 7.3% of the nitroaniline residues released during the incubation period.

DISCUSSION

We have shown that lysosomal enzymes can cleave a terminal 4-nitroaniline residue from four different side chains incorporated into N-(2-hydroxypropyl) methacrylamide copolymers. The length and composition of these degradable side chains is quite varied, and there is no discernible feature distinguishing degradable from resistant copolymers. Comparison of the results obtained with copolymers 15 and 16, or with 11 and 12, shows that small changes in side

chain composition, even the substitution of a single amino acid, converted a degradable polymer into a resistant one. Most of the copolymers used are susceptible to cleavage by chymotrypsin (7,9), an enzyme that preferentially hydrolyses peptide linkages in which a hydrophobic amino acid contributes the carboxyl moiety. As in the present study, the results obtained with chymotrypsin revealed that side chain composition affected hydrolysis rate. In general the rate of hydrolysis by chymotrypsin increased with increasing length of side chain, an effect attributed to reduced steric hindrance from the more distant main polymer backbone.

The degradation of copolymer 11 by lysosomal enzymes showed two slightly different pH optima, depending on the duration of incubation, but both were in the range pH 5.5 - 6.5. Measurements of the internal pH of lysosomes indicate values of 5-6 (11,12), and lysosomes contain many different hydrolytic enzymes (13,14), including a number of endo- and exo-peptidases that have been shown to display a wide range of individual pH optima (from 2.5 to 8.0) when incubated with a variety of substrates. Although we have demonstrated that a mixture of lysosomal enzymes isolated in the form of Tritosomes can degrade synthetic copolymers, it would be unwise at this stage to speculate as to which enzyme or enzymes are involved in the liberation of 4-nitroaniline. The side chains may well be cleaved in more than one position before free 4-nitroaniline appears. It is unclear why the initial rate of release of 4-nitroaniline at higher pH is more rapid; possibly one of the enzymes involved loses activity during the relatively long incubation period employed.

It has been stated (15) that there are two primary objectives in chemotherapy: to enhance the specificity of drug action and to increase the duration of action. We believe that synthetic macromolecular carriers have the potential to provide an efficient drug delivery system. The finding that lysosomal enzymes (the intracellular enzymes that a carrier complex injected intravenously would encounter) can cleave some peptide side chains attached to a non-biodegradable synthetic polymer is a new and important observation.

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