Studies on Functionally Terminated Poly(vinyl Alcohol) Polymers

by

Gunilla E. Meinander

A thesis
presented to the University of Manitoba
in partial fulfillment of the
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in
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STUDIES ON FUNCTIONALLY TERMINATED POLY(VINYL ALCOHOL) POLYMERS

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ABSTRACT

Synthesis and characterization of functionally terminated poly(vinyl alcohol) polymers have been investigated.

Carboxyl and amino terminated poly(vinyl alcohol)s were prepared via chain transfer reactions during the polymerization process. However, these terminal groups were found to be unreactive because of the nature of the polymer. By introducing a ten carbon hydrophobic spacer arm between the carboxyl group and the polymer backbone the reactivity of the terminal functional group could be enhanced.

In another attempt to reduce the influence of the polymer backbone on the functional group a poly(vinyl alcohol) - poly(ethylene glycol) graft copolymer, with a terminal functional group introduced on the poly(ethylene glycol) portion of the polymer was prepared.

ACKNOWLEDGEMENTS

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Chapter I

INTRODUCTION

In recent years much interest has been focused on the preparation and properties of conjugates of synthetic polymers with biologically active compounds. Drugs have been conjugated to water soluble polymers to achieve slow release of the biologically active substance in the body, and targeting to specific tissues where the drug is needed [1]. Drugs, in particular enzymes, which often cause severe allergic reactions when introduced into the body have been attached to soluble polymers via stable linkages [1-5]. These conjugates are nonimmunogenic and nonantigenic, which means that they have lost the ability to induce production of antibodies and do not combine with antibodies specific for the unconjugated enzyme. They can thus be safely administered to a patient without fear of allergic reactions. Conjugates of polymers to allergens have been shown to have a suppressive effect on ongoing allergic reactions towards the unconjugated allergens [6-9]. These properties make the polymer-allergen conjugates potential candidates as drugs in the treatment of allergic diseases.

Several different types of water soluble polymers have been used as the polymer part of the conjugates, e.g., poly-

saccharides [10-13], polyamino acids [14], gamma globulins [15], poly(vinyl pyrrolidone) [16-18], and poly(ethylene glycol) (PEG) [3-5]. Conjugates with PEG coupled at one end to the attached allergen have been shown to readily suppress ongoing allergic reactions [5-7]. Similar immunological studies with poly(vinyl alcohol) (PVA) coupled to small allergens along the backbone of the polymer chain via pendant reactive groups have given promising results [19-22].

The purpose of the present work was to study the synthesis of and to characterize PVA having a single reactive functional group attached at the end of the polymer backbone. Such a compound would be of interest as the polymer part of conjugates to molecules having biological activity, such as allergens.

Because of the nature of the PVA, functional end groups cannot be incorporated directly into the polymer, but have to be introduced either during the polymerization process or into the precursor polymer poly(vinyl acetate) (PVAc). Hence the terminal group has to be stable to the conditions of hydrolysis of PVAc to PVA. Nonhydrolysable functional groups were incorporated into PVA by chain transfer during the polymerization process. However, these end groups were found to be unreactive because of the influence of the backbone hydroxyl groups, when attached directly to the polymer. An enhanced reactivity of the terminal carboxyl group was obtained by introducing a hydrophobic spacer arm between the polymer backbone and the end group.

A terminal carboxyl group attached to PEG is more reactive than one attached to PVA due to the different immediate environment. Hence PVA-PEG block copolymers, where the terminal functional group is situated on the PEG portion of the polymers, were synthesized. A carboxyl terminated PVA-PEG graft copolymer was obtained by grafting vinyl acetate onto carboxyl terminated PEG. It should be possible to couple this polymer to ligands following the known procedures for conjugation of carboxyl terminated PEG to ligands.

After a presentation of the recent literature on the topics of importance for this work, the general methods used and the problems encountered during the course of the work with the polymers are discussed. This chapter is followed by a detailed description and a discussion of the results of the work. In the last chapter the experimental details of the analytic and the synthetic procedures are summarized.

Chapter II

LITERATURE REVIEW

In this chapter the literature on previous work, which gives the background for the present work is reviewed. As the present work involves the synthesis of a water soluble polymer which may be suitable for coupling to a ligand for use in immunological studies, the literature on the properties and applications of such polymer conjugates is reviewed, with an emphasis laid on the polymer-allergen conjugates. The coupling reactions of PEG, the most frequently used polymer in recent studies on polymer-allergen conjugates, are reviewed in some detail, while coupling reactions involving other polymers are discussed more briefly. The vast area of coupling enzymes to solid supports as well as the techniques of peptide synthesis which are applied to coupling soluble polymers to ligands have been reviewed in detail elsewhere [23-26]. The syntheses of PVA and PEG, the polymers which have been used in the present experimental work, will be described briefly, whereas the modification of end groups of PEG for conjugation purposes is reviewed here in more detail.

2.1 POLYMER-DRUG CONJUGATES

Proteins have been covalently coupled to both soluble and insoluble polymers to produce immobilized enzymes, which have a vast application , e.g., as biochemical adsorbents, in specific purifications of complementary species and in biochemical analysis [27]. Polymer-protein and polymer-drug conjugates have been synthesized for application in several new areas. Insoluble conjugates, being very bulky, cannot enter the cells and thus, by attaching drugs to such polymers, one can determine if they have extracellular activity. Tritton and Yee [28] coupled adriamycin, an anticancer drug, to crosslinked agarose and were able to demonstrate that the site of activity of the drug was on the surface of the cell membrane, contrary to what was believed before.

Soluble polymers have been used to replace biopolymers, for instance in the polymer portion in chromoproteides, with retention of the biological function of the molecule. Thus hemoglobin was covalently linked to soluble dextran [29-31], poly(ethylene glycol) [32], polyurethanes [33], and derivatives of poly(ethylene glycol) [33] for use as a blood replacement. The conjugates were able to bind oxygen reversibly. The plasma clearance half life of the hemoglobin was about four times longer for the PEG conjugated hemoglobin than for unmodified hemoglobin [32]. The clearance time corresponded directly to the effective size of the conjugate showing that the more bulky conjugates were excreted at a

slower rate. Geckeler and Mutter [34] replaced the oligosaccharide chain in Cinerubin A, an anthracycline antibiotic, with PEG, with retention of the activity of the drug.

Like the exogenous hemoglobin, enzymes used as drugs often have a very high plasma clearance rate because of rapid metabolism or an attack of proteolytic enzymes on the protein. Prolonged treatment with an exogenous enzyme also often causes antibody formation with an increased serum clearance rate and a decreased activity of the enzyme as a result. Superoxide dismutase has been used as an anti-inflammatory drug and as a radioprotectant. It is only slightly immunogenic, but has the disadvantage of having a very high plasma clearance rate, requiring readministration within short time intervals. Abuchowski and coworkers [35] coupled the enzyme to monomethoxy PEG. The conjugate had a sharply enhanced serum circulating life, was nonimmunogenic, and had a slightly improved anti-inflammatory activity. Boccu and coworkers [36] have investigated the pharmokinetic properties of PEG-superoxide dismutase conjugates and Gray and Stull [37] have investigated the radioprotective activities of PEG-superoxide dismutase and PEG-catalase conjugates. In all cases the serum circulating half lives were prolonged over that of the native enzyme.

Several enzyme-polymer conjugates, such as trypsin [38], carboxypeptidase G and arginase coupled to soluble dextran [2], acetylhexosamidase A [17], trypsin and chymotrypsin

[18] coupled to poly(vinyl pyrrolidone), and trypsin [39] and phenylalanine ammonia lyase [40] coupled to PEG have been shown to have an increased resistance towards both thermal and proteolytic denaturation and self digestion, as compared to the native enzymes. Since the conjugated enzymes have a higher stability after injection into the blood stream and are excreted at a much slower rate than the uncoupled enzyme, while still retaining the biological activity, these drugs can be administered much less frequently. The active concentration of the drug in the serum can be kept at a constant level over a long period of time using the conjugates, while the concentration level of the native enzymes varies from very high to very low, sometimes within less than one hour [36].

Some enzymes used as drugs are very immunogenic, producing antibodies after a few injections. The allergic reactions following continued administation of the drug can range in severity from a mild reaction to anaphylactic shock. The antibody production not only might cause dangerous allergic reactions, but also makes the high serum clearance rate even higher. However, if the enzyme is coupled to a nonimmunogenic soluble polymer, the immune system does not recognize the allergen, and the conjugate can safely be used without fear of allergic side reactions.

Asparaginase from various sources has been used with success in the early treatment of leukemia. However, because

of its high immunogenicity it can be administered only a few times before giving rise to antibody formation. Asparaginase and glutaminase-asparaginase were coupled to PEG and the immunogenicity and enzymatic activity of the conjugates were investigated by Abuchowski and coworkers [5,41-43], and by Inada and coworkers [4,44,45]. The conjugates have a substantially reduced immunogenicity and a serum half life several times higher than that of the native enzymes. The results when using PEG-asparaginase to treat cancer in man [5] were better than the results obtained with succinylated asparaginase [46]. The PEG conjugates also showed less immunogenicity and longer serum half life than an asparaginase-mouse albumin copolymer [47], asparaginase-poly-DL-alanine peptide [48], and glycosylated asparaginase [49].

Abuchowski and coworkers coupled PEG to uricase [50-52], which is used for the treatment of hyperuricemia and gout. Again, the conjugates showed no immunogenicity, and hence their plasma half lives were considerably longer than for the native enzyme after the first injection. On giving a single injection of the conjugate to man [52] the serum uric acid rapidly disappeared, the effect lasting for over 32 hours. Readministation thirty days later gave the same results , and no toxic side effects were observed. Nishimura and coworkers earlier demonstrated the disappearance of the binding ability of PEG-uricase towards anti-uricase serum [53].

In similar investigations, other enzymes have been coupled to PEG [3,54,55] with loss of immunogenicity and antigenicity as a result.

Polymer conjugates of proteins not only lose their ability to induce production of antibodies against the protein, but in many cases they also have been shown to be able to suppress an ongoing production of antibodies, with suppression of allergic reactions as a result. Conjugates having reduced antigenic and allergenic activities, but still retaining the immunosuppressive activities of the native antigen, may be of therapeutic use in the treatment of allergies. Several common allergens have been covalently attached to polymers, mostly PEG, and their immunosuppressive properties have been investigated. Sehon and coworkers have investigated PEG conjugates with allergens such as ragweed pollen and Timothy grass pollen antigens, ovalbumin, and bacterial allergens [7-9,56]. None of the conjugates induced antibody production against the native allergen, they did not combine with the specific antibodies in previously sensitized animals, and they all depressed markedly an ongoing IgE antibody formation in sensitized animals despite additional injections of the corresponding allergen. King and coworkers have investigated the immunological properties of the conjugates of ragweed pollen antigen E with monomethoxy PEG [57], a copolymer of D-glutamic acid and D-lysine [58], and with various alkoxy poly(ethylene glycols) [59].

They found that the immunological properties of conjugates prepared with different polymers were similar, the polymer assuming the role of reducing the antigenic valency of the conjugate, which is more notable for its immunological than for its immunosuppressive activity [59]. The immunosuppressive effects of several other conjugates have been investigated [60,61].

Small molecules or molecular groups which are the antigenic determinants of larger molecules are called haptens. Haptens such as the benzyl penicilloyl group, which is the main antigenic determinant of penicillins, and the dinitro phenyl group (DNP) have been extensively used in immunological studies as conjugates coupled to polyfunctional macromolecules such as proteins [15], polysaccharides [10-13], poly(vinyl pyrrolidone) [16] and poly(vinyl alcohol) [19-22]. The polymer-hapten conjugates induce a suppression of hapten specific IgE production, and could thus have a therapeutic use in the administration of many drugs.

2.2 COVALENT ATTACHMENT OF SOLUBLE POLYMERS TO LIGANDS

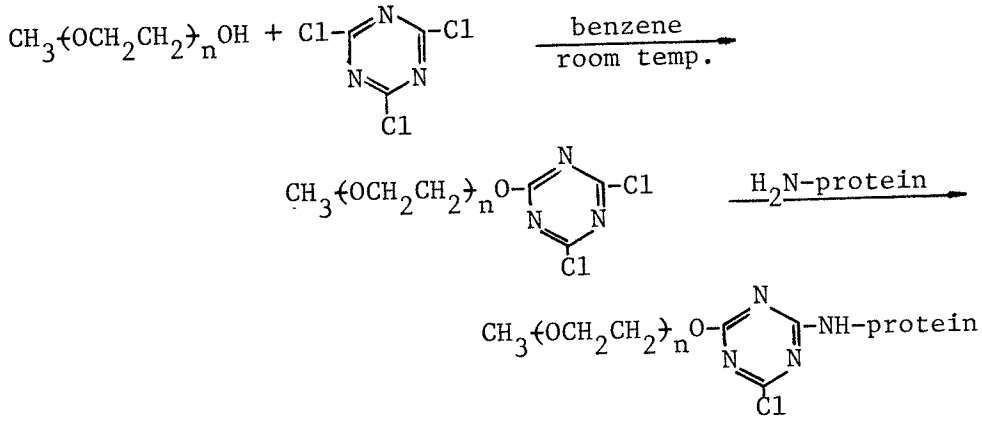
The procedures for coupling proteins and smaller molecules to polymers differ from one polymer to another. There are some common conditions that have to be fulfilled. For conjugates in which a drug is carried by the polymer, a slow release of the drug is desired, requiring a linkage that hydrolyses slowly under biological conditions. For a

polymer-allergen conjugate the linkage has to be stable under *in vivo* conditions. The linkage has to be nontoxic after normal metabolism. The coupling reaction with proteins has to be carried out in buffered cold aqueous solution, to prevent the protein from denaturing.

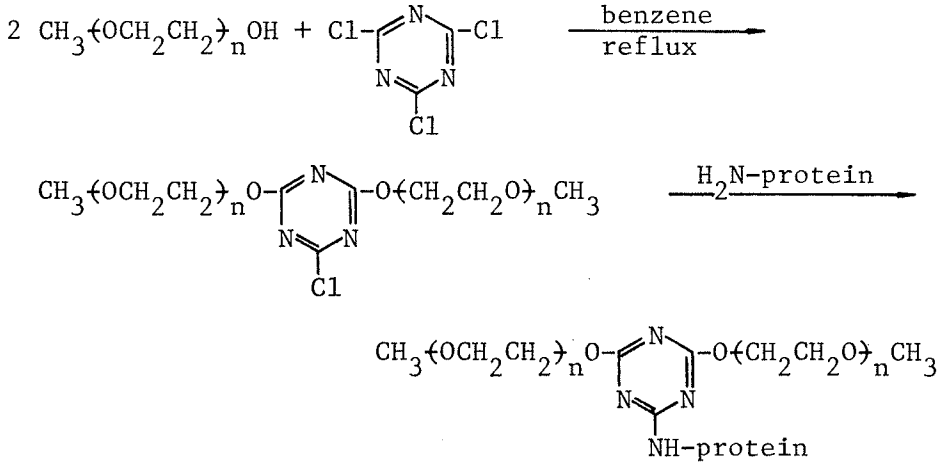
2.2.1 Poly(ethylene glycol) conjugates

The majority of the most recent investigations of polymer coupled proteins have been done on PEG-conjugates. Monomethoxy PEG, which is commercially available, is very soluble in both aqueous and organic solvents, and the single terminal hydroxyl group can easily be derivatized. The immunogenicity of PEG is also insignificant [62], in contrast to many polysaccharides and polyamino acids.

The most widely used technique to couple PEG to proteins and smaller molecules is to prepare an 'activated PEG' by reacting PEG with cyanuric chloride and then coupling the activated PEG to aminogroups on the protein [63]:



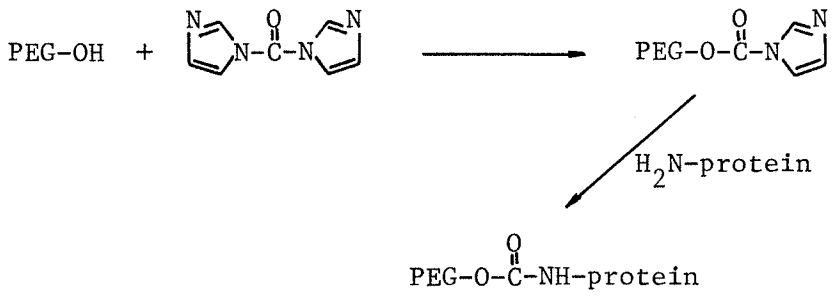
The activated PEG is prepared in an anhydrous benzene solution at room temperature. Under these conditions only one of the chlorine atoms of cyanuric chloride is substituted by PEG. The coupling reaction with the protein is carried out in aqueous solution. The amino nitrogen of the protein, being a better nucleophile than water, reacts with the second chlorine atom, while the third one is hydrolysed only at a slow rate [64]. Since there is a potential of two protein molecules reacting with the available chlorine atoms of the activated PEG, Matsushima and coworkers altered the procedure for making activated PEG in order to add two PEG strands per cyanuric chloride [44]:



Refluxing PEG with cyanuric chloride in dry benzene substitutes two of the chlorine atoms for PEG. There is now no risk of crosslinking the protein during the coupling reaction, since this activated PEG has only one reactive site.

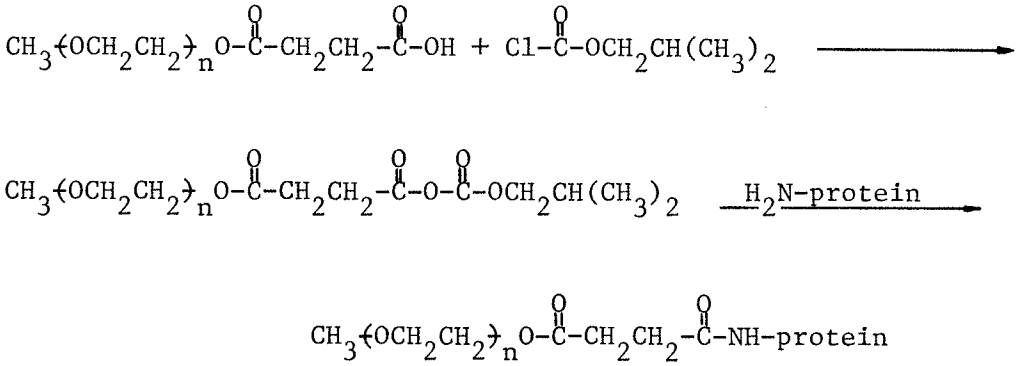
There are certain disadvantages in using cyanuric chloride as a coupling agent. Because of the high pH required for the coupling reaction and the high reactivity of the triazine activated PEG, coupling occurs not only to free amino groups of proteins, but also to aromatic hydroxyl groups and thiol groups. If thiol groups of the active center of an enzyme are modified, the biological activity of the enzyme may be lost after coupling. The triazine group absorbs light in the UV region used to monitor proteins (280 nm), thus interfering with the spectroscopic determination of the protein content of the conjugates. Cyanuric chloride is toxic <u>per se</u>, but the triazide linkage is both chemically and biochemically stable. However, the possible toxicity of the metabolism products is not yet known.

Beauchamp and coworkers have coupled proteins to monomethoxy PEG utilizing 1,1'-carbonyldiimidazole for activation of the polymer [65]. The procedure gives a conjugate with an ester linkage:



Sehon and coworkers used polymer-protein conjugates prepared with a nontoxic ester linkage [7]. Monomethoxy PEG was succinylated to give carboxyl terminated PEG

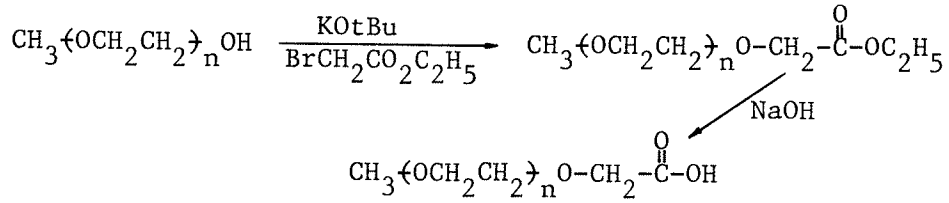
which then was reacted with isobutyl chloroformate to give a reactive mixed anhydride:



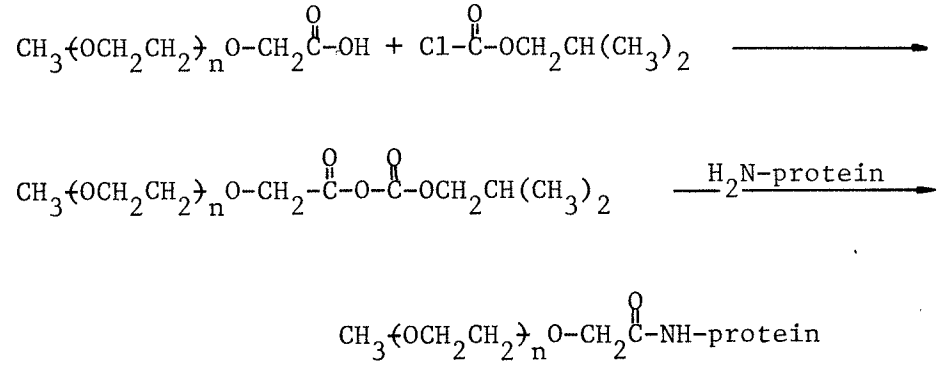
The mixed anhydride is reactive towards aminogroups, forming stable amide bonds with proteins . However, the ester link-

age between the polymer and the succinyl group is susceptible to enzymatic hydrolysis.

Ehrat and coworkers [66] have prepared a mixed anhydride attached to PEG via a stable ether linkage . The intermediate carboxyl terminated PEG was prepared by reacting monomethoxy PEG with potassium-t-butoxide and ethyl α-bromoacetate:

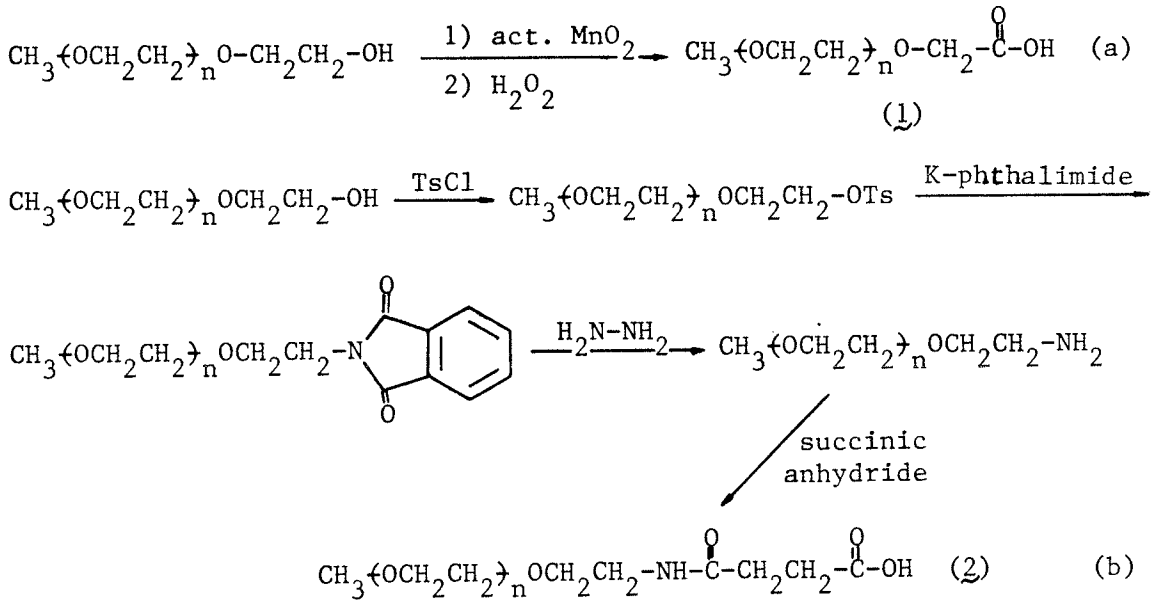


The carboxyl terminated PEG was then reacted with isobutyl chloroformate to produce the activated PEG, which was coupled to amino groups of insulin :

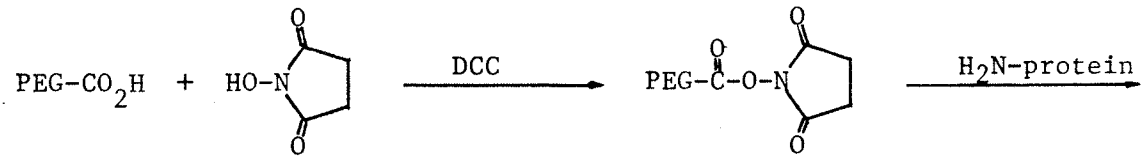


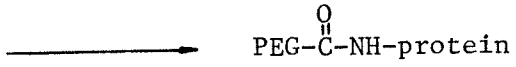
Boccu and coworkers [67] have coupled proteins to monomethoxy PEG via the reactive N-hydroxysuccinimide ester of the polymer. The intermediate carboxyl terminated polymers were prepared by two different methods, one involving a two step

oxidation of the terminal carbinol by activated manganese dioxide and hydrogen peroxide (reaction a), and the other using the method of Mutter and coworkers [68,69] for the preparation of amino terminated PEG, which then was reacted with succinic anhydride to give polymer (2) (reaction b).

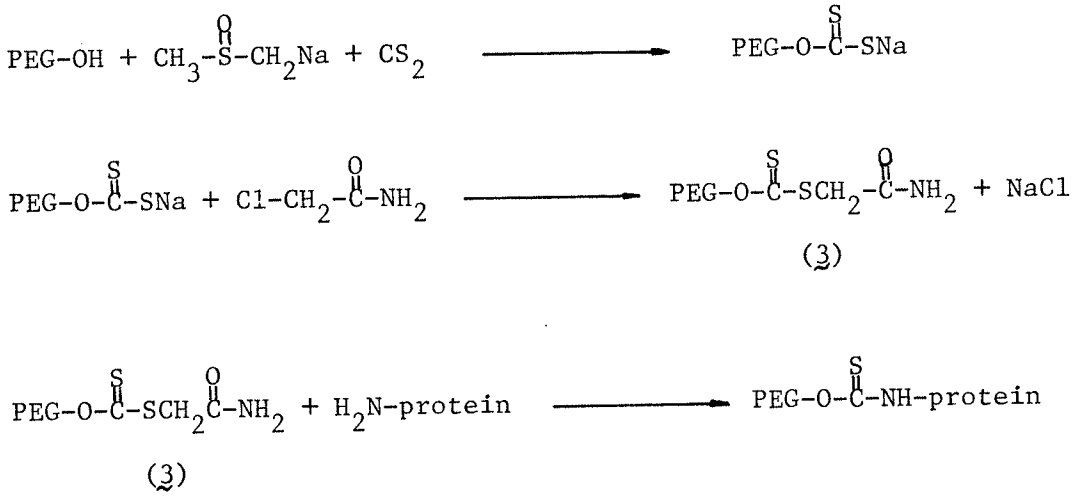


The carboxyl terminated polymers (1) and (2) were then reacted with N-hydroxysuccinimide using dicyclohexylcarbodiimide (DCC) as a coupling reagent. The succinimide ester of PEG is reactive towards nucleophilic amino groups of proteins under mild conditions (pH 8.2), leaving aromatic hydroxyl groups and thiol groups intact [67].





King and coworkers [70] have prepared an activated PEG which couples primarily to the amino groups of proteins, and to a lesser extent to hydroxyl groups. Monomethoxy PEG was converted to its S-carboxamido methyl dithiocarbonate (3) which then was coupled to the protein. The activated ester was prepared by reacting the xanthate salt of PEG with chloroacetamide. The xanthate salt of PEG was prepared by reacting the polymer with sodium methylsulfinyl carbanion.



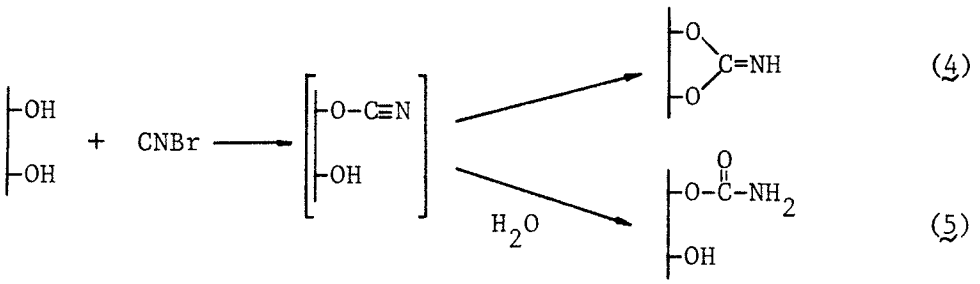
(3)

2.2.2 Polysaccharide Conjugates

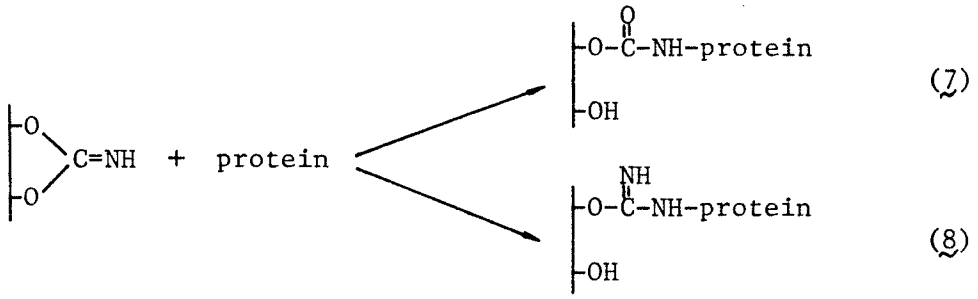
Many of the earliest conjugates prepared for *in vivo* immunological studies as well as conjugates prepared as drug carriers were made with soluble polysaccharides as the polymer portion [27]. While polysaccharides are still used as carriers for drugs to gain a longer lasting activity *in vivo*, the use of polysaccharides as the polymer portion in conjugates for immunological studies has become less significant, in favour of PEG, which is less immunogenic. The methods for coupling ligands to polysaccharides, especially the numerous different techniques of attaching enzymes to solid supports are of interest though, because some of these techniques can be applied to the synthesis of conjugates with other polymers. Since the methods of immobilizing enzymes to solid supports have been reviewed elsewhere [23-25], only the most frequently used techniques, and the techniques of special interest for the preparation of conjugates for use *in vivo* , are reviewed here.

Proteins and smaller molecules have been attached to water soluble polysaccharides either via the unmodified hydroxyl groups along the backbone of the polysaccharide or via derivatized groups of the polymers. All coupling reactions have been carried out according to the methods for immobilizing enzymes to soluble supports [23]. Soluble dextran, agarose, and other polysaccharides with unmodified hydroxyl groups as the main functional groups have usually

been activated for coupling to ligands by reaction with cyanogen bromide in alkaline solution according to the methods of Axén and coworkers [71,72]. The mechanism of the activation step is not well defined, which makes the amount of attached ligand difficult to control. The cyanogen bromide reacts preferentially with 1,2-diols forming an intermediate cyanate, which then converts either to a reactive imidocarbonate (4) or hydrolyses to an inert carbamate(5):



During the coupling step between the activated polysaccharide (4) and the amino groups of the ligand at least three different products will form, the imidocarbonate derivative (6), substituted carbamates (7), and isourea derivatives (8):



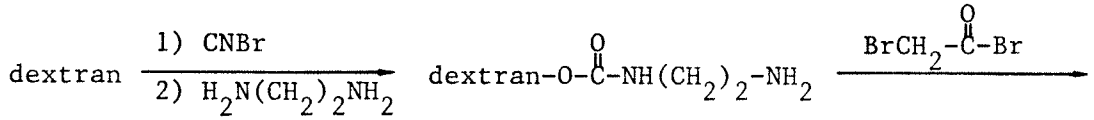
(7)

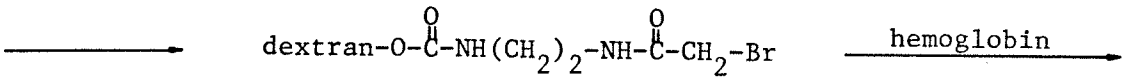
(8)

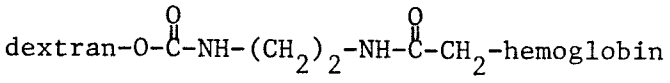
The isourea bonds of (8) are only moderately stable, which may result in a later loss of the ligand from the polysaccharide by hydrolysis [64,73].

Finlay and coworkers have modified the technique utilising cyanuric chloride to prepare an activated polysaccharide which was coupled to functional groups on a ligand [64a]. The method was similar to that used for coupling PEG to proteins (2.2.1).

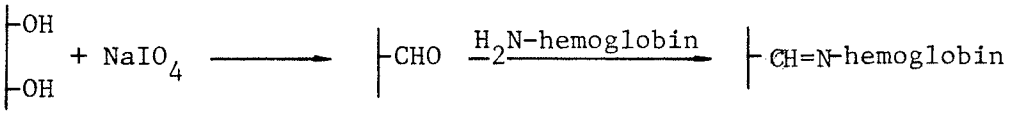
Hemoglobin has been coupled to soluble dextran by two different methods, the alkylation method and the dialdehyde method [74,29]. In the alkylation method the dextran was first converted to the amino ethylamino derivative by reacting dextran with cyanogen bromide in alkaline solution and then coupling with diaminoethane. The amino ethylamino dextran was then reacted with *α*-bromo acetylbromide giving *α*-bromo acetylamino ethylamino dextran, which was coupled to hemoglobin.

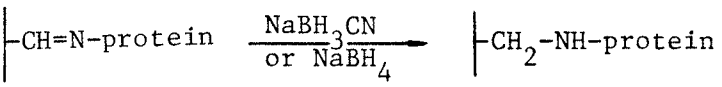






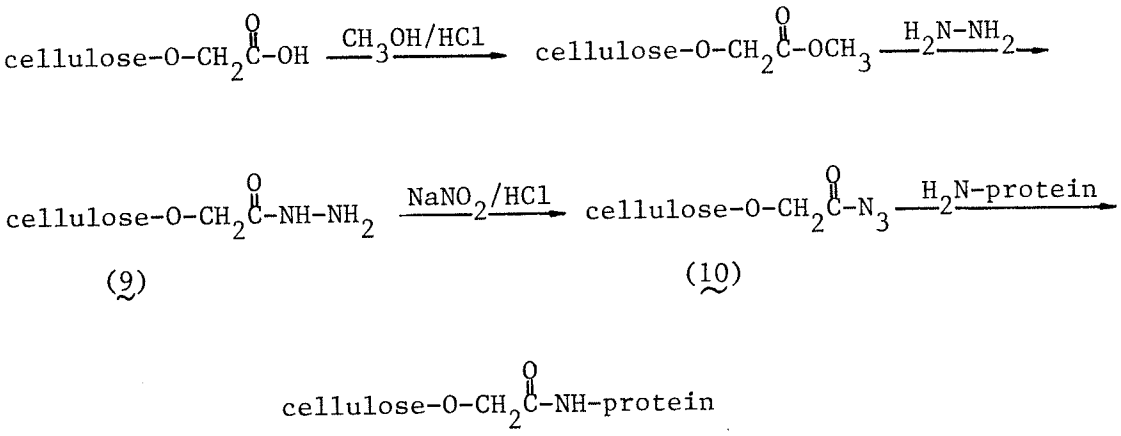
In the dialdehyde method vicinal hydroxyl groups were oxidized to aldehyde groups with sodium periodate, which then were reacted with amino groups on the hemoglobin. However , this reaction creates a Schiff's base type linkage which is susceptible to hydrolysis. In the preparation of immobilized enzymes, the coupling reaction by this method has usually been followed by a reaction of the intermediate Schiff's base with sodium borohydride to produce a stabilized secondary amine [73].





Bifunctional reagents such as bisoxiranes and divinyl sulfones, which couple simultaneously with the polymer and the ligand have been used for immobilizing enzymes on polysaccharides [75]. Because of the risk of crosslinking the polymer or the ligand these reactions are less useful.

Some of the methods used for coupling carboxymethyl cellulose (CMC) to enzymes have been applied to preparing PEG conjugates [67]. CMC itself has been used as a polymer carrier for drugs [27]. Micheel and Ewers [76] introduced the Curtius azide method of coupling enzymes to CMC , and the method was later modified by Mitz and Summaria [77]. According to this method, the methyl ester of CMC is reacted with hydrazine, and the resulting hydrazide (9) is then treated with sodium nitrite to give a reactive azido derivative (10), which is coupled to amino groups on the protein:



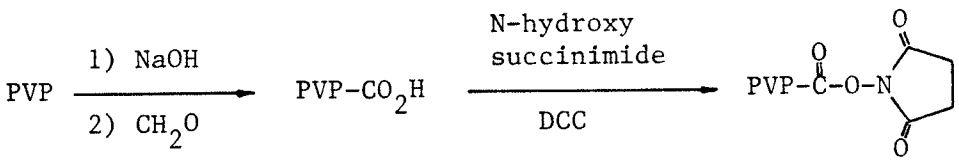
CMC and proteins have been coupled via amide bonds using coupling reagents such as carbodiimides and carbodiimidazole. CMC has been converted to an activated ester by reaction with dicyclohexylcarbodiimide (DCC) followed by N-hydroxy succinimide [57]. The activated ester forms amide bonds with amino groups of the protein. This reaction has also been applied to carboxyl terminated PEG [67].

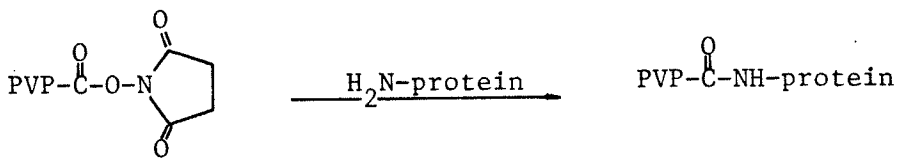
Polysaccharides have been derivatized by various methods to introduce spacer arms with terminal functional groups available for attaching to ligands [78]. The spacer arms are introduced to remove the ligands from the immediate vicinity of the polymers in order to to minimize steric interference during affinity chromatography.

2.2.3 Conjugates to synthetic polymers via pendant groups

Several different water soluble polymers and copolymers such as poly(acrylamide), copolymers of acrylamide and vinyl monomers, etc. have been used as supports for enzymes, and have been reviewed elsewhere [79]. Only those that are biocompatible and nonimmunogenic can be used in conjugates for *in vivo* use.

Poly(vinyl pyrrolidone) (PVP), an accepted plasma additive for humans, has been coupled via its activated succinimide ester to several enzymes [17,18]:

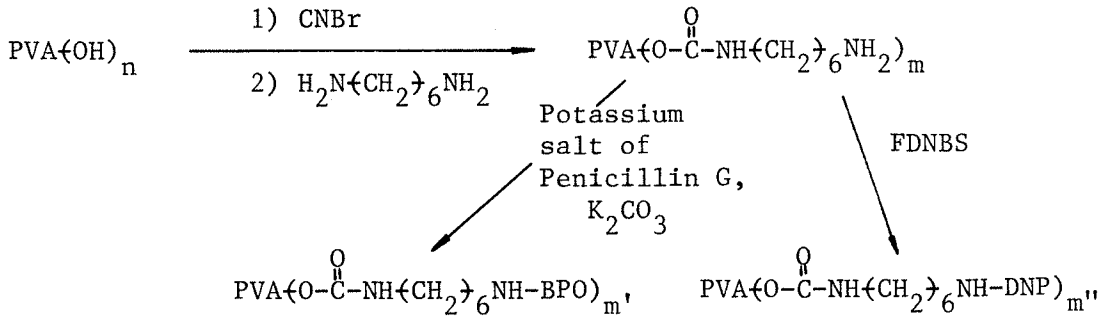




2.2.4 Polymer-hapten conjugates

Because of the obvious risks of crosslinking a polyvalent polymer during coupling to a protein, these polymers have mostly been used to prepare polymer-hapten conjugates for the purpose of immunological studies. These types of conjugates, where many small molecular groups (haptens) are attached to the same polymer chain, have been synthesized and used in immunological studies for more than two decades. The methods for attaching the haptens to the polymers have been essentially the same as those for attaching larger molecules to the polymers.

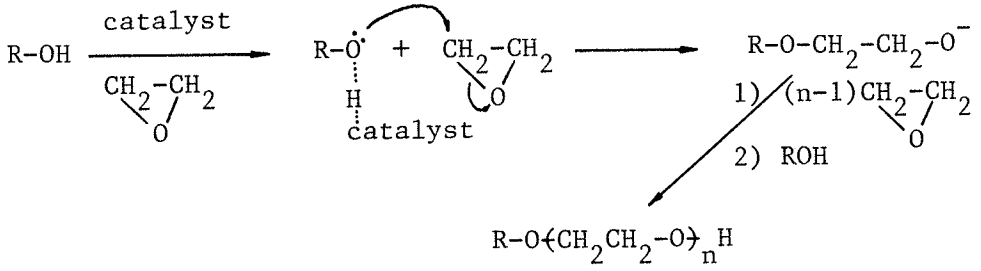
Sehon and coworkers [19-22] have prepared PVA-hapten conjugates for immunological studies. They coupled benzyl penicilloyl (BPO) and 2,4-dinitro phenyl (DNP) groups to amino groups along the backbone of PVA. The amino PVA was prepared by reacting cyanogen bromide treated PVA with 1,6-diaminohexane according to the method of Axén [71]. The potassium salt of penicillin G (BPO) and fluoro 2,4-dinitrobenzene sulfonate (FDNBS) were reacted with the amino PVA to give the conjugates:



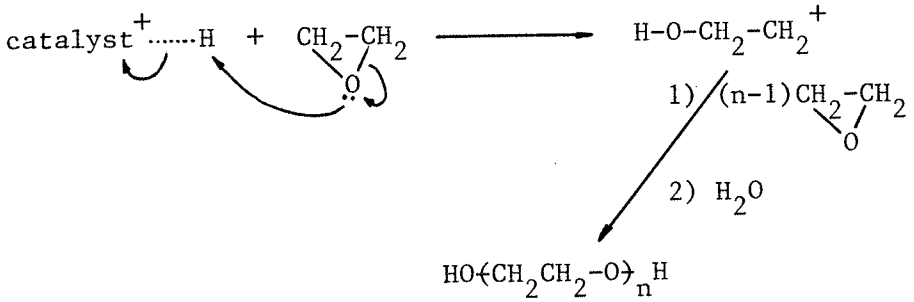
2.3 POLY(ETHYLENE GLYCOL)

Poly(ethylene glycol) (PEG) is the low molecular weight (< 5000 daltons) polymerization product of ethylene oxide. The polymer is neutral and soluble both in organic and aqueous solvents. The hydroxyl end groups are easily derivatized, which makes the use of the polymer very attractive for conjugation purposes. The commercially available monomethoxy PEG has most often been derivatized and coupled to drugs and allergens, but direct polymerization of the monomer onto the ligand is also possible in some cases.

Polymerization of ethylene oxide can proceed by an anionic , a cationic or a coordination mechanism [80]. Alcohols have been incorporated into the the polymer via an anionic polymerization:



Monomethoxy PEG is prepared in this way. The cationic and coordination polymerizations produce dihydroxy PEG:

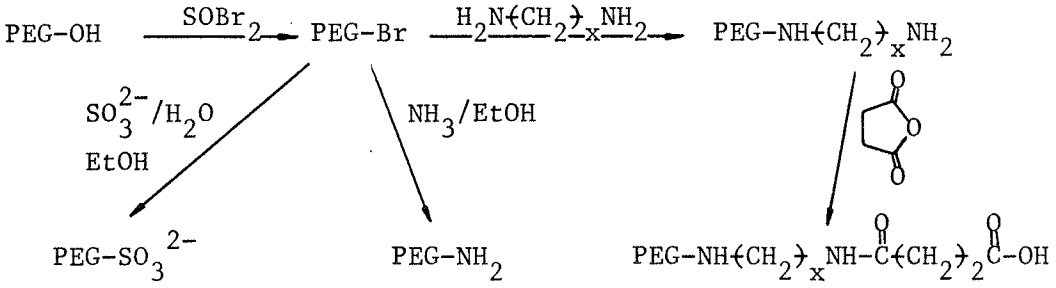


Geckeler and Mutter [34] have used boron trifluoride etherate as a catalyst in the cationic polymerization of ethylene oxide onto the aglycon of Cinerubin A.

PEG has many advantages in use as a carrier polymer for drugs and allergens. It is nontoxic and nonimmunogenic, both the unsubstituted and monomethoxy PEG are commercially available, and the terminal hydroxyl group(s) is(are) easily derivatizable. PEG dissolves readily both in organic solvents needed for derivatization, and in aqueous solution, which is required for coupling to proteins.

PEG has been modified to be used for conjugation purposes by several research groups. Carboxyl terminated PEG has been prepared both by esterification and etherification of the terminal hydroxyl group with carboxyl bearing reagents [81,82], by direct oxidation of the terminal hydroxymethylene group [67], or via amino derivatives of PEG [68,81,82] (see section 2.2.1).

Bückmann and coworkers [83] prepared an intermediate bromide terminated PEG, which was converted to carboxyl, amino, and sulphate terminated PEG:



Harris and coworkers [84] have repeated and further developed the synthesis of some of these PEG-derivatives. They have also reported the synthesis of new derivatives suitable for coupling to proteins and other molecules. PEG with terminal aldehyde groups was prepared both by oxidation with dimethyl sulfoxide (DMSO)-acetic anhydride, and by reaction with bromoacetaldehyde diethylacetal:

$$\text{PEG-CH}_2\text{-OH} + \text{BrCH}_2\text{CH(OC}_2\text{H}_5)_2 \xrightarrow[2)\ \text{HCl}]{1)\ \text{KOtBu/C}_6\text{H}_6} \text{PEG-CH}_2\text{CH}_2\text{CHO}$$

The PEG-CHO was converted into the amino or alkylamino derivative by reductive amination of the aldehyde group:

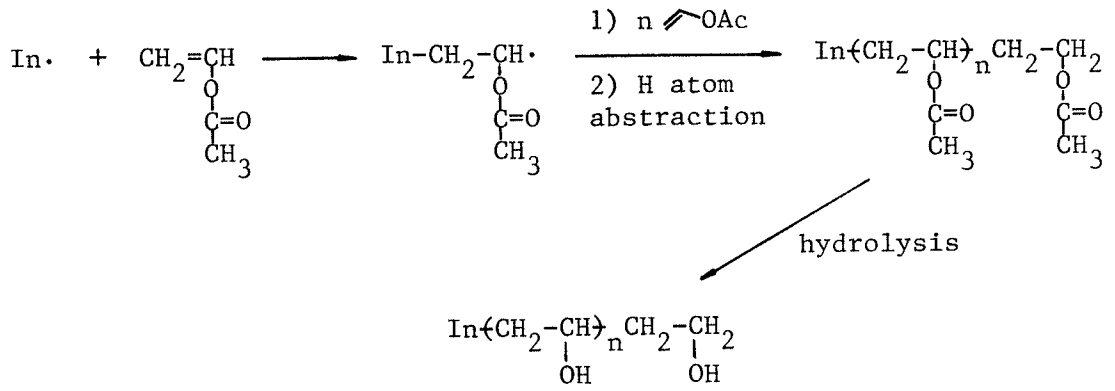
$$\text{PEG-CHO} + \text{NH}_4\text{OAc} \xrightarrow[\text{CH}_3\text{OH}]{\text{NaCNBH}_4} \text{PEG-CH}_2\text{-NH}_2$$

Pillai and coworkers have derivatized PEG with a number of acid cleavable and photo cleavable anchoring groups for application in peptide synthesis [69].

2.4 POLY(VINYL ALCOHOL)

Poly(vinyl alcohol) (PVA) is the hydrolysis product of poly(vinyl acetate) (PVAc), which is obtained by polymerization of vinyl acetate [85]. The polymerization proceeds via a radical mechanism initiated by a free-radical initiator:

$$\text{In} \longrightarrow \text{In}\cdot$$



PVA has both advantages and disadvantages when used as the polymer part in conjugates with allergens and drugs. Sehon and coworkers have shown that PVA-hapten conjugates have a greater potential for suppressing ongoing hapten specific allergic reactions than conjugates with PEG or other polymers [19-22]. PVA is biocompatible and nonallergenic. PVA is soluble in water, but insoluble in most organic solvents, which makes derivatization difficult. The polymer does not contain a derivatizable end group with a functionality different from the rest of the chain, as does PEG. Hence, commercial preparations of PVA can be used only for conjugation to haptens along the backbone of the chain. The backbone hydroxyl groups of PVA undergo many of the reactions that simple, nonpolymeric alcohols do, although often with a low degree of conversion and randomly along the chain of the polymer [85].

Incorporation of a single reactive end group into PVA has to be carried out at the polymerization stage, either by initiating the polymerization of vinyl acetate with a functionalized initiator, or by polymerizing the monomer in the

presence of a functionalized chain transfer agent. Different initiator systems for vinyl polymerization have been extensively investigated [86]. Extensive studies have also been made of chain transfer reactions in vinyl polymerization. Henrici-Olivé and Olivé have written a review on this topic [87].

Analyses of functional end groups incorporated into PVA either by the initiation or by the chain transfer method have been made either by titration or by a "reverse dye partition test" developed by Palit and coworkers [88]. However, most analyses of chain transfer products have involved only a molecular weight determination, required for the determination of chain transfer constants [87].

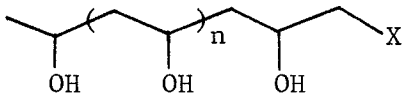
Chapter III

GENERAL METHODS

Before discussing the results of the present work the theory of polymerization and end group incorporation into PVA to produce desirable polymers will be described. Some general problems encountered when working with polymeric material, as well as the methods of characterization and analysis of the polymers will be described.

3.1 POLYMERIZATION AND END GROUP INCORPORATION INTO PVA

Immunological studies of polymer-allergen conjugates have demonstrated that the optimum chain length of the linear polymers, giving the largest immunosuppressive effects, is about 70-100 monomer units, corresponding to a molecular weight of 3000-4000 daltons for PVA [7]. The objective of the present work was therefore to synthesize a linear polymer of this size containing a single terminal functional group with a reactivity different from that of the backbone hydroxyl groups, to be utilized in the preparation of PVA-allergen conjugates:



where n = 70-100

The backbone hydroxyl groups can be derivatized and coupled to haptens [19-22], but using derivatized backbone hydroxyl groups to couple the polymer to proteins would lead to undesirable crosslinking in the product, which would affect the biological activity of the protein by producing conjugate aggregates that are too large or even insoluble. Although reactions of the backbone hydroxyl groups can be carried out in such a way as to produce PVA with a very low degree of derivatization, even with an average of only one derivatized group per chain, the result would be a heterogeneous product containing some PVA chains with many derivatized groups and some with only a few or none at all. The chains containing more than one derivatized hydroxyl group would then produce crosslinked material.

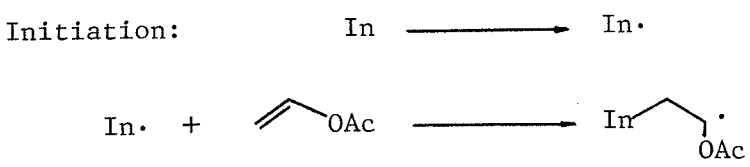
Unlike PEG, which contains reactive functional end groups distinct from the rest of the polymer chain, commercial PVA cannot be used for the preparation of terminally functionalized PVA for coupling to ligands. The backbone hydroxyl groups interfere with any reaction of the terminal primary hydroxyl group, making it impossible to derivatize only the terminal groups of the polymer. Thus a single terminal functional group has to be incorporated into the polymer during the polymerization of the monomer. The terminal group, either as such or further derivatized, will form the linkage between the polymer and the ligand in the conjugate, and therefore has to be stable to <u>in vivo</u> conditions so that

release of free ligand does not occur. This excludes the use of any potentially hydrolysable linkages, such as ester linkages. The terminal functional group as well as its possible degradation products after metabolism have to be non-toxic.

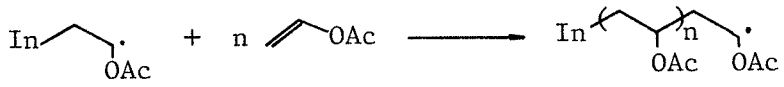
Incorporation of the end group into the polymer can be carried out during the free radical polymerization of vinyl acetate either by initiating the reaction with a functionalized initiator, or by chain transfer of the growing polymer to a solvent, which then starts the polymerization of new chains. The active group which is incorporated into the polymer has to be stable to the subsequent hydrolysis of the PVAc to PVA, or alternatively is to be generated from its precursor during the hydrolysis.

The mechanism of the polymerization of vinyl acetate is well known [85]. A functional initiator is incorporated into the polymer as the first step of the polymerization:

Initiation:



Propagation:

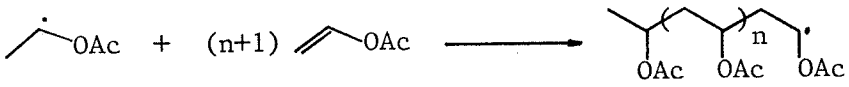


In = initiator

Ac = acetate

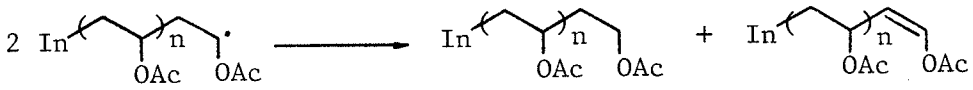
Termination can occur by reaction of two polymer radicals with one another either by disproportionation or by combination, or via hydrogen abstraction from monomer:

The monomer radical starts a new chain, which does not contain any initiator:

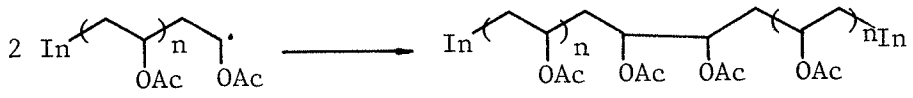


Termination by disproportionation of two polymer radicals leads to polymer with one end functionalized, while termination by combination yields a polymer with both ends functionalized:

1. Disproportionation:



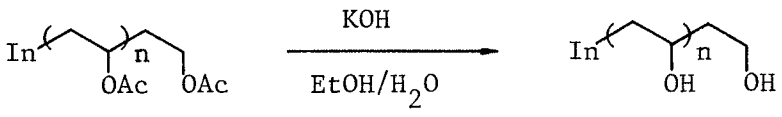
2. Combination:



The termination mechanisms depend on the polymerization conditions. At 60 °C or lower reaction temperature and at a

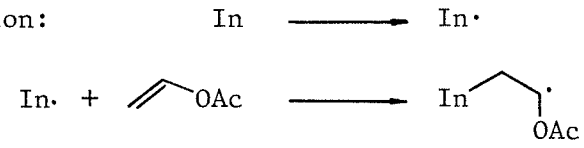
low monomer conversion PVAc radicals are assumed to terminate almost exclusively by the disproportionation mechanism and by abstraction of hydrogen from monomer [87]. Termination by combination is most likely to occur at high reaction temperatures and at high monomer conversion.

Hydrolysis of the PVAc gives the initiator terminated PVA:

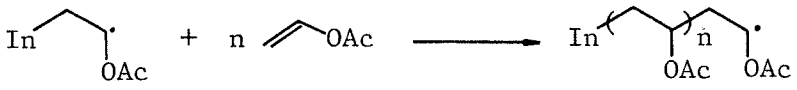


When the polymerization is carried out in a solvent, the solvent can be incorporated into the polymer. The polymerization starts in the usual manner:

Initiation:



Propagation:



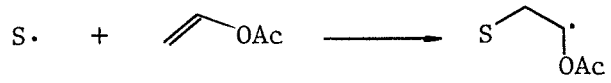
The polymer radical can abstract a hydrogen from the solvent, which then starts a new chain:

Chain transfer:

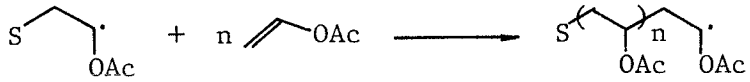
SH = solvent

S = solvent radical

Initiation by the solvent radical:

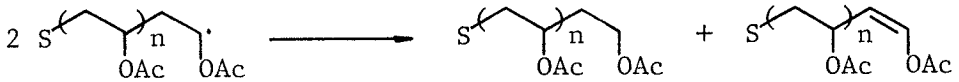


Propagation:

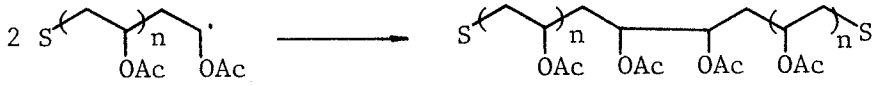


Termination:

1. Disproportionation:

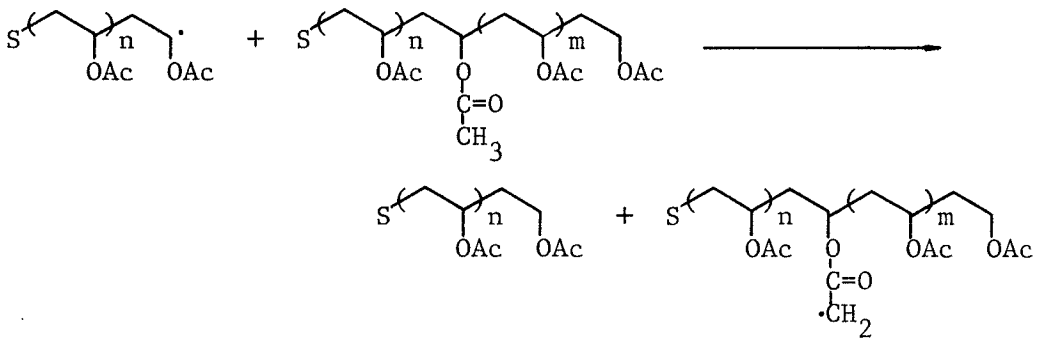


2. Combination:

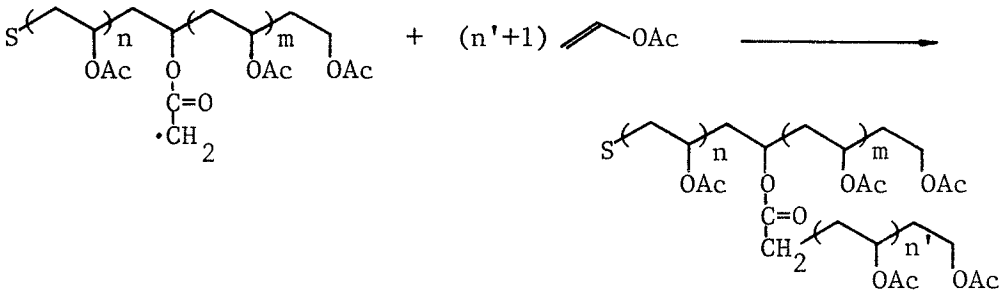


In addition to chain transfer to solvent and monomer, a growing polymer can also abstract a hydrogen from another polymer chain, producing a site for branching. The most likely site for attack is on the methyl carbon of the acetate group [89]:

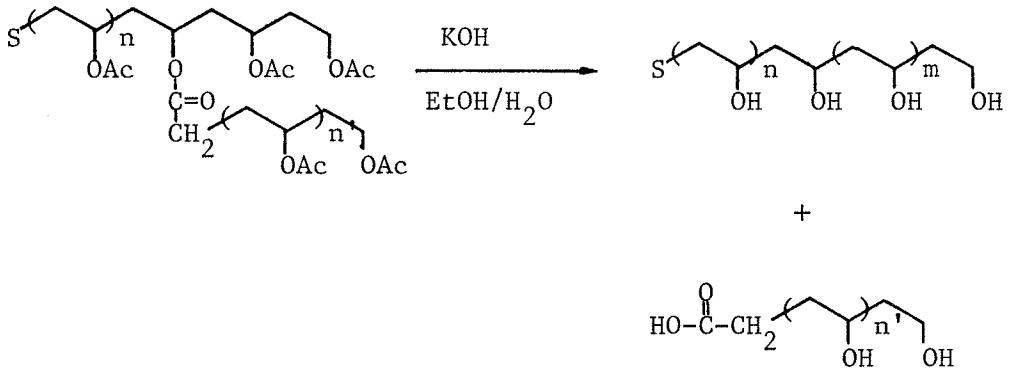
Chain transfer:



Propagation and termination:



Chain transfer to this site produces a branch which is cleaved during hydrolysis of the PVAc to PVA:



Chain transfer to the methine and methylene carbons along the backbone of the chain produces branched PVA. The amount of chain branching during polymerization is considerable at

high reaction temperatures and high conversion of the monomer, but insignificant at low conversion [87,89].

The frequency of chain transfer to solvent is determinad by the magnitude of the chain transfer constant C_S of the solvent relative to the chain transfer constant of the monomer C_M. The chain transfer constant C is defined as the ratio of the rate constant of the chain transfer reaction, k_{tr}, and the rate constant of the propagation, k_p, [87]:

propagation: $P_n^{\cdot} + M \xrightarrow{k_p} P_{n+1}^{\cdot}$

chain transfer to solvent:

$$P_n^{\cdot} + SH \xrightarrow{k_{tr}} P_n + S^{\cdot}$$

chain transfer to monomer:

$$P_n^{\cdot} + M \xrightarrow{k_{tr}'} P_n + M^{\cdot}$$

$$C_S = k_{tr}/k_p \quad ; \quad C_M = k_{tr}'/k_p$$

where $P_n^{\cdot}$ = polymer radical

P_n = polymer

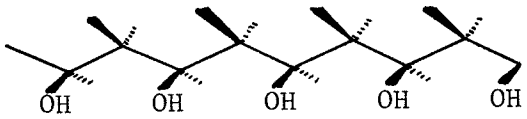
M = monomer

If C_M is higher than C_S essentially all chain transfer is going to occur to monomer and to polymer, so that only an insignificant amount of the solvent is incorporated into the polymer. If C_M is lower than C_S the degree of polymerization (DP) of the monomer is determined by the magnitude of the chain transfer constant C_S and the relative ratio of solvent to monomer [87]:

$$1/DP = \text{constant} + C_S[S]/[M]$$

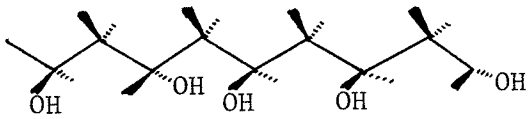
The larger C_S , the more the molecular weight of the polymer is suppressed. By varying the concentrations of monomer and solvent the molecular weight of the polymer can be adjusted within certain limits. The value of the chain transfer constant of vinyl monomer has been determined by several research groups to be between $2.0 \cdot 10^{-4}$ and $2.5 \cdot 10^{-4}$ [87]. Chain transfer constants to numerous solvents used in vinyl acetate polymerization have also been determined [87,90-92].

PVA can exist in three different steric configurations, the isotactic, atactic, and syndiotactic forms [93]. In the isotactic polymer the chiral centres at the methine carbons have the same configuration throughout the polymer chain :



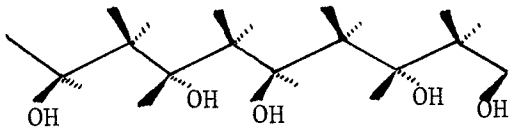
Isotactic

In the atactic polymer R and S configurations of the chiral centres are randomly distributed along the chain,



Atactic

and in the syndiotactic polymer the R and S configurations alternate :

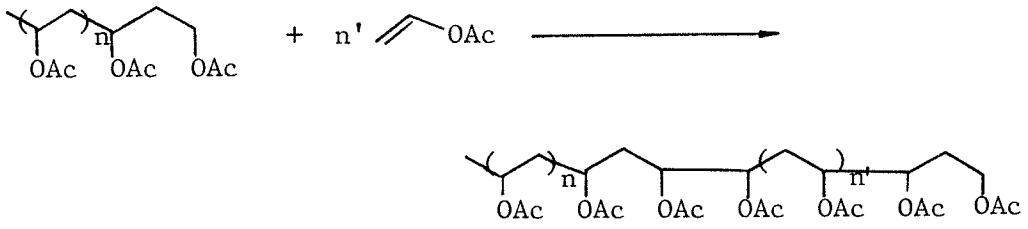


Syndiotactic

PVA obtained from polymerization of vinyl acetate usually has an atactic stereoregularity with isotactic segments.

Depending on the polymerization temperature, the amount of isotacticity can be as much as 55%, the highest values being obtained at low temperatures [89]. PVA of different tacticity can be prepared by free radical polymerization of other vinyl esters or by ionic polymerization of vinyl ethers and divinyl monomers, and hydroloysis of the resulting polymers [93]. The tacticity can be determined both from the NMR and IR spectra of the polymers.

The stereoregularity affects the solubility of the 100% hydrolysed polymer[93]. A syndiotactic stereoregularity favours intermolecular hydrogen bonding, with low solubility in water as a result. Atactic and isotactic PVA are more soluble. The solubility in water is increased by a high content of 1,2-glycol linkages, which are formed by occasional "head to head" additions in the polymerization process:



These linkages change the spatial conformation of the chain and thus disrupt the pattern of hydrogen bonding between the polymer chains. PVA obtained by polymerization of vinyl acetate at 60°C has an average of 1.5 mol% of 1,2-glycol linkages [93]. Residual acetate groups increase the solubility in water for the same reasons.

PVA has a low solubility in those organic solvents which can form hydrogen bonds to the polymer, and is insoluble in other organic solvents.

3.2 ISOLATION AND PURIFICATION OF THE POLYMERIC MATERIAL

The polymeric material had to be carefully purified from any contamination of low molecular weight material, which would interfere with the end group analyses of the products obtained in the coupling reactions.

The PVAc was normally purified by several precipitations from concentrated solutions of the polymer, and lyophilization from t-butyl alcohol or benzene. After hydrolysis of the PVAc's the PVA solutions were purified from low molecular weight material either by dialysis or by gel chromatography. The effective radius of a linear PVA chain is much larger than that of a folded protein molecule of the same molecular weight, allowing the use of relatively large pore dialysis tubing (Spectrapor 1 or 2). However, small molecular weight material was lost during the dialysis.

Since a substantial loss of the polymeric material sometimes occured, either through holes in the dialysis tubing or through the tubing closures (Fisher Scientific), purification by gel chromatography (Sephadex G 25) was preferred, especially in later work. The eluate was conveniently monitored for PVA by a spot test using KI/I_2:boric acid, which

gives a dark blue colour with the polymer [85]. The elution of PEG was monitored by spotting the fractions on TLC plates and developing them in an I_2 atmosphere. After purification the aqueous polymer solutions were lyophilized to dryness.

Isolation of a possible conjugate from the unreacted ligand was carried out using either dialysis or gel chromatography. Passing the reaction mixture through the Sephadex gel often gave an occlusion of the unreacted ligand in polymeric micelles, with incomplete separation as a consequence. Dialysis for several days against running distilled water always led to complete removal of the low molecular weight ligands from the conjugates.

3.3 CHARACTERIZATION OF THE POLYMERS

Characterization of the polymeric material obtained in this study was made by molecular weight determination and end group analysis. The molecular weight determination of PVA made by viscometry is described in the next section. The molecular weight of PEG was determined by NMR.

3.3.1 End group analysis

The standard methods of end group analysis used in this work were titration, electrophoresis, Sanger's [94] and fluorescamine tests [95], IR and NMR spectroscopy and isotope labeling [96]. These methods are well known and will

not be described in detail. Some general remarks on the complications due to the nature of the polymer will be made here, however, before describing the results of this work. A "reverse dye partition test" described by Palit and co-workers [88] to determine end groups of PVA was also attempted, but no quantitative results were obtained. The work related to this test will be described in a separate chapter.

End group analysis of polymers is possible only with the most sensitive analytical methods because of the small concentration of the functional end group in a polymer sample. In PVA of the average degree of polymerization 100 (M_n = 4400), the terminal carbonyl carbons would represent only 0.5% of the total number of carbon atoms, and 0.6% of the weight. Therefore a large amount of sample is required for analysis. Here again the limitations are the poor solubility of PVA, and possible interference of the polymer with the analysis method due to the high viscosity of the solution.

Not only the small relative amount of end groups in a sample, but also the spatial conformation and the structure of the polymer results in a possible interference with the analysis of the end group. An analysis based on a chemical reaction may fail because of steric hindrance due to coiling of the polymer chain around the potentially reactive end group. Small molecules or ions on the other hand might have access to the interior of the coil, as many of our results

indicate. Thus titration of carboxyl terminated PVA was successful using large amounts of polymer and sensitive equipment. Electrophoresis of the same polymer, to determine the total charge on the polymer, was unsuccessful, presumably because of tight ion pairing of counter ions inside the polymer coils, causing the polymers to appear electrically neutral.

Chemical analyses such as the Sanger's [94] and fluorescamine tests [95] for primary amino groups require that relatively large reagent molecules reach the amino end group within the polymer coil. Both tests are used for quantitative determinations of free amino groups of proteins. The amino groups in proteins do not react to 100% with the reagents, however, apparently due to steric hindrance. Most of the free amino groups are likely to be situated at the surface of the folded protein molecule, avoiding the more hydrophobic interior, and are thus able to react, while those amino groups which are located inside the protein coil apparently are too sterically hindered to do so. A terminal primary amino group of a linear polymer may seem not to be sterically hindered towards reaction. However, coiling of the polymer chain around the end group may shield the reactive site from the relatively large reagent molecules, thus preventing a reaction.

End group analysis by spectroscopic methods turned out to be more successful. Even though the intensity of the signal

is low, end groups of PEG and PVAc can be detected by NMR. Using ^{13}C NMR the terminal carbonyl carbon could be detected in chloroform solutions, while the carbonyl carbon was initially not observable in the spectra of the same polymers in D_2O. Nor could the terminal carboxyl group of PVA in D_2O be detected. This was later found to be due to minute amounts of paramagnetic metal ions present in the D_2O. After removing these ions by shaking the polymer solution with acetyl acetone : toluene (1:1) [97] the carboxyl group could be detected.

The IR spectrum of PVA shows no absorption in the region 1600 - 1800 cm^{-1},which makes it possible in principle to detect a carboxyl group by the carbonyl absorption around 1740 cm^{-1}. The spectra have to be recorded in D_2O. The D_2O interferes in the region just above the carbonyl region, complicating the interpretation of the spectra. The regions below 1300 cm^{-1} and between 2100 cm^{-1} and 2700 cm^{-1} are totally obscured by the D_2O.

In acidic D_2O the carbonyl absorption of the carboxyl group appears at 1750 cm^{-1}, while the carboxylate anion generated by addition of NaOD to the solution absorbs at 1400-1600 cm^{-1} [98].

The carbonyl absorption of carboxyl terminated PVA of molecular weight 4000 was too weak to show unambiguously the presence of the end group. The only method by which the

presence of the carboxyl end group could be established unambiguously proved to be radioactive labeling of the group.

3.3.2 Molecular weight determinations

The molecular weights of polymers can be measured by several different techniques such as gel filtration, osmometry, ultra centifugation, light scattering, end group analysis, viscometry, etc. The molecular weight measured is an average weight because of the heterogeneous nature of the polymer samples. Measurement of the colligative properties and end group analysis of a polymer give the number of moles of polymer per unit weight of the sample, ie. the number average molecular weight M_n is obtained. M_n is defined as

$$M_n = w/N = \sum_{i=1}^{\infty} M_i N_i / N \qquad (1)$$

where $N = \sum_{i=1}^{\infty} N_i$

w = the weight of the sample

N = number of moles

N_i = the number of molecules with mass M_i

The weight average molecular weight M_w is obtained by light scattering measurements. M_w is defined as

$$M_w = \sum_{i=1}^{\infty} w_i M_i / \sum_{i=1}^{\infty} w_i = \sum_{i=1}^{\infty} N_i M_i^2 / \sum_{i=1}^{\infty} N_i M_i \qquad (2)$$

where $w_i = N_i M_i / w$

In a polymer sample which is heterogeneous, containing polymers of different chain lengths, the number average molecular weight does not coincide with the weight average mo-

lecular weight. When determining M_w the heavy molecules carry a larger weight in M_w, while all molecules are equally weighted in M_n. The ratio M_w/M_n, called the polydispersity, is frequently 1.5 - 2.0 in vinyl acetate polymers, but can be as large as 20.0 for very heterogeneous samples [85].

A simple and rapid technique to obtain the molecular weight of a polymer is to measure the viscosity of the polymer in solution. The intrinsic viscosity is related to the molecular weight of a linear polymer by the Mark-Houwink equation [99]

$$[\eta] = KM_v^a \tag{3}$$

where $[\eta]$ = the intrinsic viscosity, K and a are constants dependent on the type of polymer and on the solvent used, and M_v is the viscosity average molecular weight, defined by

$$M_v = [\sum_{i=1}^{\infty} w_i M_i^a]^{1/a} = [\sum_{i=1}^{\infty} N_i M_i^{(1+a)} / \sum_{i=1}^{\infty} N_i M_i]^{1/a} \tag{4}$$

The intrinsic viscosity is obtained experimentally by measuring the relative viscosity of the polymer solution as a function of the concentration. The reduced viscosity (η specific/C) and the inherent viscosity (ln η relative/C) depend linearily on the concentration, and both quantities give the intrinsic viscosity when extrapolated to infinite dilution [99]. Values of the constants K and a can be determined from a plot of the logarithm of M_n versus the logarithm of the intrinsic viscosity of different standard sam-

ples. If the polymer samples are homogeneous, each containing chains of the same length only, then $M_n = M_v$ and a and K are obtained directly from

$$\log[\eta] = \log K + a \cdot \log M_n \quad (5)$$

$$\log M_n = (1/a)\log[\eta] - (1/a)\log K \quad (6)$$

However, usually M_v is higher than M_n. Flory and Leutner [100] calculated the polydispersity M_v/M_n of PVA samples obtained by polymerization of vinyl acetate using the relation

$$M_v/M_n = [(1+a)\Gamma(1+a)]^{1/a} \quad (7)$$

where a is the constant from Mark-Houwink's equation and Γ is the gamma function. Inserting this equation into equation (3) and taking the logarithm gives

$$\log[\eta] = \log K + a\log M_n + \log[(1+a)\Gamma(1+a)] \quad (8)$$

$$\log M_n = \frac{1}{a}\log[\eta] - \frac{1}{a}(\log K + \log[(1+a)\Gamma(1+a)]) \quad (9)$$

From a plot of log M_n versus log $[\eta]$, a is obtained from the slope and K from the intercept of the line with the y axis.

Values of the constants K and a can be obtained from the literature for most well known polymers [101]. However, since the values of the constants K and a found in the literature were inconsistent for low molecular weight PVA in H_2O [88,100,101], these constants were determined by measuring the viscosity of commercial PVA samples of low molecular weight, with the result $K = 7.1 \cdot 10^{-4}$ and a = 0.66 for aqueous solutions at 20.0 ± 0.1 °C.

The molecular weights of standard samples used for calculating the relationship between the intrinsic viscosity and the molecular weight are usually determined by some other method than viscometry. The molecular weight of the commercial samples used in this study had been determined by viscometry to an accuracy of 5% [101 b]. Because of the nonexistence of commercial standard PVA samples to be used as calibrants, and beause of the low moleular weight of the polymers, determination of the moleular weights of these samples ould not be obtained commercially by other methods (vapour phase osmometry or gel permeation chromatography). The precision in determining the molecular weights of polydisperse polymers is not better than 20% due to the uncertainty in the Mark-Houwink constants.

The viscosity average weights determined for PVA synthesized in the course of this work were converted to the number average molecular weights using the relation $M_v / M_n = 1.84$ which was obtained from equation (7).

The molecular weights of most of the PVAc's prepared were calculated using the DP obtained from the molecular weight of the corresponding PVA's, determined by the method above. In cases where the PVAc was not hydrolysed to PVA the molecular weight was determined by viscometry of acetone solutions of the PVAc at 18.0±0.1°C using equation (3), with $K=2.45 \cdot 10^{-4}$ and a=0.67 [101 a]. The polydispersity $M_v / M_n = 1.85$ was obtained using equation (7). Very low molecular weight PVAc (DP<30), and PEG, were characterized by NMR.

Chapter IV

THE REVERSE DYE PARTITION TEST

In this chapter the reverse dye partition test for quantitative analysis of carboxyl groups of water soluble polymers is presented as described in the literature [88]. Since all attempts to quantitatively determine the number of end groups of carboxyl terminated PVA synthesized in the present work were unsuccessful, the method was abandoned. The experimental details of this investigation are described.

Palit and coworkers have developed different dye techniques for the analysis of end groups of polymers [102]. In the dye interaction technique a dye interacts with ionic groups of the polymer in an organic solvent. The interaction with the dye causes a change in its absorption spectrum, which can be measured quantitatively. Micro amounts of basic groups have been detected using dyes such as erythrosin, eosines, and rhodamine 6 Gx [102].

In the dye partition technique, the partition of a dye between an organic phase and an aqueous phase is measured. The ionic dye, which must be only negligibly soluble in the organic solvent, is extracted into the organic phase by the polymer bearing an end group with a charge opposite to that

of the dye. The polymer must be soluble only in the organic solvent, to prevent any interaction with the dye in the aqueous layer. Cationic dyes, such as methylene blue and pinacyanol have been used to determine anionic end groups, while disulfine blue, an anionic dye, has been used to analyse cationic end groups [102,103].

For water soluble polymers Palit and coworkers used a "reverse dye partition test" [88,104-108] claimed to be specific for negatively charged end groups. The dye disulfine blue VN 150, bearing negatively charged sulphonate groups, was used in this test. A "dye reagent" was prepared by shaking a chloroform solution of dodecyl ammonium hydrochloride ($DA-NH_3Cl$) [88] or poly(methyl methacrylate) having a positive thiourea end group [104] with an aqueous solution of the dye. The negatively charged dye forms a complex with the positively charged end groups, and the complex dissolves in the chloroform layer. In the analysis, aqueous solutions of hydrophilic polymers with negatively charged end groups are shaken with this dye reagent, thus transferring part of the dye into the aqueous layer. The optical density of the chloroform layer containing the rest of the dye is measured against a blank obtained by shaking a pure water solution with the dye reagent, to get a correction for the hydrolysis of the dye complex. According to Palit and coworkers [104], an amount of the dye equal to the amount of negatively charged end groups of the polymer is transferred into the

aqueous layer in addition to the dye transferred due to hydrolysis. They have proposed the following mechanism [104]:

Formation of the "dye reagent":

$$DA\text{-}NH_3Cl \ (org) + dye\text{-}SO_3H \ (aq) \longrightarrow DA\text{-}\overset{+}{N}H_3 \ \overset{-}{S}O_3\text{-}dye \ (org) + HCl \ (aq)$$

When the dye is shaken with water part of the complex breaks down as a result of hydrolysis:

$$DA\text{-}\overset{+}{N}H_3 \ \overset{-}{S}O_3\text{-}dye \ (org) + H_2O \longrightarrow DA\text{-}NH_2 \ (org) + HSO_3\text{-}dye \ (aq) + H_2O$$

According to Palit and coworkers, in the presence of polymers with a negatively charged end group, the following reactions take place quantitatively, in addition to the above mentioned hydrolysis:

$$DA\text{-}\overset{+}{N}H_3 \ \overset{-}{S}O_3\text{-}dye \ (org) + \sim\sim\sim\overset{-}{A} \ Na^{+} \ (aq) \longrightarrow DA\text{-}NH_2 \ (org) + Na \ SO_3\text{-}dye \ (aq) + \sim\sim\sim AH \ (aq).$$

where $\sim\sim\sim A^{-}$ is a water soluble polymer with a negatively charged end group.

This mechanism, however, does not show a quantitative relationship between the amount of dye transferred from the organic solution into the aqueous and the amount of negatively charged polymer present. The dye, being negatively charged, cannot interact directly with the negatively charged polymer, but only with the positive counter ions. A calibration curve is prepared using either sodium lauryl sulphate (NaLS) or sodium laurate (NaL), which can enter the chloroform layer. A quantitative relationship between the amount of NaLS

or NaL present in the test solution and the amount of dye displaced from the organic phase can be explained by the following mechanism:

$$DA\text{-}\overset{+}{N}H_3\ \overset{-}{S}O_3\text{-dye (org)} + \text{Na LS (aq)} \longrightarrow DA\text{-}\overset{+}{N}H_3\ \overset{-}{L}S \text{ (org)} + \text{Na } SO_3\text{-dye (aq)}$$

This mechanism involves a direct displacement of the negatively charged dye by the NaLS to form a $DA\text{-}\overset{+}{N}H_3\ \overset{-}{L}S$ complex. Carboxyl terminated PVA can not enter the chloroform layer, and thus can not react according to this mechanism. It was found that NaLS, within a narrow concentration range, followed a quantitative relationship relative to the amount of dye displaced from the organic phase, but no quantitative relationship between the concentration of polymer in a sample and the amount of dye transferred from the organic phase was obtained.

Palit and coworkers claim to have successfully used the reverse dye partition test extensively in determining carboxyl and sulphate end groups of water soluble polymers, but scanning the literature we found no other research group using this test.

We attempted, using Palit's procedure, to determine the amount of carboxyl groups in terminally carboxylated PVA and in succinylated PVA, containing an average of seven carboxyl groups per polymer chain, determined by titration. Neutral PVA was used as a reference and NaLS as the standard. Some typical results are shown in Figure 1.

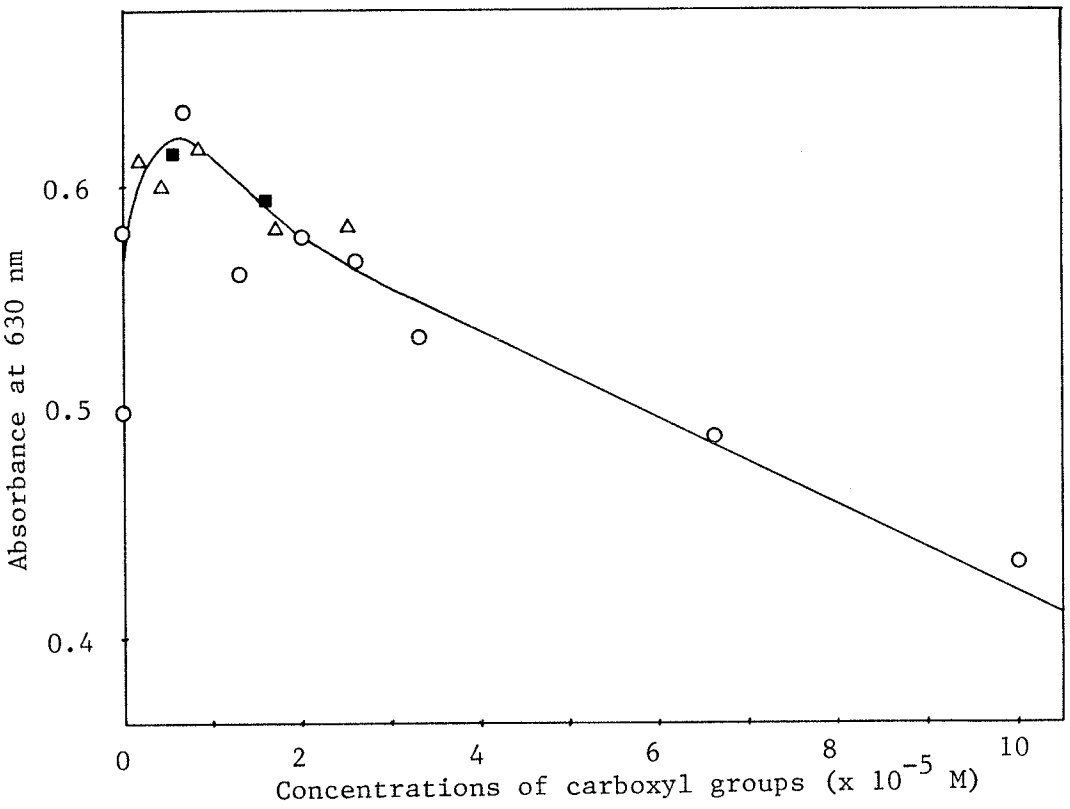


Figure 1: The reverse dye partition test
o NaLS, ▽ succinylated PVA (concentration is given as the number of carboxyl groups in the sample), ■ PVA-CO_2H.

When the dye reagent was shaken with an equal volume of distilled water ("blank") the absorbance of the chloroform layer should have been higher than when the reagent was shaken with an aqueous solution of NaLS or carboxyl terminated PVA, the difference being the measure of carboxyl groups present. However, for low concentrations of the anionic polymers ($<2 \cdot 10^{-5}$M), the absorbance of the organic solution obtained from the blank was in most cases lower than the absorbance of the organic dye reagent layer obtained from these dilute polymer solutions, indicating that more dye was transferred from the organic layer to the pure water of the blank than to the aqueous polymer solutions. Readings obtained from parallel samples of the blanks were often widely scattered, making quantitative analysis difficult. Increasing the polymer concentration above $2 \cdot 10^{-5}$M did cause a small decrease in absorbance of the organic layer which appeared to be reasonably linear in polymer concentration. However the large scatter in the data and the insensitivity of the technique make the method useless for quantitative purposes. At high polymer concentrations the solutions often formed emulsions which were impossible to break by centrifugation, and therefore the absorptions could not be measured. Samples of neutral PVA in large concentrations were also found to transfer more dye from the organic phase than the aqueous blank did. This latter observation raises the question of whether the dye transfer is caused by association with ionic groups on the polymer or simply by asso-

ciation with the polymer itself. We have also noted that the absorbance of the organic dye solutions is temperature dependent changing by approximately 10% over 35 °C (Table

TABLE 1

Temperature dependence of the dye test

	Absorbance		
Sample	0 °C	25 °C	35 °C
1	0.645	0.660	0.579
2	0.689	0.696	0.605

1). Careful temperature control was thus essential to prevent the large temperature effects from masking the small changes in dye concentration. After attempting to analyse a few of the synthesized carboxyl terminated polymers by the reverse dye partition test we abandoned the technique.

Chapter V

SYNTHESIS OF FUNCTIONALLY TERMINATED PVA

Functionally terminated PVA, which is to be utilized for coupling to proteins and smaller molecules, must have an end group that makes the coupling reaction feasible to carry out. Since the most reactive groups on the surface of proteins are amino and carboxyl groups and, to a lesser extent, thiol and aromatic hydroxyl groups, suitable functional groups on the polymer are carboxyl or amino groups, which can be coupled to the proteins using the well known procedures of amide bond formation [23,26].

In this study we have attempted to prepare carboxyl terminated PVA both by functional initiation of vinyl acetate polymerization and by chain transfer to growing polymer. A preparation of amino terminated PVA was attempted using the chain transfer technique.

5.1 CARBOXYL TERMINATED PVA VIA FUNCTIONAL INITIATION

Synthesis of carboxyl terminated PVA via polymerization of vinyl acetate was attempted by two methods using functionalized initiators. In the first method the terminal carboxyl group was incorporated into the polymer by initiation of the polymerization by carboxyl radicals, while the

second method incorporated terminal hydroxyl groups, which then had to be converted to carboxyl groups.

5.1.1 Initiation by potassium permanganate/oxalic acid

Palit and coworkers have studied different initiator systems for vinyl polymerization which provide the polymers with carboxyl end groups [109-111]. They polymerized vinyl monomers in aqueous solution containing potassium permanganate/oxalic acid as the initiator system [110,111], and used their dye tests to establish the presence of carboxyl groups. The permanganate is thought to start the reaction by reacting with monomer to produce MnO_2 , which in turn reacts with oxalic acid to produce carboxyl radicals, carbon dioxide and a Mn(III)-oxalate complex [$Mn(C_2O_4)_2$] [110]. The polymerization is assumed to be initiated by "active" carboxyl radicals, formed in the decomposition of the complex, while carboxyl radicals remaining stabilized in the solution account for the continued polymerization which occurs after all the Mn(III)-oxalate complex is consumed [110]. It is not known whether the active carboxyl radical that initiates the reaction is $^{\bullet}COO^{-}$ or $^{\bullet}OOCCOO^{-}$. The latter produces hydrolysable carboxyl end groups, which would be removed during the hydrolysis of PVAc to PVA. Palit and coworkers, however, were not able to hydrolyse the carboxyl end group from poly(methyl methacrylate) produced with this initiator [110].

We polymerized vinyl acetate in a deoxygenated aqueous solution containing potassium permanganate and oxalic acid, to 31% conversion. The molecular weight (M_n) of the resulting PVAc was determined to be 243,000, corresponding to a DP of 2,800, 28 times higher than was desirable (see 3.1). Attempts to produce lower molecular weight polymer by increasing the amount of initiator from 0.01% $KMnO_4$ and 0.1% oxalic acid to 0.35% $KMnO_4$ and 3.5% oxalic acid resulted only in a poorer yield of polymer with essentially the same molecular weight.

The polymer was not analysed further. Because of the difficulties in controlling the degree of polymerization we abandoned this polymerization method.

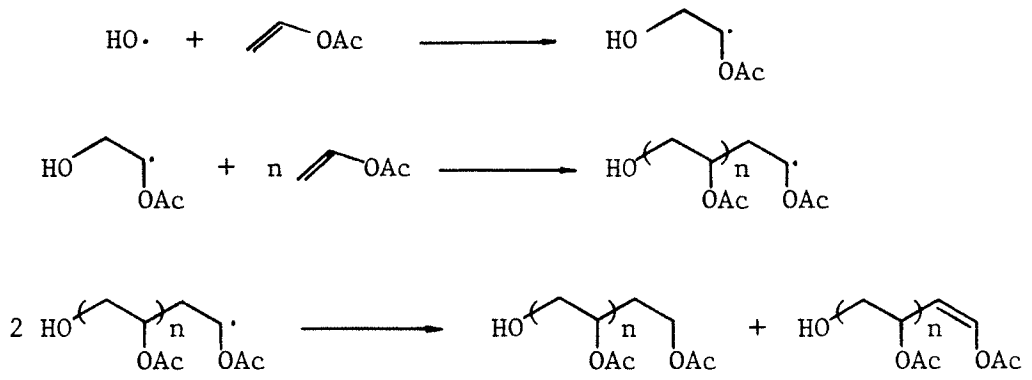
5.1.2 Initiation by Fe^{2+}/H_2O_2

Fenton's reagent, $FeSO_4/H_2O_2$, has been widely used to initiate free radical polymerizations [86,112]. The initiation is accomplished by HO radicals, produced by the reaction of ferrous ions with the peroxide [86]:

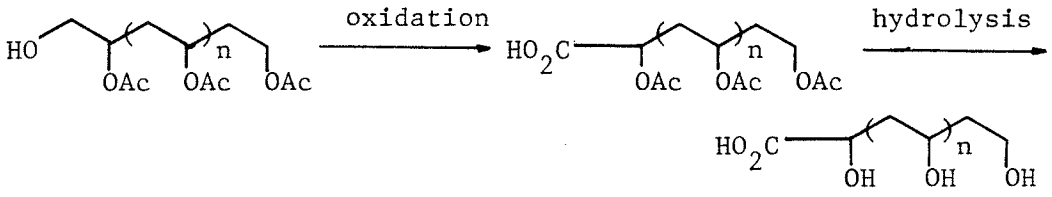
$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO\cdot + HO^-$$

The hydroxyl radicals can be consumed by ferrous ions, but in the presence of excess vinyl monomer most radicals initiate the polymerization, producing hydroxyl terminated polymer:

$$Fe^{2+} + HO\cdot \longrightarrow Fe^{3+} + HO^-$$



Palit and coworkers [113,114] and Evans [115] have reported the presence of terminal hydroxyl groups in polymers initiated by Fenton's reagent. If the terminal primary hydroxyl of the PVAc is oxidized to a carboxyl group, hydrolysis of the resulting polymer would give carboxyl terminated PVA:



In the present study vinyl acetate was polymerized in bulk with $FeSO_4$ and aqueous H_2O_2 as initiator. The polymerization gave PVAc with M_n = 42,200 (DP=490), in a 48 % conversion. Because of the undesirable high molecular weight of the polymer we did not attempt to oxidize the product.

Polymerization of vinyl acetate both in bulk and in aqueous solution with no other method of control over the degree of polymerization than varying the amount of initiator used and the time of reaction, produced PVAc of such high molecular weight that it was not useful for this study. A more effective control over the DP of the polymer was necessary. Polymerizing the monomer in a solvent which, by acting as a chain transfer agent, can suppress the growth of the individual chains would allow a more effective control of the DP. Hence, we abandoned the technique of producing functionally terminated PVA by functionalized initiation of the polymerization, and concentrated our efforts on the chain transfer technique.

5.2 FUNCTIONALLY TERMINATED PVA VIA CHAIN TRANSFER

Synthesis of functionally terminated PVA via chain transfer of polymerizing vinyl acetate to functionalized chain transfer agents provides good control over the DP of the polymer. Using this technique we attempted to prepare hydroxyl, carboxyl, and amino terminated PVA. It is also possible to obtain carboxyl terminated PVA in the absence of chain transfer agents if chain transfer occurs to the acetyl group of the polymer during the polymerization of the vinyl acetate. This results in a hydrolysable branch which gives a carboxyl terminated PVA when the PVAc is hydrolysed to PVA (see 3.1.). This reaction is described first, followed by a

description of the reactions used for preparing functionally terminated PVA via functionalized chain transfer agents.

5.2.1 Chain transfer to polymer

Chain transfer of radicals to polymer, producing branched PVAc, can occur to a considerable extent when the polymerization is carried out at a high temperature and to a high monomer conversion (section 3.1.). The value of the chain transfer constant to polymer has been determined by several research groups [87], but there is a disagreement among the various results. A general result, however, is that the chain transfer constant to polymer is higher than to monomer by a factor of about 5-10 at 50 °C. Chain transfer to the methyl carbons of the acetate groups, producing the hydrolysable branches, occurs 40 times more frequently than chain transfer to the backbone carbons [116,117]. A majority of the branches will therefore produce carboxyl terminated PVA when the PVAc is hydrolysed.

Vinyl acetate was polymerized in bulk at 60 °C with dibenzoyl peroxide (dBPO) as initiator. After 0.5 hr the solution became very viscous, and the reaction was stopped by the addition of hydroquinone. The yield of PVAc was 16%, and the number average molecular weight of the hydrolysed polymer was 39,000 (DP=890). Hydrolysis was expected to produce only a small percentage of carboxyl terminated PVA chains. Attempts were made to separate the carboxyl termi-

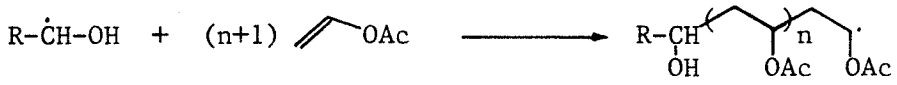
nated PVA from the noncarboxylated PVA on a DEAE-cellulose anion exchange column, but no polymer was adsorbed from a 0.08 M phosphate solution buffered at pH 7.7. Attempts were then made to separate the carboxylated PVA first by saturating the column with SCN^- ions, and then by chromatographing the tetrabutyl ammonium salt of the carboxylated PVA. Both the thiocyanate and the tetrabutylammonium ions should be easy to displace, and thus facilitate the formation of tight ion pairing between the ion exchanger and the carboxylate ions of the polymer. However, all attempts to adsorb the carboxyl terminated PVA on the column were unsuccessful.

In this reaction the degree of polymerization was as difficult to control as in the previous ones. Although the reaction was stopped at only 16% conversion, the M_n of the resulting PVA was about 10 times larger than desired. Attempts by others to polymerize vinyl acetate in bulk using azobisisobutyronitrile (AIBN) or photo initiation also resulted in polymers of high molecular weight [118]. Because of the high molecular weight of the polymer obtained in our investigation it was not further analysed.

5.2.2 Preparation of hydroxyl terminated PVAc and PVA

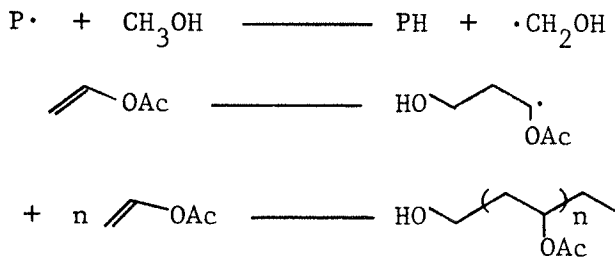
Polymerization of vinyl acetate in the presence of alcohols produces hydroxyl terminated PVAc. The chain transfer to the alcohol occurs by hydrogen abstraction from the carbon α to the hydroxyl group [119]:

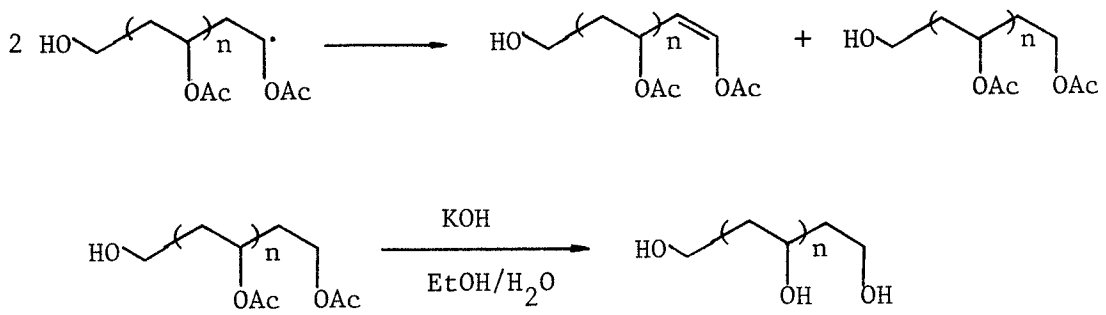
When the alcohol radical starts a new chain, the hydroxyl group, being either secondary or primary, forms the end group of that chain:



The value of the chain transfer constant C_S of methanol in vinyl acetate polymerization at 60 °C is in the range $6 - 11 \cdot 10^{-4}$ [90,119], while the chain transfer constant to monomer C_M is $2 \cdot 10^{-4}$. Thus the tendency of the radicals to transfer to methanol is at least three times higher than the tendency to transfer to monomer. If sufficient methanol is present, chain transfer occurs almost exclusively to methanol. The rapid rate of chain transfer to methanol also reduces the average chain length of the polymer. Several investigations of the polymerization and molecular weight suppression of PVAc by methanol are reported in the literature [120-123].

Methanol gives rise to PVAc and PVA terminated by primary hydroxyl groups:





Both the hydroxyl terminated PVA and PVAc were of interest. The hydroxyl terminated PVA could be used as a neutral reference polymer in the analyses of terminal carboxyl and amino terminated polymers. The initial plan regarding the hydroxyl terminated PVAc was to oxidize the end group to give carboxyl terminated PVA after hydrolysis. In later work it was used for the preparation of other PVAc derivatives.

Vinyl acetate was polymerized at 60 °C for 24 hrs with dBPO as initiator in various amounts of methanol, to obtain polymers of different DP. The results are shown in Table 2

TABLE 2

Polymerization of vinyl acetate in methanol

Batch #	[S]/[M]	DP	1/DP
B-11	0.8	220	0.0045
B-12	9.1	109	0.009
B-13	11.4	87	0.012
B-22	43	23	0.023
B-23	114	13	0.077

DP of B-11 - B-22 was determined by viscometry
DP of B-23 was determined by NMR

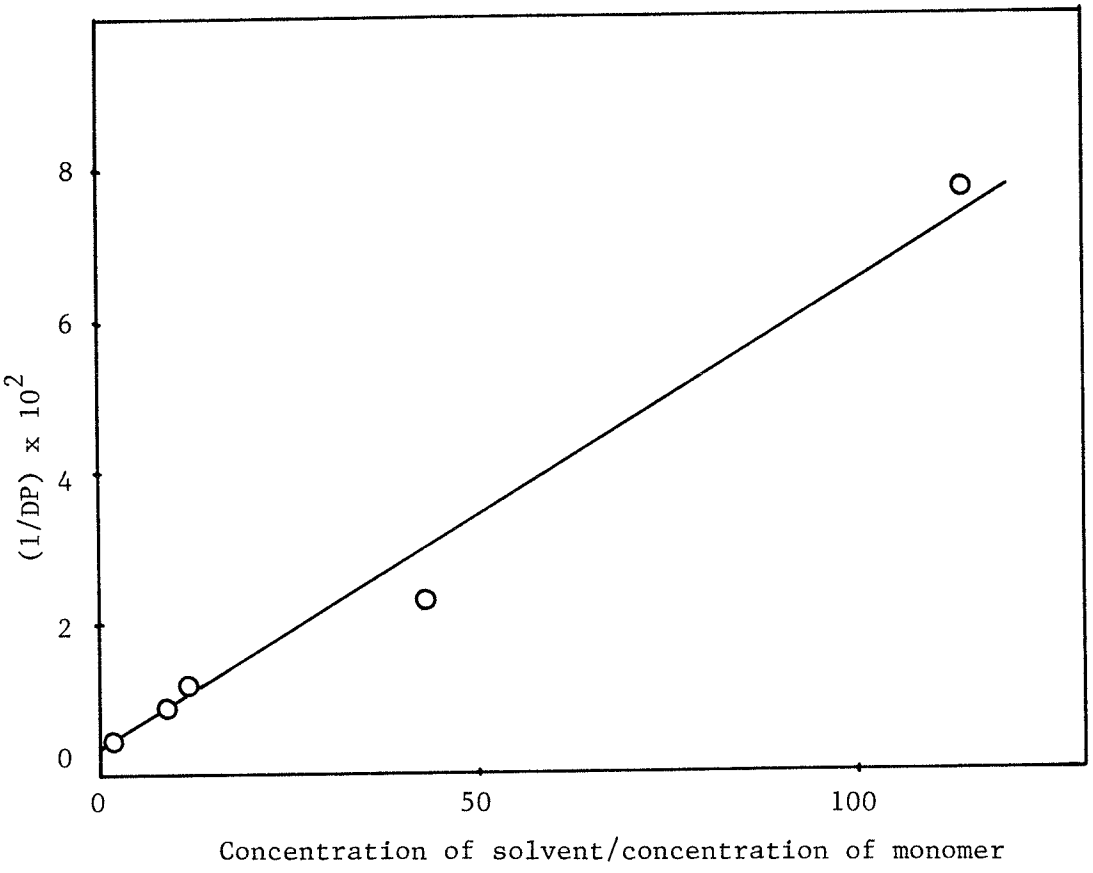


Figure 2: DP of PVAc as a function of monomer concentration in methanol

and Figure 2.

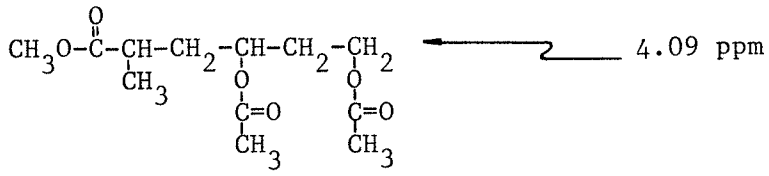
The polymers with DP=109 and 87 were of suitable size for the preparation of conjugates, while those with lower DP were more useful for the determination of the presence of the terminal functional group.

End group analysis of the hydroxyl terminated PVAc was performed by NMR. The expected average number of hydroxyl groups per polymer chain is less than one, since nonhydroxylated chains are produced by initiation by the dBPO or by initiation by monomer radicals, produced by chain transfer to monomer. The majority of the chains with a single terminal hydroxyl group are initiated by a methanol radical, and are terminated either by a chain transfer reaction or by disproportionation between two polymer radicals. A small fraction of the chains is expected to be dihydroxylated as a result of termination by combination of two hydroxyl terminated polymer radicals (see section 3.1.).

All of the termination reactions, except the combination of polymer radicals, give rise to terminal $-CH_2-OAc$ groups. Hence, the number of these terminal groups can exceed the number of terminal hydroxymethylene groups in a polymer sample.

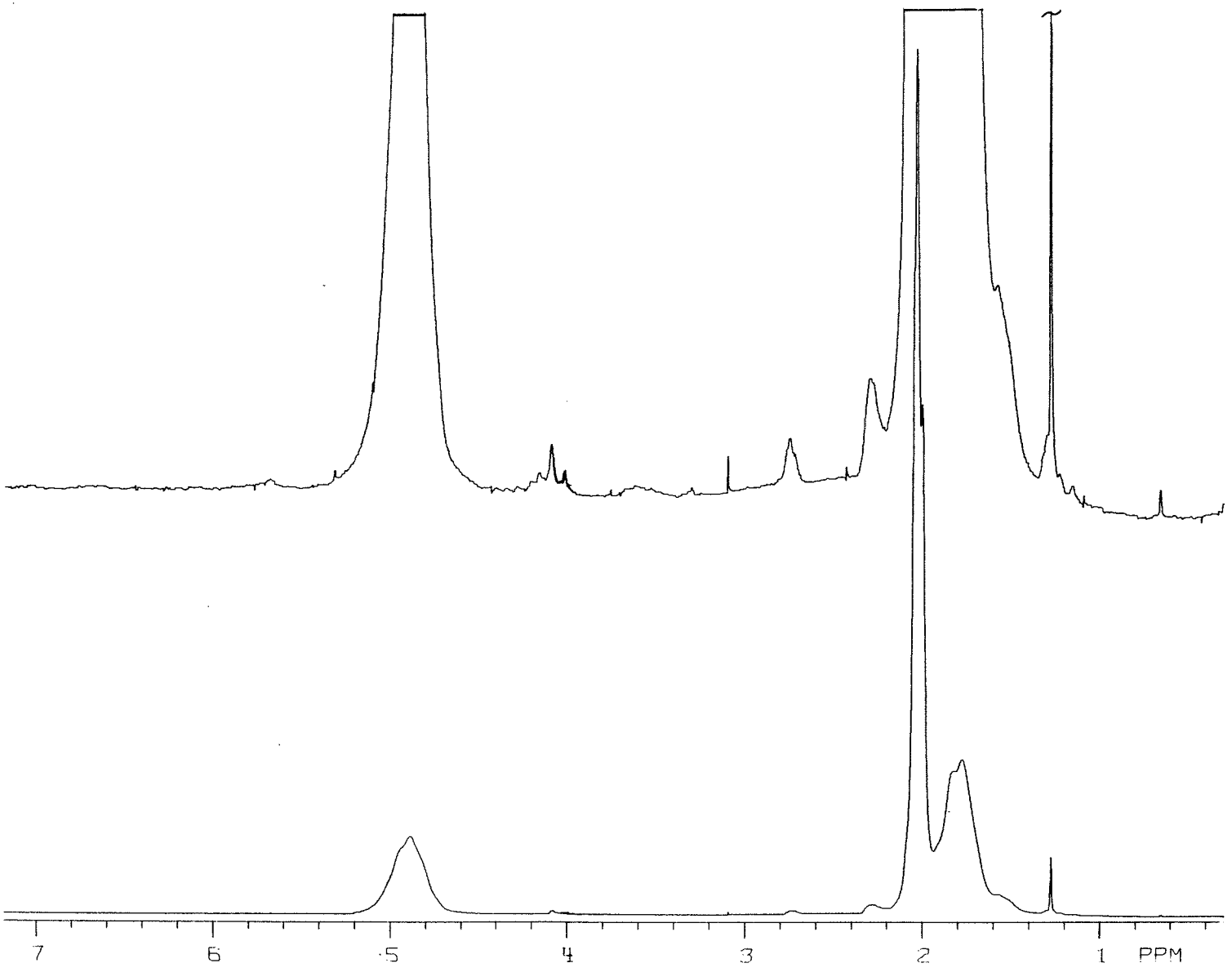
Brosse and coworkers have investigated the polymerization of vinyl acetate in methanol with hydrogen peroxide as initiator [121-123], and analysed the polymer by NMR [123].

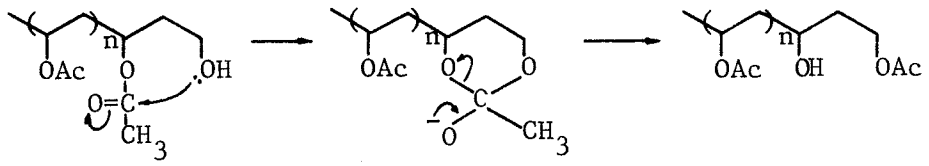
They attributed a triplet at 4.03 ppm to a hydrogen α to a secondary hydroxyl group, assumed to have been formed by partial hydrolysis of the acetate groups during reaction. A small peak seen at 3.6 ppm was attributed to hydrogens α to a terminal secondary hydroxyl group. However, Moskalenko and coworkers [124], who telomerized vinyl acetate with methyl esters of propionic and isobutyric acids, assign triplets at 4.02 - 4.15 ppm in the ^{1}H NMR spectra of these telomers to the methylene hydrogens next to a terminal acetate group, ie.



The ^{1}H NMR spectrum of the hydroxyl terminated PVAc prepared in the present work showed a triplet of low intensity at 4.08 ppm, integrated to 0.9 ± 0.1 per polymer chain (Figure 3). We assign this triplet to the methylene hydrogens of the terminal acetoxy methylene group. A small unresolved peak at 3.8 ppm was attributed to the multiplet from the methine hydrogen next to a secondary hydroxyl group in the chain. This could be formed by transesterification of a terminal hydroxyl group with backbone acetate groups:

Figure 3: ^{1}H NMR spectrum of PVAc-OH in $CDCl_3$


7
6
5
4
3
2
1
PPM



There are several indications that the assignment of the resonance peak at 4.03 ppm by Brosse and coworkers is incorrect. A methine proton *a* to a secondary hydroxyl group along the PVAc chain should appear as a quintet and not a triplet, because of the spin-spin splitting by the protons of both adjacent methylene groups. The peak was observed at 4.03 ppm (benzene solution), which is the expected chemical shift for the methylene hydrogens of the acetoxy methylene group. The intensity of this peak increased when the amount of H_2O_2 in the polymerization was increased. The concentration of acetoxy methylene end groups is expected to follow a similar pattern. H_2O_2 is a good chain transfer agent ($C = 3000 \cdot 10^{-4}$) [123], so increasing amounts of the reagent suppress the molecular weight of the polymer and thus gives rise to more end groups. Decreasing the amount of vinyl acetate in the polymerization results in a higher ratio solvent:monomer, which has the same effect on the molecular weight. In view of the above discussion the peak at 4.03 ppm is more likely to be the methylene hydrogens of the terminal acetoxy methylene group.

Attempts were made in the present work both to acetylate [125] and to silylate [126] the hydroxyl group of the polymer following standard procedures. If the ^1H NMR resonance peak at 4.08 ppm of hydroxyl terminated PVAc were caused by hydrogens *a* to a hydroxyl group, the peak position would shift upon acetylation or silylation. No shift in the position or change in the intensity of the peak was observed, while the silylated PVAc-OH showed a small cluster of peaks around 0 ppm. This provides additional evidence that the triplet at 4.08 ppm is due to the methylene hydrogens of a terminal acetoxy methylene group.

Since no triplets caused by the methylene hydrogens of a terminal hydroxy methylene group were found in the spectra, we assumed that a large fraction of the terminal hydroxyl groups were transesterified, giving rise to secondary hydroxyl groups. The NMR resonance peak of the methine hydrogen *a* to the secondary hydroxyl group causes a quintet which is broad, and therefore of low peak intensity and difficult to observe. Such a multiplet of very low intensity was seen at 3.8 ppm in some of the spectra of PVAc-OH, but was absent in the spectra of acetylated and silylated PVAc-OH. The secondary hydrogen is expected to have a lower reactivity than a terminal primary hydrogen, which could explain the low intensity of the silyl peak.

The results of the NMR analysis of the hydroxyl groups were not unambiguous, but further work including the prepa-

ration of derivatives of the hydroxyl groups confirmed their presence. The polymer obtained after hydrolysis was used as a reference polymer in the analyses of the PVA's.

Attempts were made to oxidize the hydroxyl terminated PVAc by using both Jones' reagent, CrO_3/H_2SO_4 in acetone [127], and a pyridinium:dichromate complex according to the procedure of Cornforth and coworkers [128]. The polymers were hydrolysed immediately after the oxidations, and were analysed by ^{13}C NMR. No carbonyl peak was detected in the spectrum of either polymer in D_2O.

5.2.3 Preparation of carboxyl terminated PVA

Carboxyl terminated PVA was prepared by chain transfer of polymerizing vinyl acetate to chain transfer agents containing the carboxyl group or a precursor to the carboxyl group. Hydrolysis of the resulting PVAc gave PVA-CO_2H. Three different chain transfer agents were used: Methyl propionate, acetonitrile, and 11-bromoundecanoic acid.

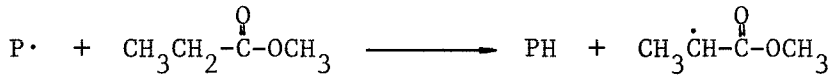
5.2.3.1 Methyl propionate as chain transfer agent

When vinyl acetate is polymerized in the presence of esters the the polymer radicals can abstract hydrogens from all methylene carbons on both sides of the functional group [119]. In methyl propionate the most probable site for hydrogen abstraction is at the only CH_2 carbon, adjacent to

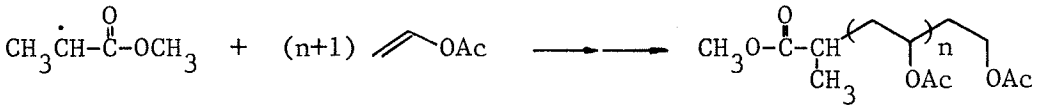
the carbonyl group. Moskalenko and coworkers have prepared telomers of vinyl acetate with methyl propionate [124] and observed no hydrogen abstraction from the methoxy group of the ester.

Chain transfer at the site *a* to the carbonyl produces ester terminated PVAc which upon hydrolysis is converted to carboxyl terminated PVA. The mechanism is as follows:

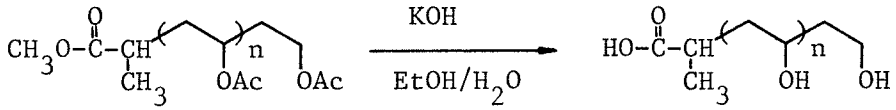
Chain transfer:



Propagation and termination:



Hydrolysis:



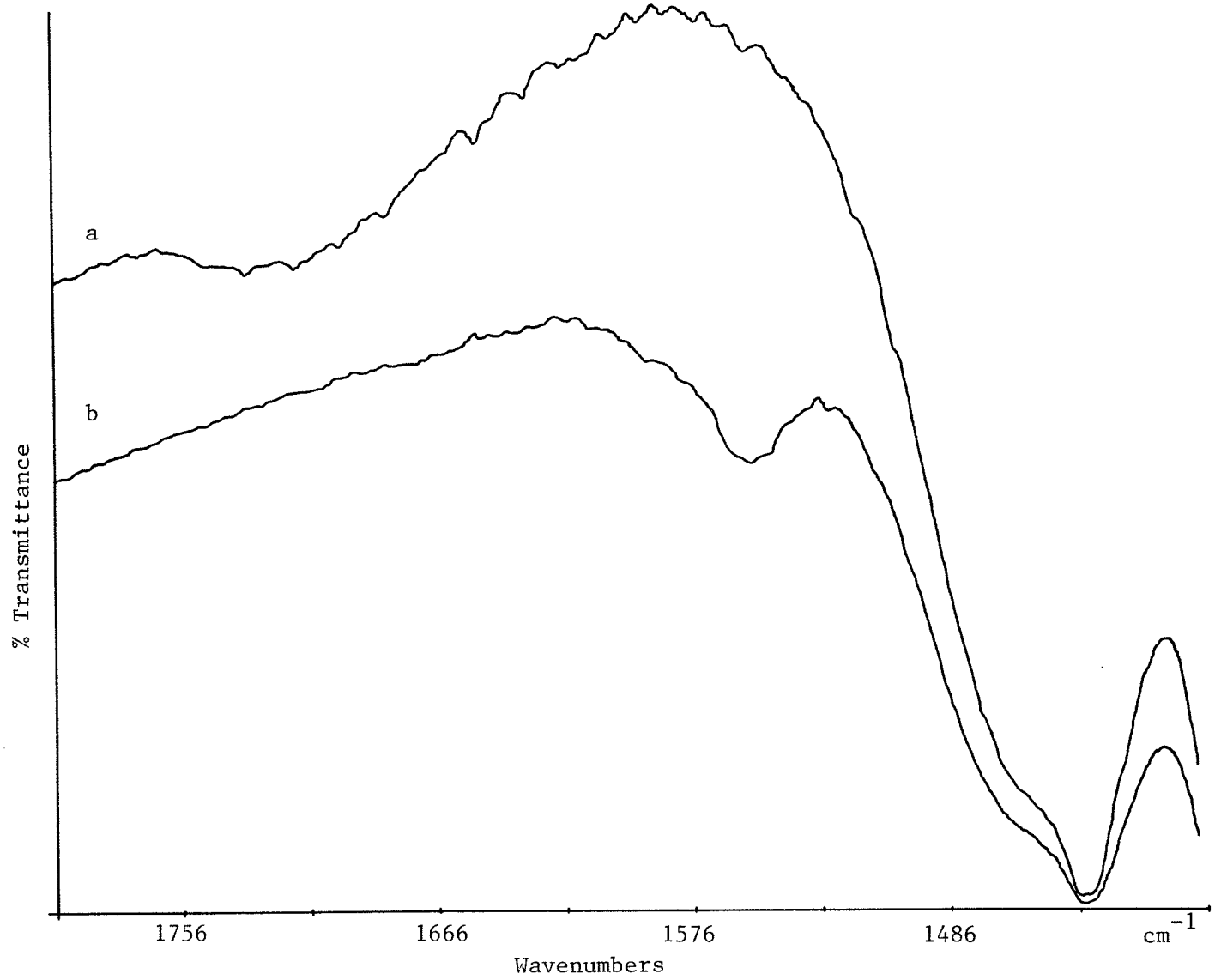
The chain transfer constant of methyl propionate in vinyl acetate polymerization is $23\text{-}26 \cdot 10^{-4}$ [90,92,129], which is about 13 times that of the monomer. As a consequence most of the chain transfer takes place to methyl propionate.

Since most of the chains are terminated either by chain transfer to the ester or the monomer, or by disproportionation of two polymer radicals, there should be an average of one or slightly less than one carboxyl group per chain, with a small amount of noncarboxylated and dicarboxylated material.

Vinyl acetate was polymerized in methyl propionate at 60 °C in a solvent:monomer ratio of 1:1 with dBPO as initiator. Reaction for 24 hrs gave a 60% conversion of the monomer. After hydrolysis PVA with an average molecular weight of M_n =3800 (DP=88) was obtained. The number of carboxyl groups in the PVA, determined by titration was 1.2±0.3 per chain. This value, being higher than one, may indicate that the estimation of the molecular weight of the PVA was too high (20%).

Although a large amount of the polymer was used in the titrations, the pK_a of the carboxyl group could not be determined because of the small amount of end groups present in a sample. Thus, we tried to obtain further evidence of the presence of carboxyl groups. The IR absorption of the carbonyl stretching was barely observable at 1740 cm^{-1} in acidic D_2O . This peak was not present in the spectrum of the basic solution, which showed a peak at 1550 cm^{-1} , attributed to the carboxylate anion [98] (Figure 4).

Figure 4: IR spectrum of PVA-CO_2H in D_2O/HCl (a) and D_2O/NaOD (b).


a
b
% Transmittance
1756
1666
1576
1486
cm-1
Wavenumbers

A ^{13}C NMR of a D_2O solution of the polymer exhibited a carbonyl carbon peak of low intensity at 178 ppm relative to TMS, and was observed only after paramagnetic ions present in the D_2O had been removed by shaking the sample with an equal amount of acetylacetone/toluene [97] (Figure 5).

Attempts to detect the presence of the carboxyl group by electrophoresis and dye tests were unsuccessful.

The experiment gave a polymer of desired size, but the analysis of the terminal carboxyl group was not unambiguous because of the minute amounts of the group in a polymer sample. To establish both the presence and the amount of carboxyl groups in the polymer we decided to prepare ^{14}C-carbonyl labeled carboxyl terminated PVA.

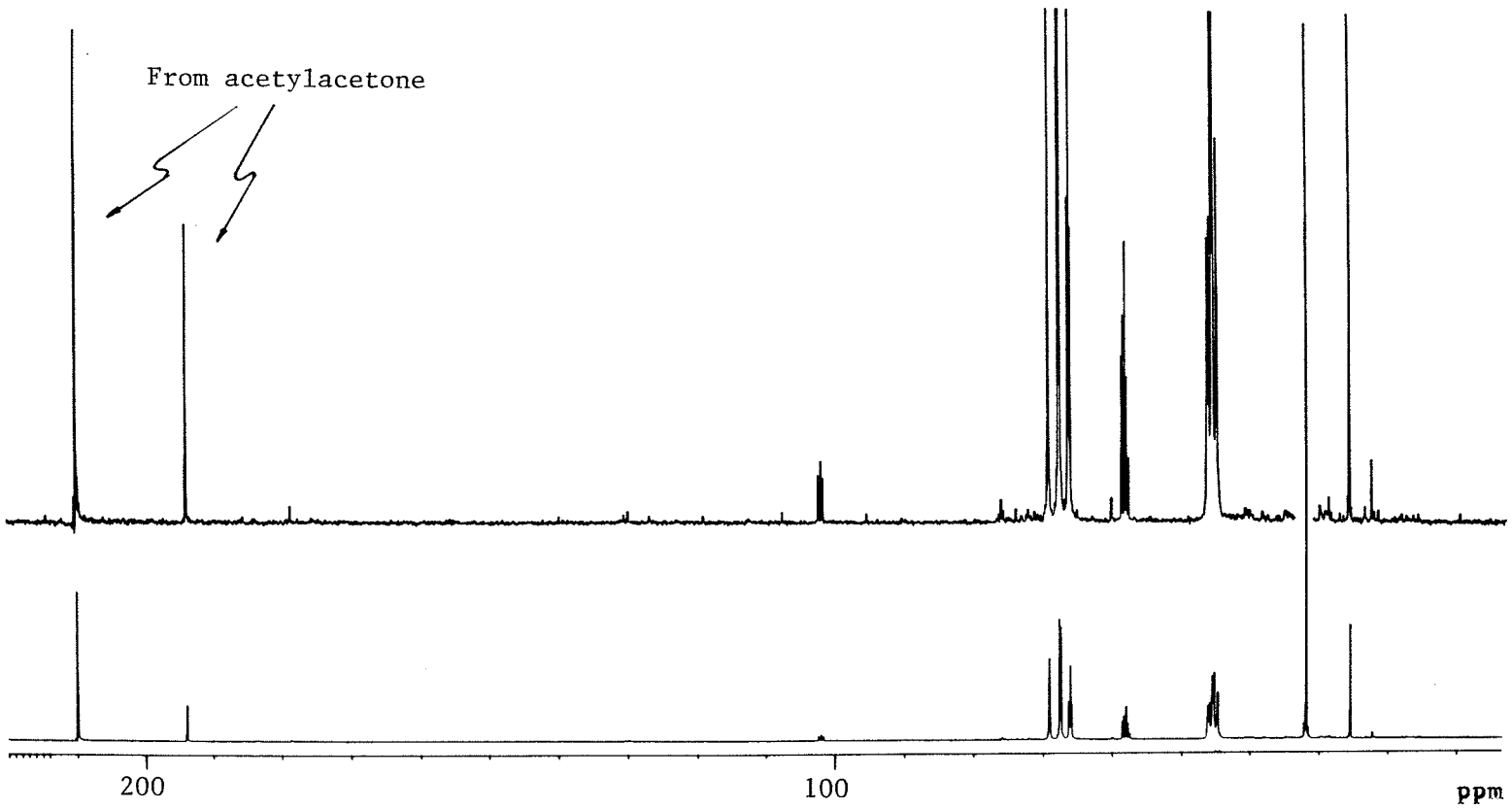
5.2.3.2 ^{14}C-carbonyl labeled carboxyl terminated PVA

Isotopic labeling of terminal groups of polymers has been carried out for the determination of the number of end groups present [96,130]. Bevington and coworkers have prepared vinyl polymers with radioactive end groups to investigate the mechanisms of polymerizations [131].

The ^{14}C-carbonyl labeled PVA was synthesized via chain transfer of polymerizing vinyl acetate to ^{14}C-carbonyl labeled methyl propionate.

a. Preparation of ^{14}C-carbonyl labeled methyl propionate

Figure 5: ^{13}C NMR of PVA-CO_2H
prepared via chain transfer to methyl propionate.


From acetylacetone
200
100
ppm

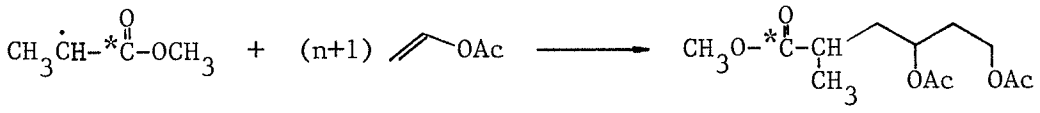
The ^{14}C-labeled methyl propionate was prepared by trans esterification of ^{14}C-carbonyl labeled sodium propionate with unlabeled methyl propionate using p-toluene sulphonic acid as catalyst.

b. Preparation of ^{14}C-carbonyl labeled carboxyl terminated PVA

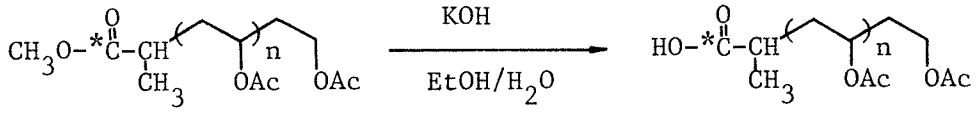
Chain transfer of ^{14}C-carbonyl labeled methyl propionate to polymerizing vinyl acetate produces PVAc with a terminal labeled ester group, which upon hydrolysis forms the terminal labeled carboxyl group of PVA:

Chain transfer:

Propagation and termination :



Hydrolysis:



The isotopically labeled polymer was prepared by polymerizing vinyl acetate with the radioactively labeled methyl propionate at 60 °C for 24 hrs in a solvent to monomer ratio of 1:1, with dBPO as initiator. The PVAc was separated from

unreacted methyl propionate and monomer by evaporation under reduced pressure followed by repeated precipitation from benzene/pentane, and finally by repeated lyophilization from t-butyl alcohol until the specific activity, measured with a liquid scintillation counter, was constant. After hydrolysis the polymer was dialysed for five days against running distilled water, and was lyophilized. The degree of incorporation of ^{14}C-labeled carbon into the polymer is shown in

TABLE 3

Activity of the labeled PVAc-CO_2CH_3 and PVA-CO_2H

Batch #	$[\eta]$[a] (ml/g)	DP	Activity [MBq/mol] PVAc	Activity [MBq/mol] PVA
1	25.02	89	26.5	24.2
2	24.40	86	28.8	24.4
3	24.58	87	21.5	18.9
		average:	25.6	22.5
		calc[b]		15.1

a) Intrinsic viscosity for PVA in water at 20 °C
b) Based on one labeled carbonyl per chain

Table 3. Since the radioactive carbon of the end group is not lost during the hydrolysis step, the PVAc should have the same activity as the PVA. However, higher average activities per chain were measured for the PVAc than for the PVA. This should not be due to residual labeled methyl propionate in the PVAc, since precautions were taken to remove it completely from the polymer. It may indicate some incorporation of the methyl propionate via chain transfer to the

methoxy carbon, resulting in loss of propionate on hydrolysis.

The calculated activity of the PVA, if every chain were terminated with propionate, was 15.1 MBq/mol (0.408 mCi/mol). The average measured activity was 22.5 MBq/mol (0.609 mCi/mol), indicating an average of 1.5 carboxyl groups per chain. Titration of unlabeled polymer gave an average of 1.2±0.3 CO_2H/chain.

Matsumoto and coworkers [92], studying chain transfer reactions in vinyl polymerization, found 0.9 - 1.5 titratable groups per PVA chain, prepared via chain transfer to methyl propionate, the higher values obtained for polymers of higher monomer conversion. The higher values (>1) were assumed to be caused by the error in the average molecular weight determined on the unfractionated material obtained from the polymerizations to higher conversions. Our results are in good agreement with their study.

As this experiment established the presence of the carboxyl group unambiguously, polymers prepared with methyl propionate as chain transfer agent were used in attempts to couple PVA to ligands.

5.2.3.3 Acetonitrile as chain transfer agent

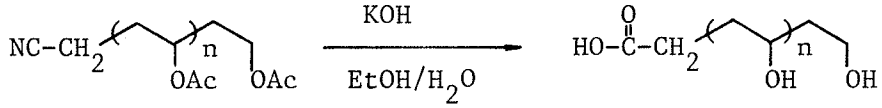
Chain transfer of polymerizing vinyl acetate to acetonitrile gives nitrile terminated PVAc. The terminal nitrile

group is hydrolysed to a carboxyl group during the conversion of the PVAc to PVA. The chain transfer constant of acetonitrile in vinyl acetate polymerization is $10 \cdot 10^{-4}$ [90], a value lying between that of methanol and methyl propionate. The chain transfer again occurs by proton abstraction from the carbon α to the functional group. The acetonitrile radical then starts a new polymer chain, which can be converted to carboxyl terminated PVA:

Chain transfer:

Propagation and termination:

Hydrolysis:



Vinyl acetate was polymerized in acetonitrile at 60 °C with dBPO as initiator in a solvent to monomer ratio 10:1. Reaction for three days gave a monomer conversion of 33%. The number average molecular weight of the hydrolysed polymer was 3300 (DP=75).

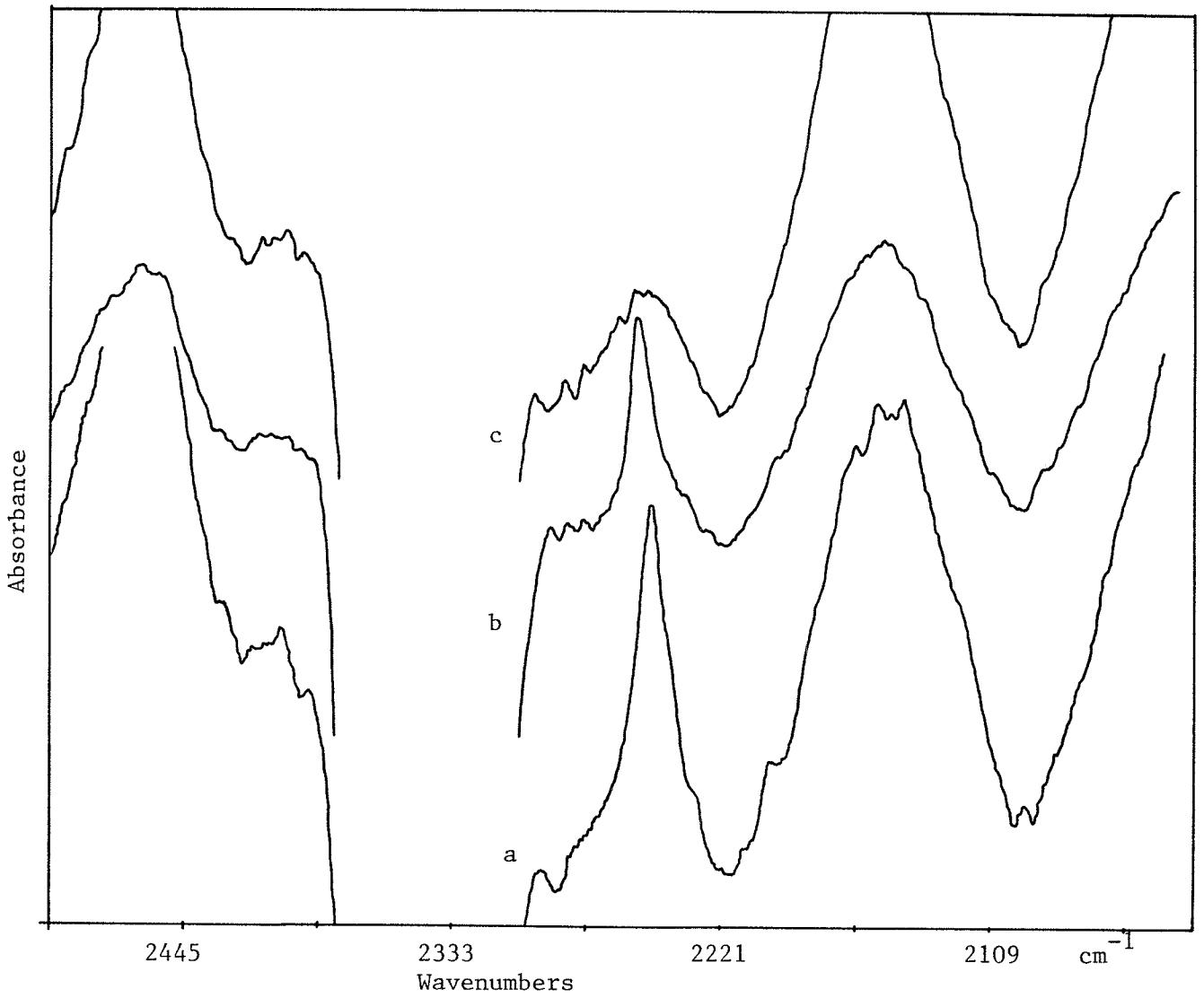
End group analysis of the nitrile terminated PVAc was carried out by IR. After comparing the spectrum of the pure nitrile terminated PVAc in chloroform with the spectrum of a PVAc sample with a small amount of added acetonitrile, a weak absorption band at 2250 cm^{-1} was assigned to the C≡N strech (Figure 6). The nitrile absorption of pure acetonitrile occurred at 2253 cm^{-1} .

After hydrolysis the polymer titrated for 1.1 acidic groups per polymer chain. A very weak IR absorption band was detected at 1740 cm^{-1} in acidic D_2O

5.2.3.4 11-bromoundecanoic acid as chain transfer agent

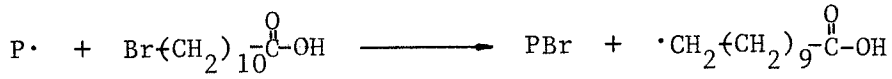
The difficulties in spectroscopic detection of the terminal carboxyl group of PVA indicated that the carboxyl group might be bound as an intramolecular lactone or orthoester, which also would render the carboxyl group unreactive. In order to remove the terminal carboxyl group from the immediate vicinity of the polymer chain we attempted to polymerize vinyl acetate in the presence of 11-bromoundecanoic acid as chain transfer agent. The chain transfer constant of 11-bromoundecanoic acid has not been reported in the literature, and therefore an estimation of the constant was made by comparison to constants of similar molecules. There are two possible sites of chain transfer in the molecule: by hydrogen atom abstraction on the carbon α to the carboxyl group and by abstraction of a bromine atom. The chain

Figure 6: IR spectrum of nitrile terminated PVAc
a = PVAc-CN; b = PVAc-OH + CH_3CN; c = PVAc-OH.


Absorbance
c
b
a
2445
2333
2221
2109
cm^{-1}
Wavenumbers

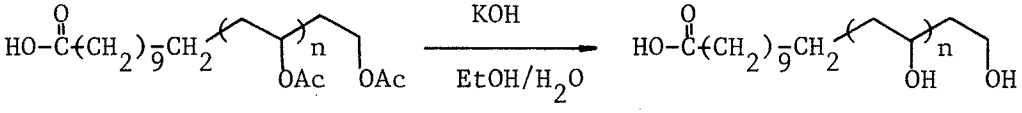
transfer constant for n-butyl bromide, where the transfer occurs by abstraction of the bromine atom, is $50 \cdot 10^{-4}$ [90] and that for acetic acid, where the transfer occurs *a* to the carboxyl group, is $10 \cdot 10^{-4}$ [90]. Chain transfer to a methylene carbon *a* to a carboxyl group of a carboxyl acid could not be found in the literature, but it is likely to occur more often than to the methyl carbon of acetic acid. However, the chain transfer constant to the bromine is high enough that the major fraction of the chain transfers should occur at that site, producing carboxyl terminated PVA with a spacer arm between the functional group and the polymer backbone:

Chain transfer:



Propagation and termination

Hydrolysis:



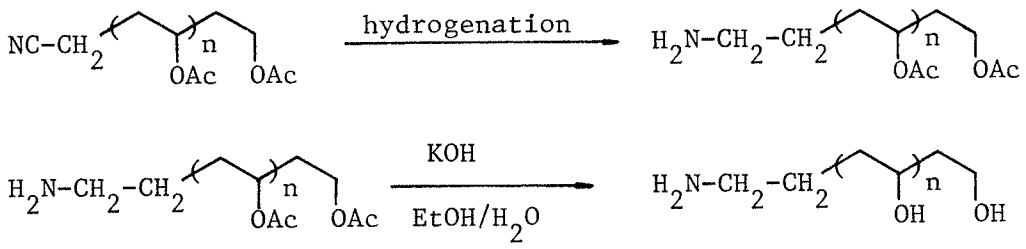
The polymerization was carried out for four days at 60 °C with dBPO as initiator using a monomer:chain transfer agent ratio of 1:2. However, we were unable to isolate any polymer from the large excess of solid chain transfer agent.

5.2.4 Preparation of amino terminated PVA

Attempts were made to prepare amino terminated PVA via chain transfer to acetonitrile and subsequent hydrogenation, and by chain transfer to an amide. However, problems were encountered with the analysis of the terminal groups because of low reactivity, presumably because of hydrogen bonding to the backbone hydroxyl groups. In order to facilitate the analysis, attempts were made to prepare telomers of vinyl acetate with n-butyl amide as telogen.

5.2.4.1 Reduction of nitrile terminated PVA

If the nitrile group of nitrile terminated PVA, prepared by polymerizing vinyl acetate with acetonitrile as chain transfer agent, is hydrogenated before hydrolysis to PVA, an amino terminated PVA is obtained:

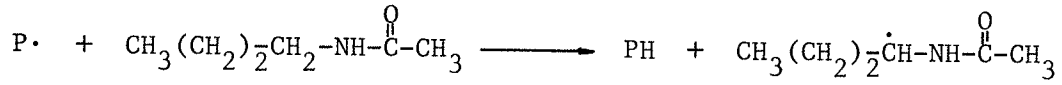


Attempts were made to hydrogenate the nitrile group of nitrile terminated PVA, prepared as in section 5.2.3.3., in acetic acid/water with palladium on charcoal as catalyst.

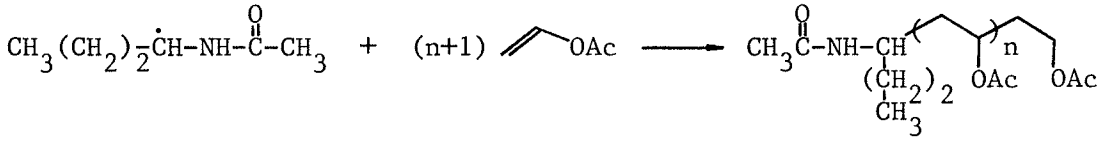
After hydrolysis, dialysis and lyophilization the polymer titrated for 1.1±0.3 acidic groups per chain. The pK_a could not be determined from the titration results, so it is not clear whether an ammonium ion or a carboxyl group was titrated. The pK_a of 4-hydroxy-n-butyl ammonium ion, which resembles the terminal group of the PVA-NH_3, is 10.35 [132]. This value falls within the pH region covered during the titration (pH 2-10.8). Negative results were obtained using Sanger's and fluorescamine tests for amino groups. Whether this was due to low reactivity of the shielded amino end groups towards the reagents or to absence of amino groups could not be determined.

5.2.4.2 Chain transfer to n-butyl acetamide

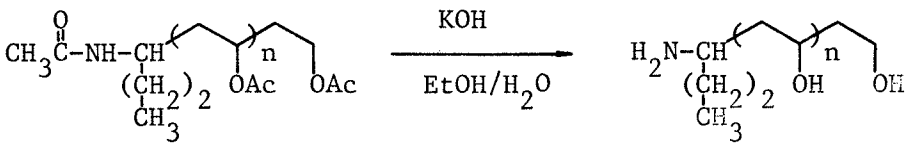
Chain transfer of a PVAc radical to n-butyl acetamide occurs by proton abstraction from the carbon *a* to the nitrogen atom [119]:



The amide radical starts a new chain which will be amide terminated:



Hydrolysis gives an amino terminated PVA:



The chain transfer constant of n-butyl acetamide is $40 \cdot 10^{-4}$ [90].

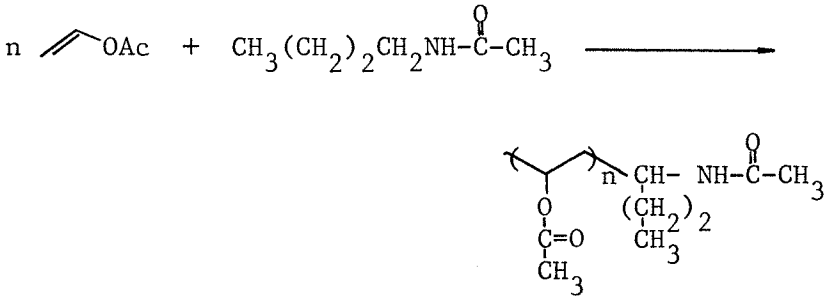
Vinyl acetate was polymerized in n-butyl acetamide, prepared by acetylation of n-butyl amine with acetic anhydride, at 60°C with dBPO as initiator in a solvent to monomer ratio of 2.5:1. Reaction for 24 hrs gave a 30% monomer conversion. The hydrolysed polymer had an average molecular weight M_n = 4,000 (DP=91). Sanger's and fluorescamine tests for amino groups gave negative results.

In order to assure the hydrolysis of the terminal amide group the polymer was heated for 45 min on a steam bath at pH 12. However, the polymer obtained gave the same results on analyses for amino end groups.

The analyses of amino end groups all involved attempted chemical modifications of the terminal group. IR spectroscopy was not used because of the large hydroxyl absorption of the polymer which obscures the region of the $-NH_2$ absorption. The negative results of the analyses of amino end groups for all amino terminated polymers prepared presumably reflect the inability of the terminal group to react because

of intramolecular hydrogen bonding to the backbone hydroxyl groups of the polymer, and because of steric hindrance due to the random coiling of the polymer chain around the group. Attempts were therefore made to prepare telomers of vinyl acetate with n-butyl acetamide to facilitate detection of the amine groups.

The monomer was polymerized in n-butyl acetamide in a monomer to solvent ratio of 1:30. After removal of excess monomer and amide under high vacuum the liquid residue was analysed by NMR. The spectrum showed two overlapping singlet peaks at about 2.0 ppm of similar intensity, which were assigned to the two different acetate groups of the adduct:



An amount of solid polymer, too small to allow determination of the molecular weight by viscosity, was isolated and analysed. Sanger's test for amines gave negative results.

Since the presence of amino end groups in the polymers could not be determined unambiguously, the amino terminated polymers were not used in coupling reactions to ligands, except for one unsuccessful attempt in which dicyclohexyl car-

bodiimide was used as coupling reagent. Coupling reactions were instead carried out with the better characterized carboxyl terminated PVA.

Chapter VI

COUPLING REACTIONS

The coupling of a carboxyl or an amino terminated polymer to functional groups of a biologically active compound involves many of the same problems as in peptide synthesis. For an amide bond to form, one of the participating functional groups has to be activated for reaction. In the conjugation of a synthetic polymer to a biologically active compound this activation is suitably carried out on the polymer bound functional group. This can be done without the risk of denaturing or otherwise rendering the biological component inactive. Activation of a functional group on a delicate biologically active compound might lead to a drastic decrease in the activity of the molecule, giving a biologically useless conjugate.

The activation step often has to be carried out in organic solvents to avoid hydrolysis of the active group. This is a major drawback when working with PVA because of the poor solubility of this polymer in most organic solvents.

Some of the coupling reactions were performed on small model compounds prior to using the functionalized polymers. Monitoring the reaction is easier with low molecular weight compounds and, furthermore, working with polymers involves

the risk of trapping unreacted material within polymeric micelles or vesicles during the workup, with false analysis results as a consequence.

6.1 PREPARATION OF MODEL AMINE

Since coupling of a functionalized polymer to a protein involves reaction primarily with the amine groups of lysine residues of the protein, α-dinitrophenyl lysine (α-DNP-lysine) was first chosen as a model compound for the protein part of the conjugate. The substance has a bright yellow colour, and can be quantitatively analysed by its absorption at 425 and 450 nm.

The α-DNP-lysine was synthesized via the ε-benzoyl lysine (ε-Bz-lysine), which was prepared following the procedure of Okuda and Zahn [133] via the reaction of benzoyl chloride with the copper(II) complex of lysine, in which the α-position is protected. After deprotection, the ε-Bz-lysine was dinitrophenylated by the method of Sanger [94 a]. Finally the ε-Bz group was removed by refluxing the compound in acetic acid/hydrochloric acid for three days. The correct structure of the product was confirmed by NMR spectoscopy.

The α-DNP-lysine proved to be very sparingly soluble in neutral and basic aqueous solutions, which were needed for the coupling reaction. ε-DNP-lysine (Sigma) was more solu-

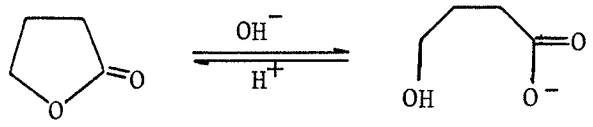
ble in aqueous solutions of suitable pH, and was therefore later used as a model compound for proteins instead of the stucturally more logical but effectively insoluble α-DNP-lysine.

6.2 LACTONE INVESTIGATIONS AND APPLICATIONS TO PVA-CO_2H

It is possible that the carboxyl end group of PVA forms an intramolecular lactone with the backbone hydroxyl groups. A lactone is as such, without further activation, susceptible to nucleophilic attack by amines, which would lead to formation of amide bonds. The lactone formation and ring opening of the model compounds γ-butyrolactone and γ-methyl-γ-butyrolactone were investigated. The reactivities of the lactones with benzylamine and 1,6-diaminohexane were studied before applying the same reactions to carboxyl terminated PVA and α-DNP-lysine.

6.2.1 Ring opening and closure of model lactones

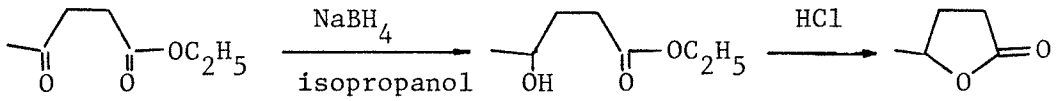
The ring opening of γ-butyrolactone in basic solution was followed by NMR. The lactone remained in its lactone form after standing for three days at pH 7.8. At pH 9 the ring gradually opened, and after standing for 0.5 hr in a solution of initial pH 12.2 and final pH 9.9 the ring was totally opened:



Stable at pH≤7.8 Stable at pH ≥9.9

The kinetics of the opening of the ring was studied by following the rate of addition of sodium hydroxide to an aqueous solution of the lactone required to keep the pH constant at 10.0±0.5 at 24.5 °C and at 0 °C. The pseudo first order rate constant was found to be $1.7 \cdot 10^{-4}$ s^{-1} at 24.5 °C and $3.0 \cdot 10^{-5}$ s^{-1} at 0 °C, suggesting a slow ring opening. Details can be found in the experimental section.

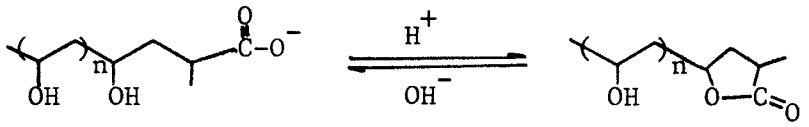
γ-Methyl-γ-butyrolactone, the second model lactone, was prepared by sodium borohydride reduction of ethyl levulinate, which was followed by lactonization [134]:



The structure of the lactone was confirmed by NMR. The IR carbonyl absorption appeared at 1785 cm^{-1}.

The lactone ring was stable in acidic solution but opened in strongly basic solution. When titrating the open form with acid, it started to lactonize at pH 1.2 at room temperature. Heating the sample for 5 minutes on the steam bath closed the ring totally.

These results suggest that carboxyl terminated PVA may exist in a lactone form at least in acidic solutions:



Basic solution Acidic solution

From the carbonyl IR absorption of the carboxyl terminated PVA at 1740 cm^{-1} in acidic and neutral solution it cannot be determined unambiguously whether the absorption is due to a lactone or to a carboxylic acid. The carbonyl absorption of the five membered ring of γ-methyl-γ-butyrolactone appeared at 1785 cm^{-1} in chloroform, while that of the six membered ring δ-valero lactone appears at 1735 cm^{-1} [134]. The carbonyl absorption of carboxylic acids in solution varies between 1730-1760 cm^{-1} [135].

6.2.2 Direct coupling of lactones to amines

A lactone ring can serve as an "activated ester" of the terminal carboxyl group, susceptible to nucleophilic attack by amino groups, because of the good leaving group properties of the intramolecular alkoxide ion. Thus, we investigated the possibilities of a direct reaction of this activated group to amino groups, without any conventional activation of the carboxyl group.

The model reactions of the two low molecular weight lactones with benzylamine and 1,6-diaminohexane were carried out on a small scale in NMR tubes where the disappearance of starting materials and appearance of the product could be monitored. The results of the model reactions are summarized in Table 4 and the results of the coupling attempts

TABLE 4

Direct coupling of model lactones to amines

Lactone	Amine	Solvent	Reaction cond. temp / time	Product
a	c	DMF	r. t./3 days	yes
a	d	borate buff. conc. solution	steam/0.5 h	yes
b	c	phosph. buff. conc. solution	steam/0.5 hr	yes
b	c	phosph. buff. dilute solution	steam/0.5 h	no
b	c	phosph. buff conc. solution	r. t./15 h	yes[e)]

a=γ-methyl-γ-butyrolactone
b=γ-butyrolactone
c=benzylamine
d=1,6-diaminohexane
e) the reaction was carried out on a large scale.
r. t. = room temperature

with PVA are summarized in Table 5

TABLE 5

Direct Coupling reactions of PVA-CO_2H to α-DNP-lysine

Reaction conditions			Amide formation
Pretreatment of PVA	Solvent	temp/time	
-	DMF	r.t./3 days	- [a]
acidified	borate buff. pH 10	r.t./15 hrs	-
boiled for 0.5 hr in pH 1	borate buff. adj. to pH 9.5	r.t./15 hrs	-
"	borate buff. adj. to pH 10	r.t./15 hrs	-
"	phosph. buff. pH 10	0-25 °C/15 hrs	-

[a] Reaction carried out on microscale in an NMR tube

The reaction of γ-methyl-γ-butyrolactone was first carried out in DMF. No reaction was observed after heating the sample on a steam bath for 0.5 hr, but amide formation occurred after reaction at room temperature for three days. The reaction was repeated for carboxyl terminated PVA and benzylamine on a NMR scale, but no change in the methylene hydrogens α to the amino group of the benzylamine indicating amide formation could be detected.

Since the coupling reactions of PVA with proteins have to be carried out in aqueous solutions to avoid denaturation of the proteins, model reactions were also carried out in aqueous buffer solutions. The lactone ring is unstable in solu-

tions of high pH, but the amine has to be unprotonated to be able to react. This requires a pH of > 10 (pK_a for ammonium salts of benzylamine = 9.35, for diamino hexane = 10.93 [132]). At this pH the nucleophilic attack of the amino group on the lactone carbonyl competes with the ring opening by hydrolysis which, however, should be relatively slow.

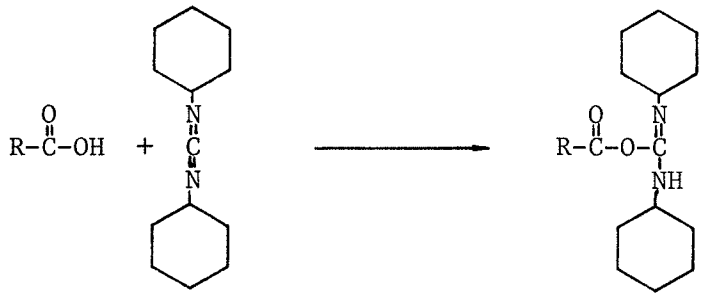
Both lactones were reacted with the amines in borate and and phosphate buffers at pH 10. In concentrated solutions of lactone and amine amide formation occurred, while no reaction could be observed in dilute solutions (Table 4).

The reactions were repeated on a larger scale with carboxyl terminated PVA and *a*-DNP-lysine. In some experiments the polymer was boiled for 5 min in acidic solution to ensure lactone formation. After reaction with *a*-DNP-lysine the polymer solutions were gel chromatographed to remove any low molecular weight material. In almost all cases the yellow *a*-DNP-lysine stayed on the column while the polymer eluted uncoloured with the void volume. In those cases where the polymer was slightly yellow when emerging from the column, the colour disappeared during the subsequent dialysis leaving the white unreacted polymer in the solution. Thus all attempts to couple PVA-CO_2H directly to *a*-DNP-lysine were unsuccessful.

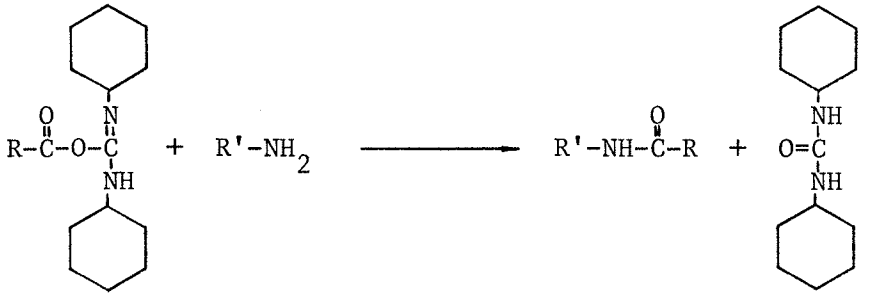
6.3 COUPLING REACTIONS USING DCC AS COUPLING REAGENT

Following the unsuccessful attempts to couple amino groups to the carboxyl terminated PVA without activation of the end group we studied model reactions using the coupling reagent dicyclohexylcarbodiimide (DCC), and then carried out the reactions under several different conditions with the carboxyl terminated PVA.

DCC is used in peptide synthesis to activate the carboxyl terminal towards reaction with the amino groups of amino acids [136]. In the first step of the reaction DCC forms an activated ester with the carboxylic acid:



The activated ester then reacts with amino groups to form an amide bond and dicyclohexylurea (DCU):



(DCU)

The DCU is practically insoluble both in water and most organic solvents and can be filtered off. The activation step can be carried out in aqueous solution without significant hydrolysis of the intermediate [136].

6.3.1 Model coupling reactions

Phenylacetic acid and 4-hydroxybutyric acid, the open form of γ-butyrolactone, were used in the model coupling reactions to benzylamine using DCC as coupling reagent. Reactions were carried out both in organic and aqueous solutions.

Phenylacetic acid and benzylamine were coupled using DCC in DMF. The structure of the resulting amide was confirmed by NMR. 4-Hydroxybutyric acid was coupled in aqueous solution to benzylamine with DCC as coupling reagent. To ensure the opening of the lactone ring before reaction the precursor γ-butyrolactone was heated in a large excess of 1 M phosphate buffer, pH 10. The amide was obtained in a low yield as detected by TLC. The reaction conditions and results are summarized in Table 6.

TABLE 6

Model coupling reactions with DCC as coupling agent

		Activation		Coupling		
Acid	Amine	Solvent	time (min)	Solvent	time (h)	Product
a	c	DMF	45	DMF	15	+ d)
b	c	Phosph. buff./ dioxane	10	Phosph. buff.	15	+ e)

Reactions were carried out at room temperature
a=phenylacetic acid
b=4-hydroxybutyric acid
c=benzylamine
d) Confirmed by NMR
e) Detected by TLC

6.3.2 Coupling attempts with PVA-CO_2H using DCC

After the successful coupling reactions of the model compounds to benzylamine with DCC, attempts were made to couple carboxyl terminated PVA to α-DNP-lysine with DCC as coupling reagent. The coupling attempts were carried out under different reaction conditions, which are summarized, together with the results, in Table 7. All coupling attempts were unsuccessful.

Attempts have been made to couple succinylated PVA to amines using carbonyldiimidazole as coupling reagent under similar reaction conditions, but these attempts were also unsuccessful [118].

TABLE 7

Coupling attempts of PVA-CO_2H with DCC

Reaction conditions				
Activation of PVA		Coupling to amine		
Solvent	time	Solvent	time	Product
DMF	1 hr	borate buff./ DMF	15 hrs	-
H_2O pH 2.5	10 min	borate buffer	"	-
H_2O pH 2.5	"	phosph. buffer	"	-
" pH 4	"	borate buffer	"	-
" pH 7	"	"	"	-
" pH 10	"	"	"	-
" pH 10	"	phosph. buffer	"	-
" pH 7 [a)]	"	borate buffer	"	-
DMSO [a)]		DMSO	"	-

For all activation reactions carried out in aqueous solutions the DCC was added in a small amount of dioxane.
[a)] the DCC was added last

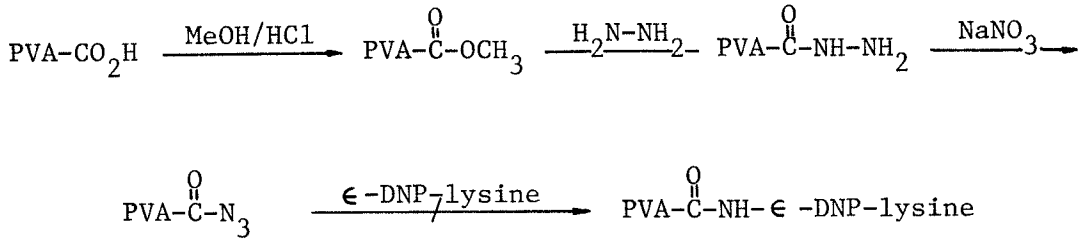
6.4 COUPLING ATTEMPTS VIA OTHER INTERMEDIATES

Several different methods of coupling the carboxyl terminated PVA to amines via activated intermediates employed in reactions for immobilizing enzymes to solid supports and in peptide synthesis were attempted. Since all these attempts were unsuccessful, only a short account of them is given here.

6.4.1 The Curtius azide method

The Curtius azide method [76,77] (see 2.2.2) was adopted for carboxyl terminated PVA. Without isolation or characterization of any of the intermediates, the polymer was es-

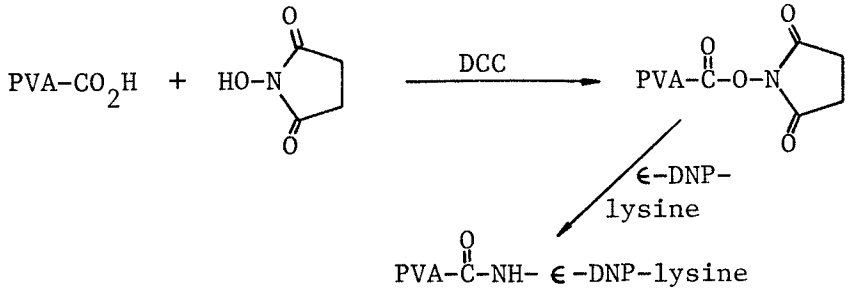
terified, reacted with hydrazine hydrate, the hydrazone converted to the azide and reacted with ε-DNP-lysine in buffered aqueous solution:



The yellow colour of ε-DNP-lysine did not remain in the polymer after dialysis.

6.4.2 Coupling via the N-hydroxysuccinimide ester of PVA

N-Hydroxysuccinimide esters of carboxylic acids are reactive towards amines, and stable enough to be isolated and stored in a dry atmosphere. Thus the activated polymer can be prepared beforehand, and stored until needed [137]. N-Hydroxysuccinimide esters are prepared by reacting the carboxylic group with N-hydroxysuccinimide in a dry solvent in the presence of DCC [137]. Following the method of Boccu and coworkers [67] carboxyl terminated PVA was reacted with N-hydroxysuccinimide in DMF using DCC as coupling reagent. Immediately after isolation of the intermediate the coupling reaction to ε-DNP-lysine was carried out in aqueous buffer solution:

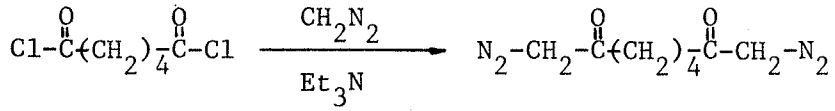


The purified polymer obtained contained only traces of ε-DNP-lysine as determined by UV spectroscopy.

6.4.3 Coupling via bifunctional linkages

Bifunctional linking reagents have been employed to immobilize proteins to solid supports [72]. Reacting one end of the bifunctional reagent with the polymer in dilute solution, to prevent crosslinking, leaves the unreacted end available for coupling to the ligand. Activation of the polymer and coupling to the ligand can thus be carried out in a single step, requiring only successive addition of the reagents.

1,6-bisdiazo adipate was prepared by reaction of adipoyl chloride with diazomethane [138]:



This bifunctional reagent was reacted with carboxyl terminated PVA in H_2O/dioxane, and then without isolation with ε-DNP-lysine in aqueous buffer. The polymer obtained did not contain any ε-DNP-lysine after purification.

Similar coupling attempts using divinyl sulfone [72] have also been unsuccessful [118].

Several reactions employed for coupling carboxyl functions on polymers onto proteins used in immobilizing enzymes require organic conditions. These reactions can not be utilized for coupling reactions to PVA because of the low solubility of this polymer in most organic solvents.

6.5 CONCLUSIONS

The chain transfer reactions of polymerizing vinyl acetate to functional transfer agents used as solvents were successful in giving the functionally terminated polymers of desired molecular weight. The presence of the end groups could not be established unambiguously by analytical methods such as FTIR, ^{13}C NMR, ^{1}H NMR, dye tests and electrophoresis. Only ^{14}C labeling of the carbonyl end group showed the presence of carboxyl groups unambiguously. The difficulty in analysing the polymers was due both to the minute amount of end groups present in a polymer sample, and to the nature of the polymer. End groups capable of hydrogen bonding such as the carboxyl and amino groups probably are tightly trapped within the polymer coil and hydrogen bonded to the backbone hydroxyl groups. The same argument explains the low reactivity of these terminal groups in the coupling reactions.

Although these polymers are functionally terminated, their low reactivity renders them useless for conjugation purposes. The next step was therefore to try to make the end group of PVA reactive by removing them from the influence of the backbone hydroxyl groups by introduction of a spacer arm between the polymer and the end group.

Chapter VII

FUNCTIONALLY TERMINATED PVA WITH SPACER ARMS

When coupling ligands to soluble supports for use in affinity chromatography, the polymer support is sometimes equipped with short side chains terminated by the functional groups onto which the ligands are coupled [78]. The side chains act as spacer arms which remove the ligand from the immediate vicinity of the polymeric support. By doing this the steric obstacles hindering the binding of the counter components to the ligand are minimized, giving a more activated polymer-ligand conjugate.

In order to prepare a reactive functionally terminated PVA with a spacer arm, the spacer arm has to be chemically different from the main polymer chain. A hydrophobic spacer arm would be preferable as it would not associate with the hydrophilic polymer backbone and would therefore be more accessible to external reagents.

A functional group on a spacer arm could be introduced into the polymer in the polymerization stage through a chain transfer reaction, but in practice it is not feasible to carry out the reaction in a large amount of a high molecular weight transfer agent. An attempt was earlier made using 11-bromoundecanoic acid as the chain transfer agent

(5.2.3.4.), but no polymer could be isolated due to the interference of the large excess of the transfer agent. Instead attempts were made to react the end group of hydroxyl terminated PVAc with molecules which would provide both the spacer arm and the terminal functional group. PVAc is soluble in most organic solvents, so it is easier to derivatize that polymer than PVA. The spacer arm has to be coupled to the polymer by a linkage which is stable to the hydrolysis conditions required to convert the PVAc to PVA, which excludes all types of ester linkages. Ether linkages, on the other hand, are stable to hydrolysis conditions.

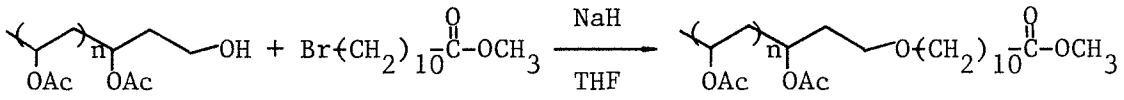
The first attempt was to react epichlorohydrin with the terminal hydroxyl group of PVAc-OH to produce oxirane terminated PVAc with a short spacer arm [72]. However, that method was abandoned because of the instability of the oxirane group towards the hydrolysis conditions employed when converting PVAc to PVA. Terminating PVAc-OH with undecanoate ester proved to be a more successful method.

7.1 UNDECANOIC ACID TERMINATED PVA

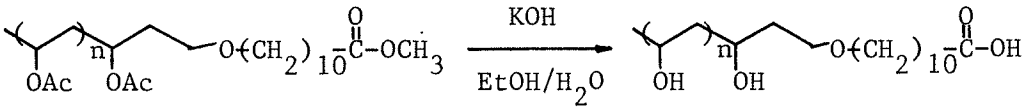
Reacting hydroxyl terminated PVAc with 11-bromoundecanoate ester gives ester terminated PVAc with a ten carbon hydrophobic spacer arm between the back bone of the polymer and the ester end group. The terminal ester group is hydrolysed during the conversion of PVAc to PVA, giving carboxyl terminated PVA with a spacer arm. The spa-

cer arm is long enough to remove the carboxyl group sufficiently from the influence of the backbone hydroxyl groups.

The methyl undecanoate terminated PVAc was prepared by reacting methyl-11-bromoundecanoate with hydroxyl terminated PVAc in the presence of sodium hydride:



Hydrolysing the PVAc to PVA gave carboxyl terminated PVA with a spacer arm:



Titration of the different batches of the product indicated 0.7-0.9 acidic groups per polymer had been incorporated, representing a 70-90% conversion. In the IR spectrum of the polymer in acidic D_2O the carbonyl absorption appeared at 1730 cm^{-1} . In the spectrum of a basic D_2O solution of the polymer this peak had disappeared, and the absorption due to the carboxylate anion appeared at 1550 cm^{-1} .

Earlier attempts to separate carboxylated PVA from non-carboxylated PVA on DEAE cellulose ion exchange columns had been unsuccessful (section 5.2.1), so no attempts were made

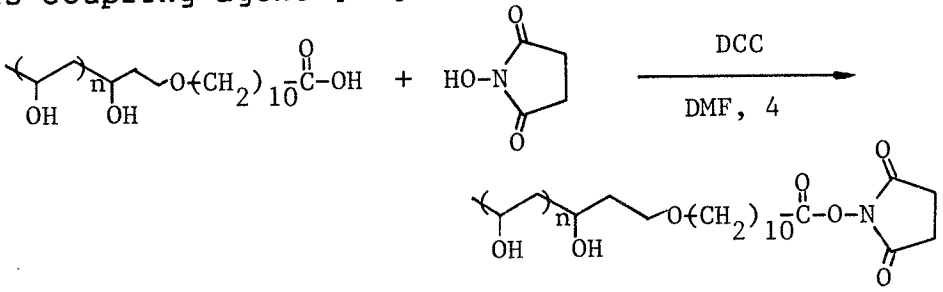
at this point to separate the noncarboxylated material from the carboxylated. The presence of noncarboxylated PVA presents no problem for subsequent coupling reactions of the carboxylated polymer to form conjugates and can be removed during purification of the conjugates.

7.2 COUPLING OF UNDECANOIC ACID TERMINATED PVA TO AMINE

Attempts to couple undecanoic acid terminated PVA directly to ε-DNP-lysine using DCC as coupling agent, employing reaction conditions as in section 6.3.2 were unsuccessful. The use of an activated succinimide intermediate in the coupling of carboxyl terminated PVA without a spacer arm to α-DNP-lysine had previously resulted in traces of amide present in the polymer (section 6.4.2). Hence this method was employed for the undecanoic acid terminated PVA.

7.2.1 Preparation of activated undecanioc acid terminated PVA

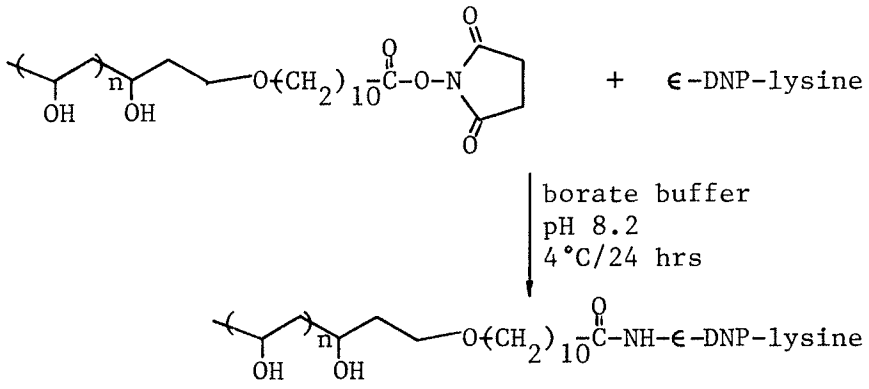
The preparation of N-hydroxysuccinimide activated PVA was carried out by reacting the undecanoic acid terminated PVA with N-hydroxysuccinimide in anhydrous conditions with DCC as coupling agent [97]:



The reaction had to be carried out in a very dilute solution because of the low solubility of PVA in cold DMF. After removal of the DCU by filtration, the polymer was isolated by precipitation with diethyl ether. Because of the poor solubility of PVA in organic solvents it could not be further purified by reprecipitation from a concentrated organic solution. The subsequent coupling step was carried out immediately without further purification or analysis.

7.2.2 Coupling of the activated PVA to amine

The N-hydroxysuccinimide ester of the undecanoic acid terminated PVA was coupled to ε-DNP-lysine in aqueous buffer solution:



After reaction at 4 °C for 24 hrs the product was isolated from excess ε-DNP-lysine by gel filtration (Sephadex G25) and by dialysis against distilled water for four days to remove any unbound ε-DNP-lysine from the polymer. After lyophilization a pale yellow polymer was obtained. The polymer contained 0.26 DNP groups per chain, as determined by the UV absorption at 425 nm. This accounts for 26% conversion of the total amount of PVA, of which only 80% was carboxylated. Based on the amount of carboxylated PVA present the conversion was about 30%. The unreacted PVA was not separated from the conjugate because of their similar physical and chemical properties.

These results confirmed the prediction that the terminal carboxyl group, when removed from the immediate vicinity of the polymer backbone, would retain its activity. The low conversion, 30% based on the carboxyl terminated polymer, could not be attributed solely to a low reactivity of the carboxyl group. The product was obtained from the carboxyl terminated PVA in two steps, of which the latter suffered from the reaction conditions which had to be used. This last step had to be carried out in aqueous solution, so a simultaneous hydrolysis of the active N-hydroxysuccinimide ester could not be avoided. An amino group, being more nucleophilic than a hydroxyl group, is more reactive towards the N-hydroxysuccinimide ester, but the relative excess of water favours the hydrolysis reaction. A very dilute solu-

tion had to be used because of the low solubility of the polymer in cold water. Equimolar amounts of the activated polymer and the amine were used in this experiment, but using a large excess of the amine should increase the yield of conjugate.

7.2.3 Attempts to couple the activated PVA to ovalbumin

Attempts were made to couple ovalbumin to the N-hydroxysuccinimide activated ester using the previous method. Ovalbumin has 19 lysine residues to which the polymer can be attached, so the polymer was added in a 19 molar excess over the protein. The coupling reaction was carried out at 4 °C for 15 hrs in borate buffer, pH 8.2. The reaction product was desalted by gel chromatography and was lyophilized. To remove the unreacted material from the possible conjugate the product was eluted through a DEAE cellulose ion exchange column. Elution with 0.008 M phosphate buffer, pH 7.7, should have allowed a fractionation of the unreacted PVA from the conjugate, with subsequent elution of the conjugate with 0.05 M acetate buffer, pH 4. The results of the elution indicated that no coupling had occurred. The PVA was eluted with the first 2-4 fractions (30 ml) of phosphate buffer, and the protein was eluted with the acetate buffer. NMR spectra of the lyophilized polymer and protein fractions confirmed that no coupling had occurred.

Although the conjugation was successful with the model compound ε-DNP-lysine, three repetitions of the above experiment showed that coupling to protein does not take place under the same conditions. Since steric hindrance is not usually a problem in immobilizing enzymes on solid and soluble supports, hindrance to coupling was not anticipated in the present experiment. However, the combined effect of the poor formation of the active ester because of solubility problems, the poor solubility of the activated ester, which forces the reaction to be carried out in dilute solution, and the steric effects on the reaction of the amino groups attached to the bulky protein with the single active end group on the polymer coil, is evidently enough to prevent the coupling from occuring. The smaller ε-DNP-lysine can reach the activated end group of the polymer and react, while it is probably more difficult for the amino groups of the protein to penetrate the PVA coils. The situation for an enzyme being immobilized on a solid support is also more favourable because of the large number of active groups along the support, while the percentage of active groups per PVA in this case is only around 1%.

Since the steric hindrance in the reaction cannot be altered, further efforts were made to minimize the other negative factors. Earlier work on PEG has established that, unlike PVA, it is easy to introduce an active end group and prepare PEG-protein conjugates. For this reason attempts

were made to prepare PVA-PEG copolymers with an active functional group on the PEG portion of the copolymer. It was anticipated that this would facilitate synthesis of conjugates yet retain the desired biological properties of having a major PVA portion in the polymer part of the conjugates.

Chapter VIII

PVA-PEG POLYMERS

If functionally terminated PVA with a PEG spacer arm is prepared advantage can be taken of known procedures to prepare PEG-ligand conjugates. Procedures that use the reactivity of the terminal hydroxyl group of PEG can not be used because of interference with the backbone hydroxyl groups of PVA. A carboxyl terminated PEG spacer arm provides the potential for coupling to ligands with the methods used for carboxyl terminated PEG.

If the PEG spacer arm can be made sufficiently long relative to the PVA chain, the solubility of the block copolymer approaches the solubility of PEG. A polymer-allergen conjugate made with this kind of copolymer would probably still have the properties of a PVA conjugate in a biological system, since the PVA terminates the polymer strands.

Attempts were made to polymerize ethylene oxide directly onto hydroxyl terminated PVAc, with limited success. Grafting of vinyl acetate on PEG proved to be more successful. Before attempting these reactions different methods of converting the terminal hydroxyl group of monomethoxy PEG to a terminal carboxyl group were reviewed.

8.1 PREPARATION OF CARBOXYL TERMINATED PEG

The general plan for preparing a carboxyl terminated PVA-PEG-CO_2H copolymer was to first prepare the hydroxyl terminated PVAc-PEG-OH copolymer. The hydroxyl terminator would be converted to a carboxyl group, and then the acetates would be removed by hydrolysis to provide PVA-PEG-CO_2H. Since the methodology for conversion of PVAc-PEG-OH to PVAc-PEG-CO_2H should be the same as for the known conversions of PEG-OH to PEG-CO_2H, these reactions were reviewed as model reactions for the copolymer system.

A direct oxidation of the terminal hydroxyl group to a carboxyl group both with chromium trioxide in the form of the pyridinium:dichromate complex [139], and with activated manganese oxide following the method of Boccu and coworkers [67] was first attempted. Both methods failed to give carboxyl terminated PEG as determined by ^{13}C NMR spectroscopy.

A more successful method was to react the terminal hydroxyl group with ethyl-*a*-bromoacetate to produce the ester terminated PEG which, after hydrolysis, gave carboxyl terminated PEG [66,83]:

$$CH_3O(CH_2CH_2O)_nCH_2CH_2\text{-}OH \xrightarrow[THF]{\text{NaH or Na/naphthalene}} CH_3O(CH_2CH_2O)_nCH_2CH_2\text{-}O^-$$

$$\xrightarrow{BrCH_2\overset{O}{\overset{\|}{C}}\text{-}OC_2H_5} CH_3O(CH_2CH_2O)_nCH_2CH_2\text{-}O\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}OC_2H_3 \xrightarrow{\text{hydrolysis}}$$

$$CH_3O(CH_2CH_2O)_nCH_2CH_2\text{-}O\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH$$

The alcoholate anion of the hydroxyl terminated PEG was generated by addition of either sodium hydride or sodium/naphthalene [140] to a dry THF solution of the polymer. When using sodium/naphthalene the reaction could be easily monitored by the disappearance of the colour of the dark blue sodium/naphthalene complex. When all of the alcohol had reacted the colour of additionally added complex remained. In the case of sodium hydride a 2-5 molar excess of the hydride over the terminal hydroxyl groups was added. The yield of ester terminated PEG was higher when the anion was generated with sodium/naphthalene. The results of the reactions are summarized in Table 8

TABLE 8

Preparation of carboxyl terminated PEG

		Analysis		
		Ester	Acid	
Batch	Solvent	^{1}H NMR [a]	^{13}C NMR [b]	titr
1	DMF		-	
2	CH_2Cl_2		-	
3	THF	0.1	-	0.1
4	THF	1	+	0.7

1 = pyridinium/dichromate
2 = MnO_2
3 = *a*-Br-acetate/NaH
4 = *a*-Br-acetate/Na/naphthalene
a) The number of CO_2Et/chain is estimated by integration.
b) The presence of a carbonyl resonance peak is indicated with a + .

The ^1H NMR spectrum of the ethyl ester terminated PEG show a singlet at 4.10 ppm which was assigned to the methylene protons next to the carbonyl. (See Figure 7). The peaks of the ethoxy group appeared at 1.30 and 4.25 ppm.

After hydrolysis the methylene protons next to the carbonyl shifted to 3.95 ppm. Integration of the methylene peaks and titration of the final acid indicated there were 0.1 carboxyl groups per chain for PEG #3 (see Table 8). PEG #4 (see Table 8) integrated for 1.0 carboxyl groups per chain and titrated for 0.7. The ^1H NMR spectra of PEG #4 are shown in Figures 7 and 8.

Attempts were made to separate the carboxylated PEG from the uncarboxylated on Dowex 1·8 - 50 anion exchanger, but with no success.

Figure 7: ^{1}H NMR of PEG-O-CH_2-(CO)-OC_2H_5 prepared with Na/naphthalene.

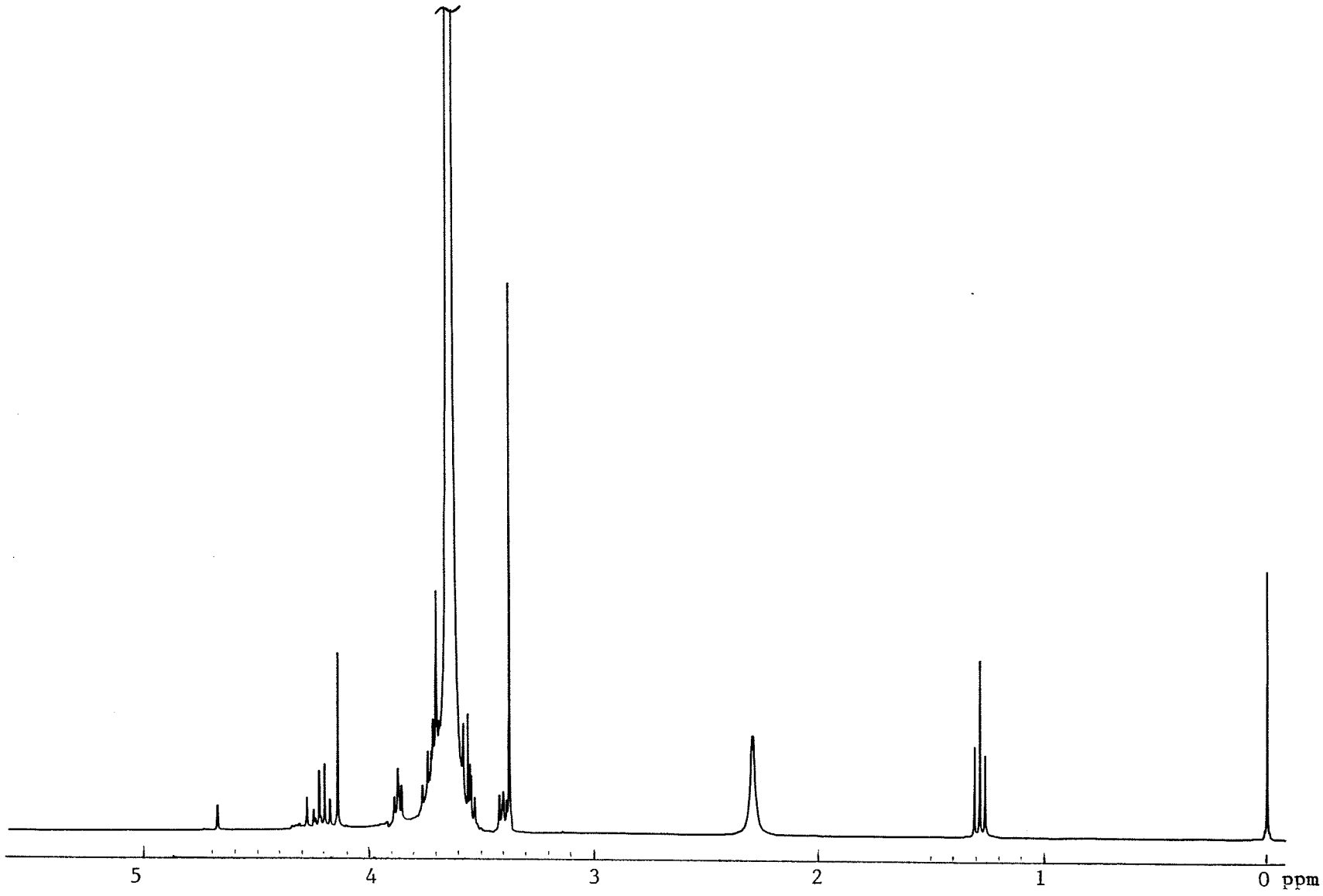
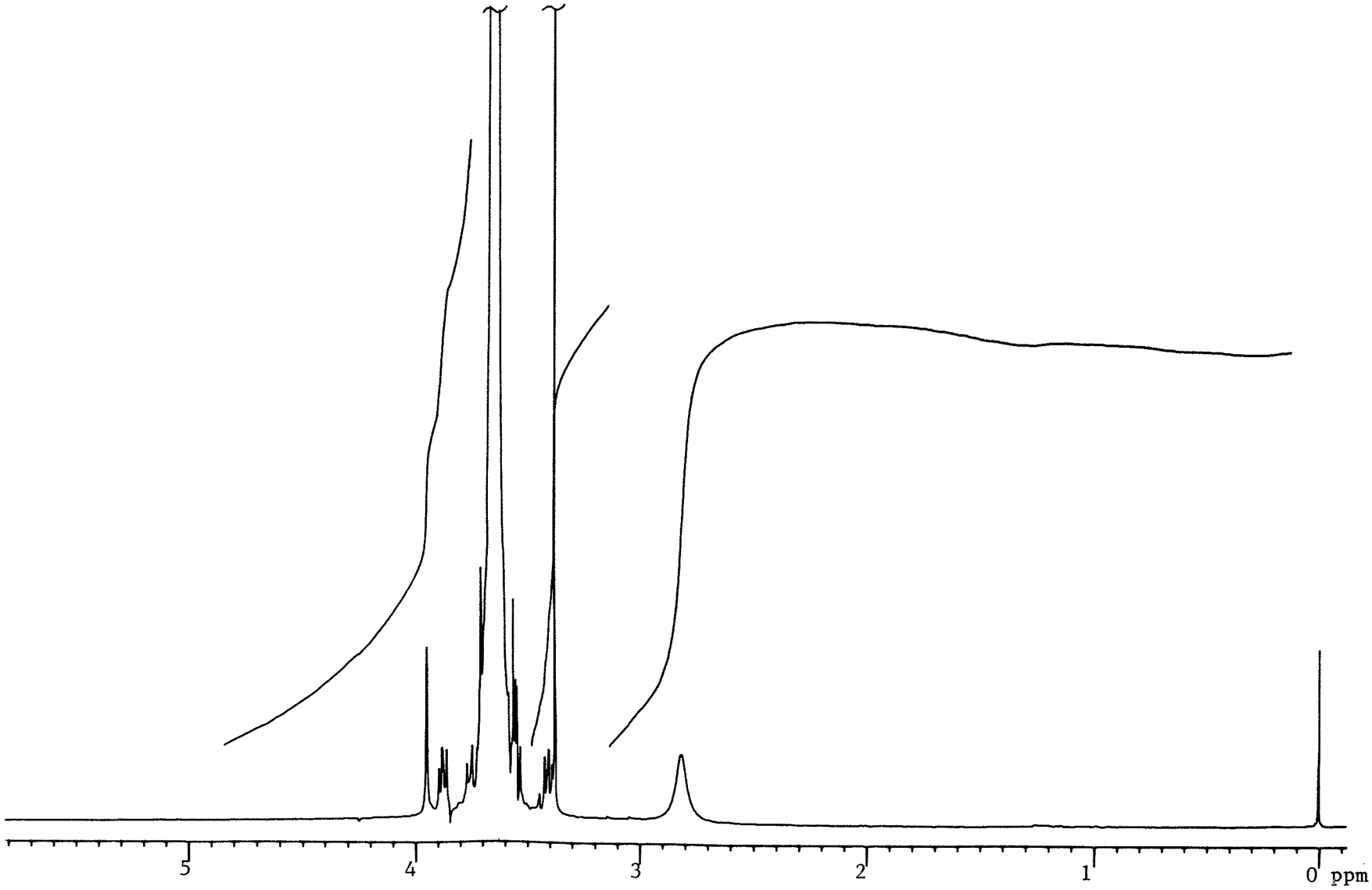

5
4
3
2
1
0 ppm

Figure 8: ^{1}H NMR of PEG-O-CH_2CO_2H prepared with Na/naphthalene.


5
4
3
2
1
0 ppm

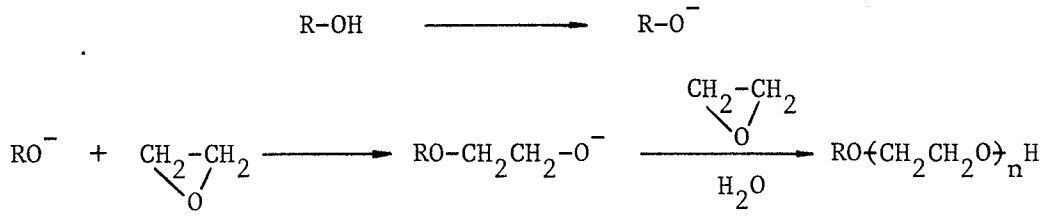
8.2 POLYMERIZATION OF ETHYLENE OXIDE ONTO PVAC-OH

An alcohol can be incorporated into PEG by an anionic polymerization of ethylene oxide in the presence of the alcohol and a catalyst [80]. The alcoholate ion, formed by the influence of the catalyst, functions as the initiator of the polymerization. Usually the alcohol is used in excess, as a solvent, and a desired amount of the alcoholate ion is generated by addition of sodium, sodium hydroxide, or other bases. A slightly different approach had to be used in this work, since the alcohol could not be used as a solvent, and a 100% conversion of the alcohol to the alcoholate was desired. Thus the polymerization of ethylene oxide onto hydroxyl terminated PVAc was attempted in THF or dioxane using four different methods, three of which proceeded via an anionic mechanism and were initiated with the alcoholate ion generated by different catalysts. The fourth method proceeded via a cationic mechanism.

Since the alcoholate ion of PVAc-OH can transesterify with the backbone acetate groups, thus transporting the negatively charged group along the chain, the single polymerized ethylene glycol strand may be located anywhere along the PVAc chain.

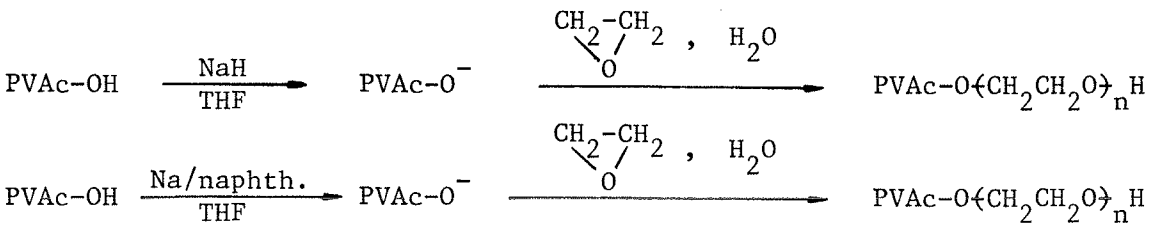
8.2.1 Sodium hydride or sodium/naphthalene as catalyst

If ethylene oxide is added to a solution containing alcoholate ions of PVAc-OH, these ions should initiate the polymerization of monomer via an anionic mechanism, and should be incorporated into the polymer [141]:



Thus attempts were made to polymerize the monomer on to the alcoholate ion of PVAc-OH, generated beforehand with either sodium hydride or sodium/naphthalene. A model reaction was carried out with benzyl alcoholate generated with sodium/naphthalene.

The alcoholate ion of the hydroxy terminated PVAc was generated by addition of sodium hydride or a THF solution of sodium/naphthalene to the THF solution of the alcohol. Ethylene oxide was added while care was taken not to introduce any humidity to the solution. The reaction was eventually quenched with water.



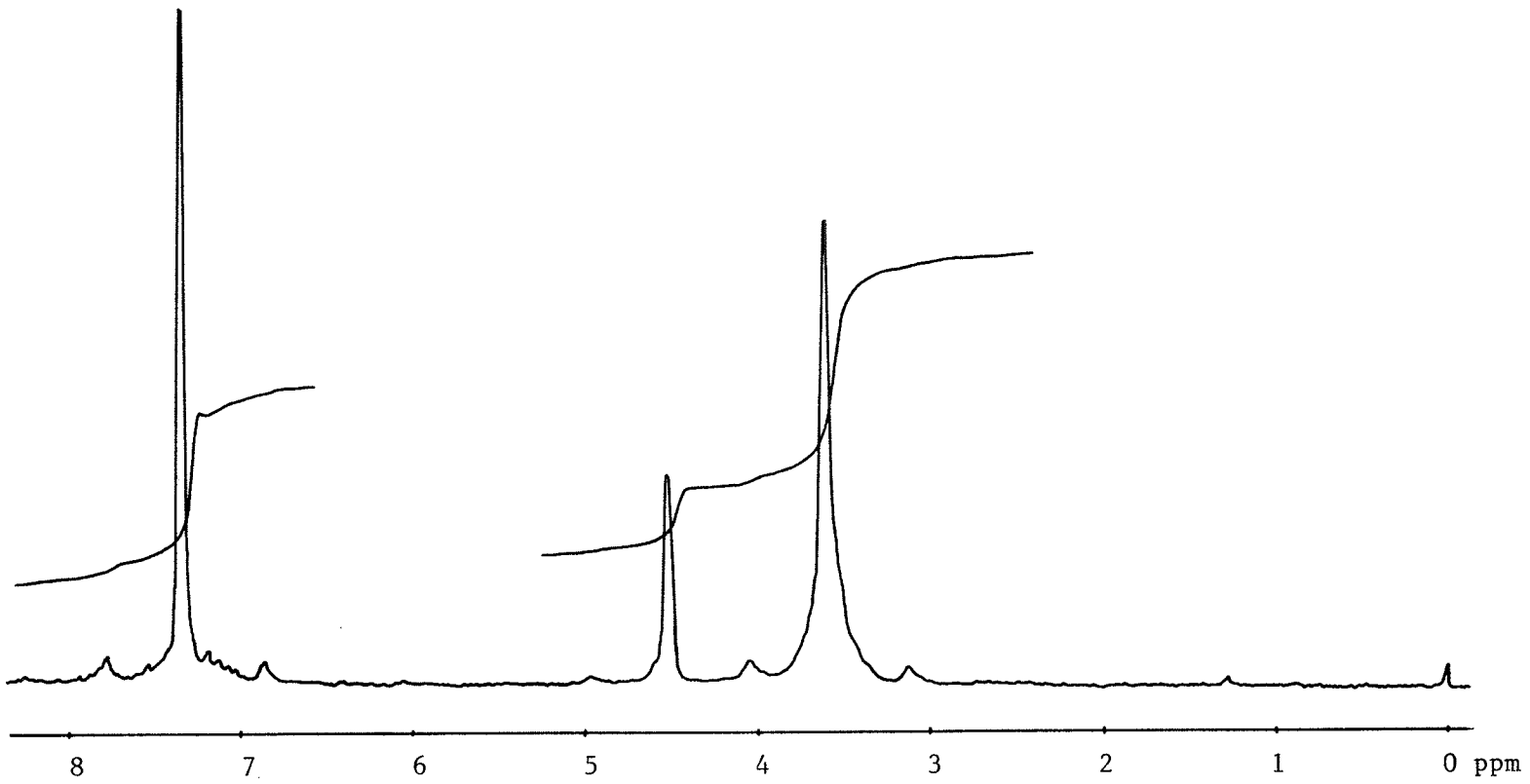
The model reaction with benzyl alcohol and sodium/naphthalene was carried out under similar conditions. The reaction data, together with the data from the other polymerization reactions, are summarized in Table 9 .

The reaction with benzyl alcohol was successful. The ratio of alcohol to ethylene oxide used in the reaction was about 1:10. The product, an oily liquid after evaporation under high vacuum, contained alcohol and ethylene glycol in a ratio 1:2 as determined by NMR (Figure 9), while TLC gave four distinct spots with R_f values less than that of benzyl alcohol, indicating 1:1, 1:2, 1:3, etc. adducts. No free benzyl alcohol or PEG homopolymer could be detected in this product.

Using the alcoholate ion of PVAc-OH, no polymerized ethylene glycol was produced. ^1H NMR of the isolated PVAc showed only a broad unresolved peak of very low intensity at the chemical shift expected for PEG (3.6 ppm). This indicated that a small part of the polymer may have initiated a reaction with ethylene oxide, but the living polymer was terminated rapidly.

The difference in reactivity of PVAc-OH and benzyl alcohol may be due to a different strength of ion pairing of the alcoholates with the counter ions and to an aggregation of the alcoholate ions in the dry DMF [141]. Although one would expect a free anion to be able to initiate the poly-

Figure 9: ^{1}H NMR of benzyl alcohol-PEG prepared using Na/naphthalene as catalyst

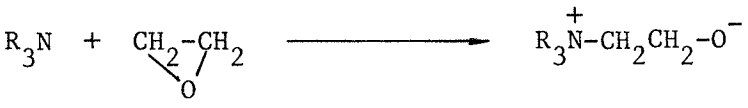

8
7
6
5
4
3
2
1
0 ppm

merization better than its ion pair, the reverse has been indicated in the polymerization of ethylene oxide [141]. An association of the oxygen atom of the monomer with the cation of the initiating ion pair is assumed to take place. If this association is made difficult to attain because of loose ion pairing with the counter ions, or by solvating these ions, the rate of the initiation of the polymerization of ethylene oxide is reduced. The propagation reaction on the other hand is enhanced by solvating the cation of the ion pair [141].

In order to solvate the alcoholate ions to enhance the propagation step, hexamethylene phosphoramide (HMPA) was added to the solutions after generating the anions with sodium/naphthalene [142]. However, not even benzyl alcohol could initiate the polymerization of ethylene oxide with HMPA present.

8.2.2 Polymerization with triethylamine as catalyst

Tertiary amines have been used as catalysts for the polymerization of ethylene oxide in the presence of alcohols [141]. The amine is thought to react with the ethylene oxide to produce a zwitterion, which then reacts with the alcohol to produce the alcoholate anion [143]:



$$R_3\overset{+}{N}-CH_2CH_2-O^- + R'OH \longrightarrow R_3\overset{+}{N}-CH_2CH_2-OH + R'O^-$$

The alcoholate ion initiates the anionic polymerization of the monomer:

$$R'-O^- + \underset{\diagdown O \diagup}{CH_2-CH_2} \longrightarrow R'O-CH_2CH_2-O^- \xrightarrow[H_2O]{\overset{CH_2-CH_2}{\diagdown O \diagup}} R'O\{CH_2CH_2-O\}_nH$$

We attempted to polymerize ethylene oxide onto PVAc-OH and onto benzyl alcohol as a model reaction using triethylamine as catalyst. The ethylene oxide was distilled into a THF solution containing the alcohol and triethylamine.

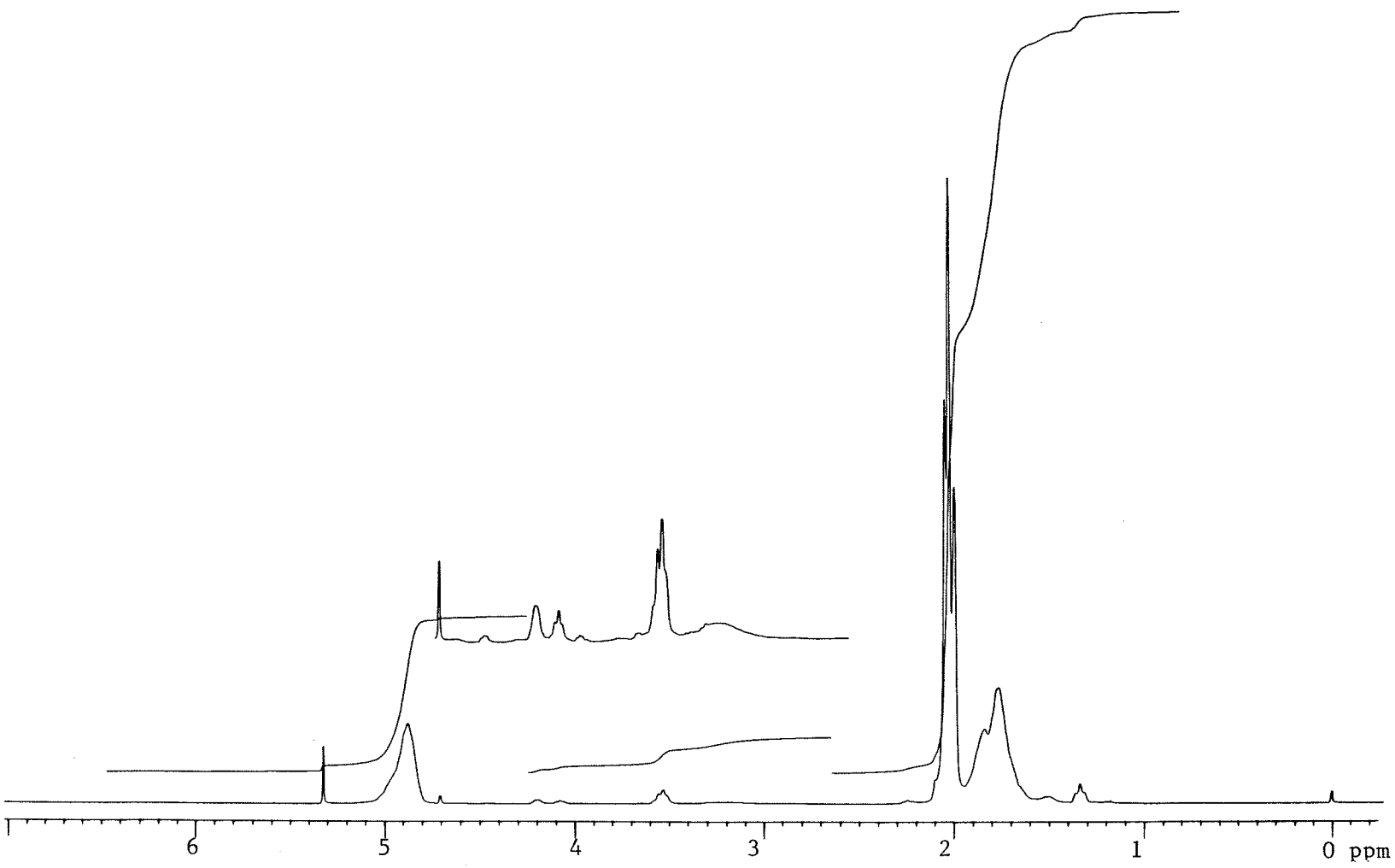
The reaction with benzyl alcohol gave a product which, after evaporation under high vacuum and removal of the quaternary ammonium alcohol on a cation exchange resin (Dowex 50W-X8), contained ethylene glycol bound to the benzyl alcohol in a ratio 4:1 (Table 9) The initial ratio of ethylene oxide to benzyl alcohol was 13:1. No free benzyl alcohol could be detected in the product by TLC. A parallel reaction of ethylene oxide and triethylamine in THF without any alcohol gave small amounts of PEG. This indicates that small amounts of homopolymer probably form together with the alcohol bound PEG in the polymerization initiated by alcohol.

Initiating the polymerization with PVAc-OH in the presence of triethylamine gave, after reaction for three days at

room temperature, a polymer with an average of one ethylene glycol unit per PVAc chain, estimated by NMR (Figure 10). Attempts to carry the propagation step further by addition of monomer and reaction for three more days at 55 °C resulted in a degradation of the PVAc, which gave a severely discoloured polymer that was not investigated further.

Figure 10: ^{1}H NMR of PVAc-PEG prepared with triethylamine

The PVAc was obtained by direct polymerization of ethylene oxide onto PVAc-OH using triethylamine as catalyst.


6
5
4
3
2
1
0 ppm

8.2.3 Polymerization with boron trifluoride etherate as catalyst

Boron trifluoride and its diethyl ether complex have been used in the cationic polymerization of heterocycles including ethylene oxide [144]. The exact mechanism involved in the initiation is not fully understood. Merrall and coworkers have polymerized ethylene oxide in the presence of alcohols [145]. They concluded that the initiating species may be complexes of the catalyst, the alcohol and the monomer, giving PEG with the alcohol incorporated as an end group. Geckeler and Mutter have polymerized ethylene oxide onto the aglycon of Cinerubin A using boron trifluoride etherate as catalyst [34]. Their product was analysed only by TLC.

Attempts were made to polymerize ethylene oxide onto PVAc-OH using the method of Geckeler and Mutter. Boron trifluoride etherate was added to a cold solution of PVAc-OH and ethylene oxide in dioxane, the flask was sealed, and the reaction mixture was stirred for 2-5 days at room temperature.

Since the BF_3 readily forms complexes with all compounds present in this system, it is difficult to predict which one or which ones are the initiating species. Thus the formation of PEG homopolymer in addition to PVAc-PEG block copolymer was expected.

Since PVAc is insoluble whereas PEG dissolves readily in water, the product was purified from PEG homopolymer by repeated precipitation from a concentrated ethanol solution by addition of water. Starting with a polymer mixture which by NMR spectroscopy contained PVAc and PEG in a ratio 2:1, most of the PEG was lost in the precipitations. Finally PVAc with only a trace of PEG was obtained (Figure 11). The results may indicate that most of the PEG was in the form of homopolymer. However, the precipitation method may have fractionated off PVAc-PEG containing a large portion of PEG. The copolymer may have solubility properties similar to PEG, thus making it difficult to separate the copolymer from the homopolymer. Because of the small amount of ethylene glycol present in the final product we did not proceed further on this line but instead concentrated our efforts on preparing a PEG-PVA-CO_2H free radical graft polymer.

Figure 11: ^{1}H NMR of PVAc-PEG-OH

The product was prepared by direct polymerization of ethylene glycol onto PVAc-OH with boron trifluorid etherate as catalyst.
a = before purification ; b = after purification

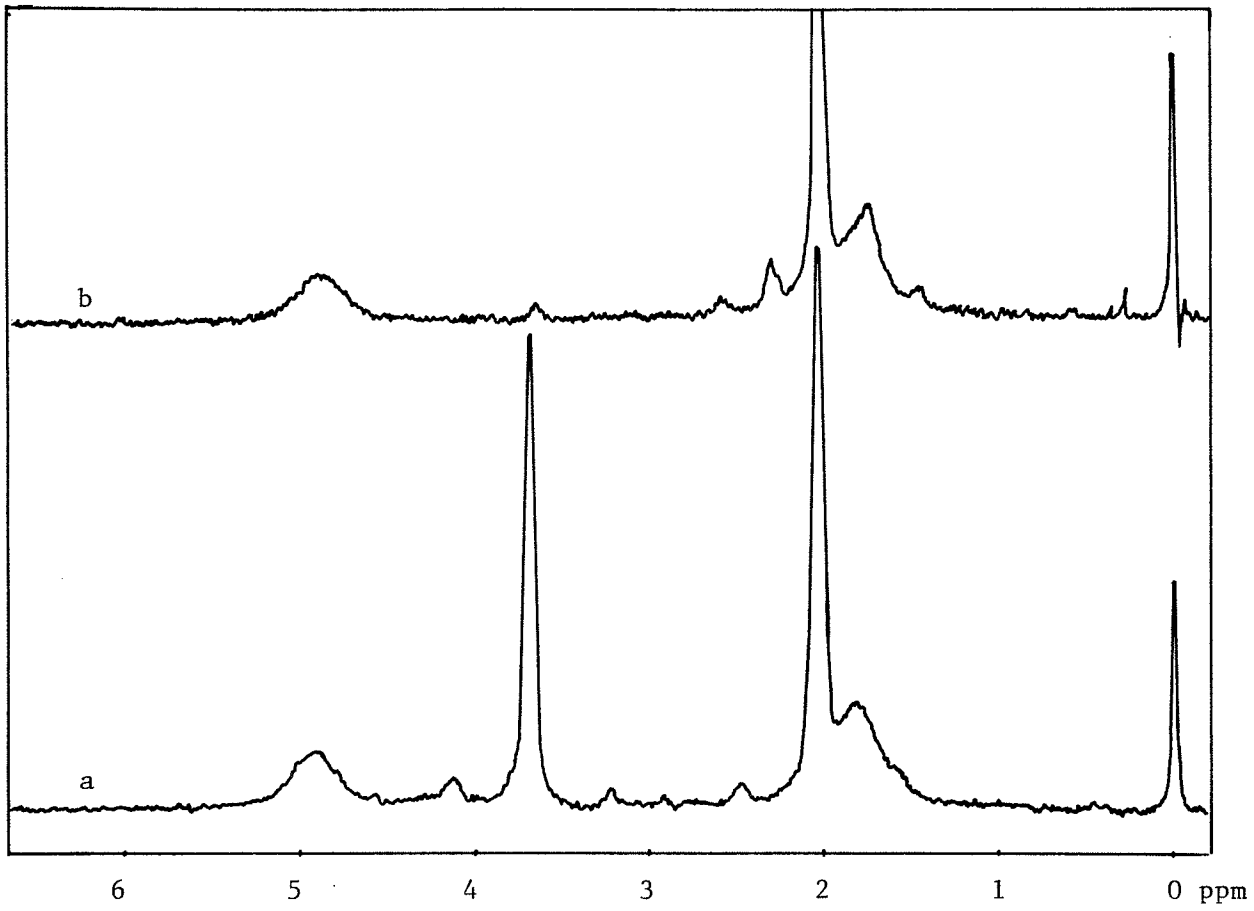

b
a
6
5
4
3
2
1
0 ppm

TABLE 9

Polymerization of ethylene glycol onto alcohols

Catalyst	Alcohol	Solvent	Product	
			alc./monom.	homopolym.
a	benzylalc.	THF	1:2	
a	"	THF/HMDA	-	no
a	PVAc-OH	THF	-	no
a	"	THF/HMDA	-	no
b	PVAc-OH	THF	-	no
c	benzylalc.	THF	1:4	yes f)
c	-	THF		yes
d	PVAc-OH	dioxane		yes

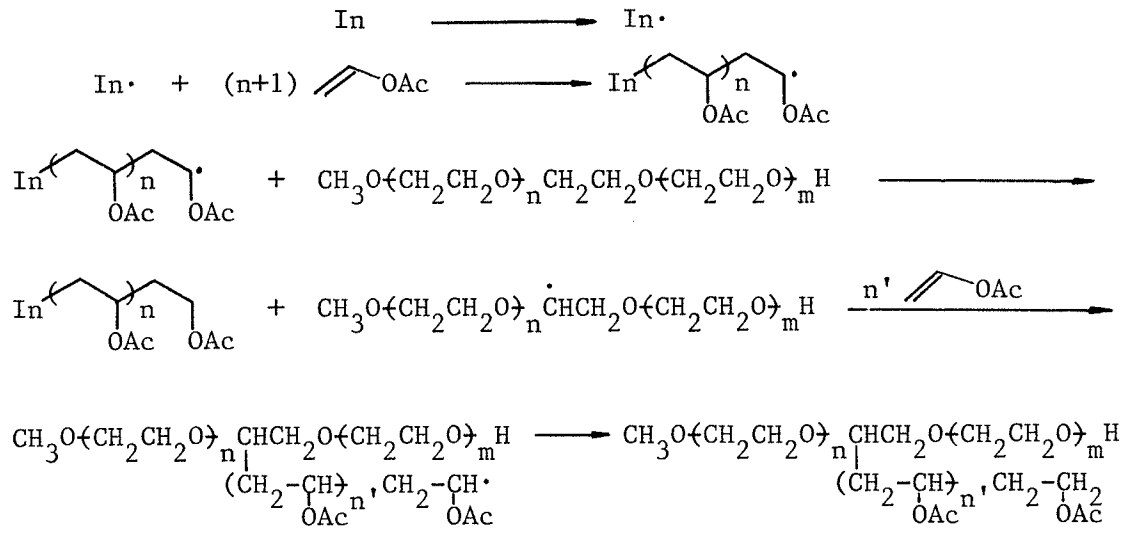
a = Na/naphthalene
b = NaH
c = triethyl amine
d = BF_3-etherate
f) Probably present in small amounts.

8.3 FREE RADICAL GRAFTING OF VINYL ACETATE ONTO PEG

Vinyl monomers have been grafted along the backbone of polymers containing pendant functional groups [146-150]. The reactions have been initiated either with a free radical initiator [151], photochemically, or by means of a chemical reaction, ie. "chemical initiation" [147,149]. Ethylene oxide has been polymerized on to the pendant hydroxyl groups of PVA to produce a PEG-PVA graft [152].

By grafting PVAc onto PEG a polymer with properties of both PVAc/PVA and PEG is obtained. After hydrolysis the bulk of the polymer resembles PVA, while the reactivity of the end group on the PEG is likely to be retained. If the grafting is carried out on carboxyl terminated PEG, the carboxyl group might be used to conjugate to ligands.

Vinyl acetate was grafted onto PEG via chain transfer of the polymerizing vinyl acetate to PEG. The sites of chain transfer start the polymerization of chains of PVAc which are attached to the PEG backbone, thus producing the grafted polymer. Initiation of the vinyl acetate polymerization was carried out in the usual way by thermolysis of dBPO:



The polymerization of vinyl acetate onto PEG-OH was carried out at 60 °C, at which temperature the PEG readily dissolved in the vinyl acetate, making the use of a solvent, which would interfere by chain transfering with the vinyl radicals unnecessary.

After an induction period of 15-20 min the polymerization started and was completed in another 15-20 min. The reaction was terminated by opening the flask to atmospheric oxygen, or by adding carbon tetrachloride. By increasing the amount of PEG and initiator relative to the vinyl acetate,

the ratio of vinyl acetate monomer units to ethylene oxide monomer units in the resulting polymer could be decreased from 8.4:1 to 1.4:1. Three batches of grafted polymer were prepared with hydroxyl terminated PEG before preparing the carboxyl terminated graft. The reaction data and results

TABLE 10

Graft polymerization of vinyl acetate onto PEG

Batch #	Vinyl acetate [g]	PEG [g]	dBPO [g]	Product (PVAc:PEG)[a]	
				initial	final
1	5	0.5	0.08	8.4:1	12:1
2	5	1.0	0.08	3.2:1	3.7:1
3	5	1.0	0.1	1.4:1	
4[b]	5	1.0	0.1	1.7:1	9:1

a) The ratio PVAc:PEG was determined by NMR before and after attempted isolation from the homopolymers.
b) Batch #4 was prepared with PEG-CO_2H

are summarized in Table 10

Only a few PVAc strands per PEG chain were expected to form via the chain transfer reaction. Along with the graft, some unattached PVAc homopolymer and some unreacted PEG were also expected.

Separation of free PEG and PVAc from the potential PVAc-PEG graft was necessary in order to characterize the graft. The usual method for such a separation is to take advantage of the selective solubilization of individual components in

different solvents. Thus the first two batches of graft were precipitated from ethanol solutions by the addition of water with the hope that the PVAc and PVAc-PEG graft would be preferentially precipitated. However, analysis of the precipitate by NMR indicated that the separation was incomplete and that the PVAc and/or PVAc-PEG was also being fractionated with preferential precipitation of higher molecular weight material only.

In order to facilitate the separation the product mixtures (batch #3 and #4) were hydrolysed to a mixture of PVA, PEG and PVA-PEG graft in order to take advantage of the greater difference in solubility of PEG and PVA. After hydrolysis and desalting, the aqueous solution of the mixture was extracted with methylene chloride with the expectation that PEG would be extracted by the organic solvent leaving the PVA and PVA-PEG graft in the aqueous solution. Although emulsion formation was a problem this could be partially overcome by centrifugation eventually giving (after several extractions and back extractions) a methylene chloride fraction of pure PEG and an aqueous solution which analysed for both PEG and PVA (NMR). The fact that the PEG detected in the aqueous fraction was indeed PEG-PVA graft and not free PEG was ascertained by TLC on alumina which showed the absence of free PEG. In addition, when an admixture of PEG and PVA was distributed between methylene chloride and water the PEG distributed itself between the organic and the aque-

ous layer in a ratio 16:1. A control extraction of the isolated graft did not redistribute the PEG, maintaining the ratio PVA:PEG (6.8:1) in the aqueous layer, while nothing could be found in the organic layer. Any free PEG would have redistributed itself into the organic phase.

An attempt was also made to separate both the free PVAc and the free PEG from the unhydrolysed polymer by column chromatography on silica. About 62% of the material was eluted with methylene chloride/t-butyl alcohol. The ratio of PVAc to PEG in that material was 7.6:1. Only traces of graft eluted using increasing ratios of t-butyl alcohol to methylene chloride. The first fraction (30 ml) eluted with pure methanol yielded graft (15% of the material) with a PVAc:PEG ratio 1:5.6, and the free PEG finally eluted in several consecutive fractions making up a total of 12% of the material.

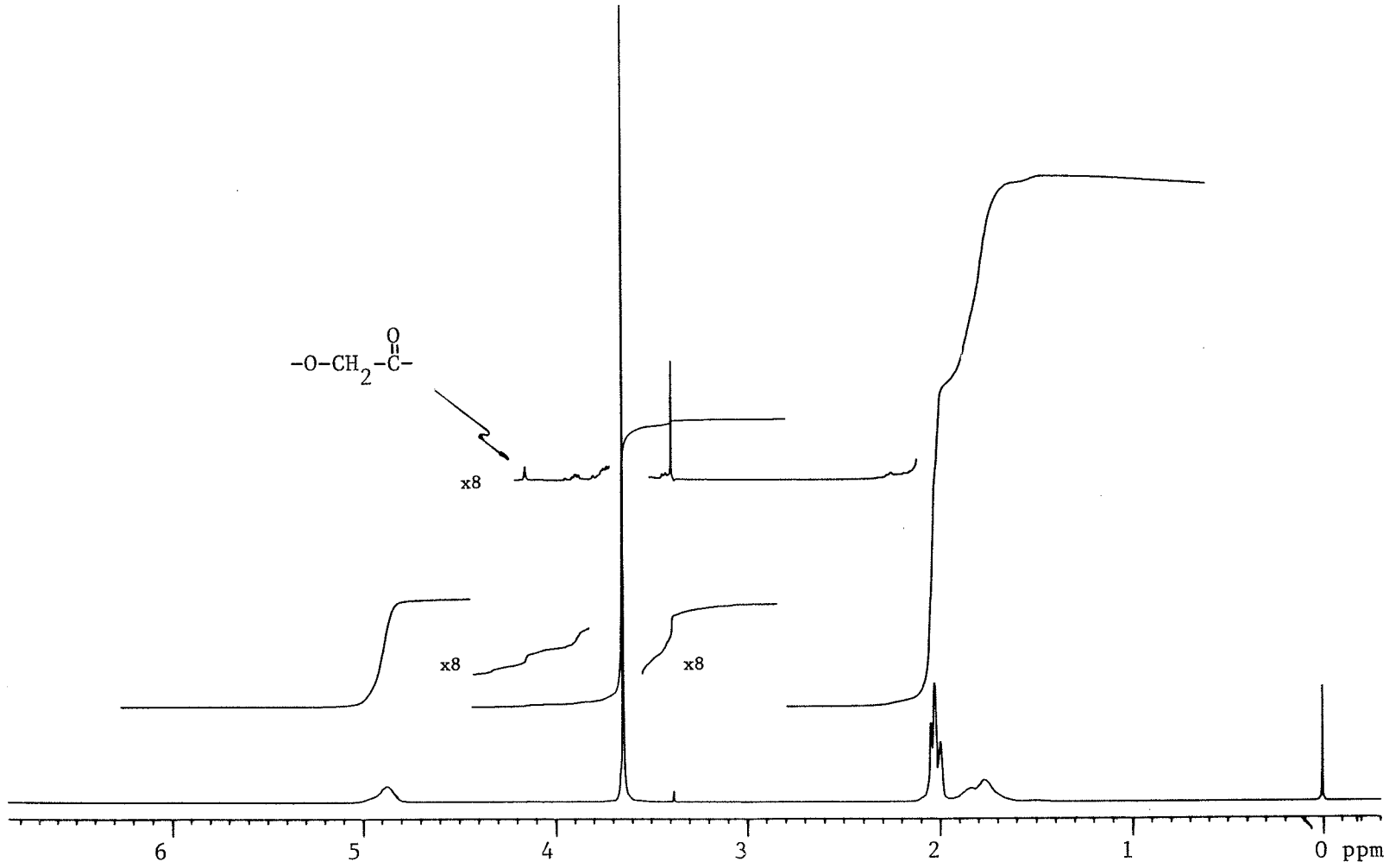
A relatively simple separation of free PEG from the PVA and PVA-PEG graft was eventually obtained by triturating the dry powdered mixture with methylene chloride. This procedure was used on the carboxyl terminated graft PVA-PEG-CO_2H. Successive triturations gave a total of 11.7% free PEG. The amount of free PEG was in agreement with the amount obtained in the separation of the unhydrolysed polymer mixture on silica. In both methods the free PVAc/PVA was not separated from the PVA-PEG graft, and thus the amount of this homopolymer could not be determined.

^1H NMR of the carboxyl terminated grafted PVAc-PEG (1:1.78) showed a singlet of low intensity at 4.15 ppm, assigned to the methylene hydrogens of the terminal carboxy methylene group (Figure 12).

The graft is not homogeneous, which was indicated during the course of separation of the homopolymers from the graft. The polymer containing more and longer PVAc/PVA chains has properties resembling PVAc/PVA more than PEG, while the polymer containing short and few or only one PVAc/PVA chain, resembles PEG in solubility.

Although the free PVA could not be separated from the graft it should not interfere with conjugate formation to the PVA-PEG-CO_2H. After formation of the conjugate with the graft it should be possible to separate any free PVA by chromatography.

Figure 12: ^{1}H NMR of PVAc-PEG-CO_2H graft polymer


-O-CH2-C-
O
x8
x8
x8
6
5
4
3
2
1
0 ppm

8.4 SUMMATIONS

Free radical grafting of vinyl acetate onto carbonyl terminated PEG to prepare a PVA-PEG-CO_2H polymer was successful. The ratio of PVA to PEG in the graft could easily be modified by varying the ratio of reactants. Thus a PVA-PEG-CO_2H polymer, in which the bulk resembles PVA, and which potentially has the reactivity of carboxyl terminated PEG, was obtained. This graft polymer may be suitable for coupling to ligands.

Chapter IX

CONCLUSIONS

Various methods of synthesizing functionally terminated PVA to be utilized for conjugation to biologically active compounds have been investigated. The initial attempts to prepare carboxyl terminated PVA by functionalized initiation of the polymerization of vinyl acetate resulted in polymers of too high molecular weight because of difficulties in controlling the degree of polymerization.

The DP was easier to control when the polymerization was carried out in the presence of a chain transfer agent, which also provided the polymer with the desired terminal functional group. However, the end group analysis of these polymers, with the functional group attached directly to the PVA main chain proved difficult. Only by ^{14}C labeling the carbonyl group of the carboxyl terminated PVA was it possible to establish unambiguously the presence of the terminal functional group. Subsequently the assignment of the very weak IR absorption peak of the carboxyl group could be made. Spectroscopic methods to determine the end groups of these polymers were often successful, while analysis methods based on chemical reactions of the terminal functional groups failed.

Attempts to couple carboxyl terminated PVA to amines using various methods were also unsuccessful. This may indicate that the terminal functional group is tightly hydrogen bonded to the backbone hydroxyl groups and is screened inside the random coil of the polymer, which renders it unreactive and thus useless for conjugation purposes.

In order to make the terminal functional group more reactive it was removed from the immediate vicinity of the PVA backbone hydroxyl groups by the introduction of a spacer arm. Attempts to introduce the terminal carboxyl group at the end of a ten carbon hydrophobic spacer arm by using 11-bromoundecanoic acid as a chain transfer agent were unsuccessful because of difficulties in isolating the polymer from the large amounts of transfer agent. It was found that hydroxyl terminated PVAc prepared by using methanol as chain transfer agent could be further derivatized. In PVAc-OH the single hydroxyl group does not associate with the backbone acetate groups, and is reactive. Thus undecanoate ester terminated PVAc was prepared by reacting the polymeric anion with 11-bromoundecanoate ester. Hydrolysis gave the undecanoic acid terminated PVA. Attempts to couple this polymer to the model amine via the N-hydroxysuccinimide ester were successful. However, attempts to couple the polymer to ovalbumin were not successful, indicating that the activated carboxyl group of the polymer was inaccessible to the amino groups of the bulky protein for steric reasons.

Finally a carboxyl terminated PVA-PEG polymer was prepared, for which the known procedures for preparing PEG-protein conjugates should be applicable. The most successful approach was to prepare a graft of PVA on carboxyl terminated PEG. The bulk of the polymer resembles PVA, while the single terminal reactive group is located on PEG. Biologically this graft should be equivalent to carboxyl terminated PVA with a PEG spacer arm. The ratio of PVA to PEG can easily be modified.

Both the conjugation of the graft to allergens and the immunological properties of these PVA-PEG allergen conjugates are currently under investigation by the Department of Immunology at the University of Manitoba.

Chapter X

EXPERIMENTAL

10.1 MOLECULAR WEIGHT DETERMINATIONS

10.1.1 PVA

The molecular weights of PVA samples were determined by measuring the relative viscosity of the aqueous solutions at 20.0±0.1 °C using an Ubbelohde viscometer. The solutions were diluted directly in the viscometer.

a. Determination of the constants **K** and **a** of Mark-Houwink's equation:
The relative viscosities of commercial PVA samples (Aldrich, M_n = 3,000, 10,000, 14,000 and 28,000) were measured and the values for K and a were determined from the graphs in Figure 13. The input data are summarized in Table 11. The values obtained were $K=7.1\cdot10^{-4}$ and a=0.66.

b. Determination of the molecular weight of PVA:
The relative viscosities of the aqueous PVA solutions were measured. M_v was obtained from the equation

$$[\eta]=7.1\cdot10^{-4}\ M_v^{\ 0.66}$$

and M_n from the relationship $M_v/M_n=1.84$, which was calculated with equation (7) of Chapter 3. Some typical results are shown in Table 12 and Figure 14.

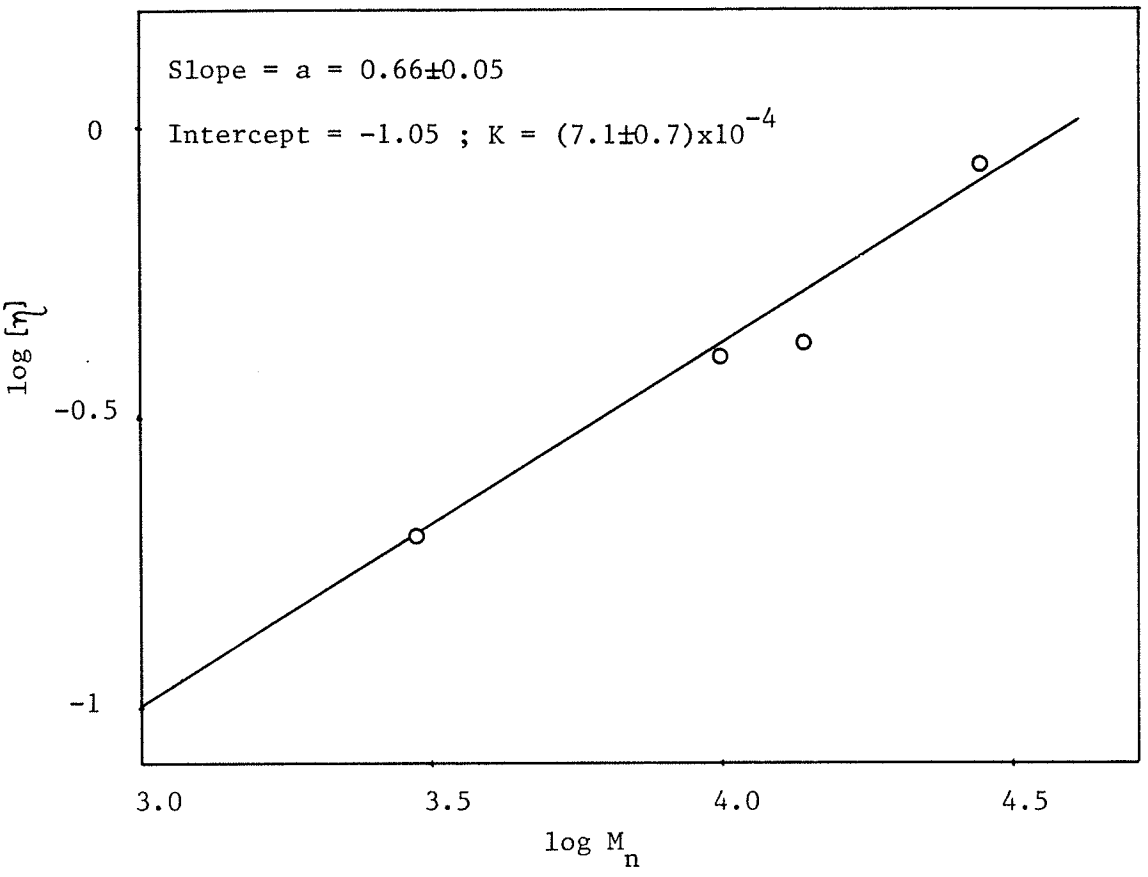


Figure 13: Determinations of constants a and K in Mark-Houwink's equation

TABLE 11

Viscosities of standard PVA samples

M	$[\eta]$(dl/g)	log M	log $[\eta]$
3,000	0.1370	3.477	-0.7093
10,000	0.4150	4.000	-0.4089
14,000	0.4120	4.146	-0.3737
28,000	0.8600	4.447	-0.0655

M = molecular weight of commercial PVA
η = viscosity

TABLE 12

Typical viscosities for PVA-OH solutions

#	C(g/dl)	ηrel	ηsp	ηsp/C	ln ηrel	ln ηrel/C
1	1.28	1.4207	0.4207	0.3287	0.3512	0.2744
2	0.96	1.3058	0.3058	0.3185	0.2668	0.2780
3	0.768	1.2389	0.2389	0.3111	0.2142	0.2789
4	0.640	1.1928	0.1928	0.3013	0.1763	0.2754
5	0.5486	1.1669	0.1669	0.3042	0.1544	0.2814
6	0.4267	1.1284	0.1284	0.3009	0.1208	0.2832

C = concentration
η = viscosity
$\eta_{rel} = \eta / \eta_0 = t/t_0$
$\eta_{sp} = \eta_{rel} - 1 = (\eta - \eta_0)/\eta_0 = (t-t_0)/t_0$
$[\eta] = \lim (\eta_{sp}/c) = \lim \{(\ln \eta_{rel})/c\} \; ; \; c \to 0$

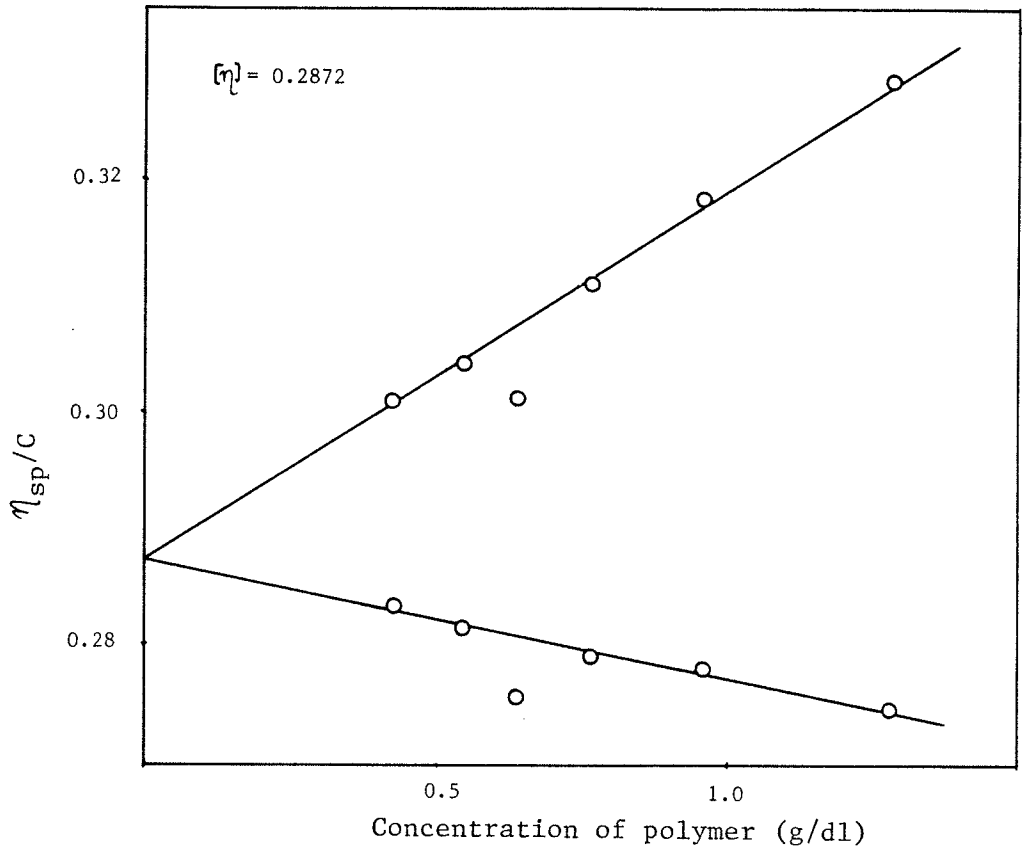


Figure 14: Determination of the intrinsic viscosity of PVA-OH

10.1.2 PVAc

The molecular weights of PVAc samples were determined by measuring the viscosity of acetone solutions of the polymer samples at 18.0 ±0.1 °C. The Mark-Houwink constants used were $K = 2.45 \cdot 10^{-4}$ and $a = 0.67$ [101].

10.2 END GROUP ANALYSIS

10.2.1 Instrumental methods

The ^{1}H NMR spectra were recorded on a Varian Anaspect EM 360 (60 MHz), Bruker WH 90 or Bruker AM 300 machine, and the ^{13}C NMR spectra on a Bruker WH 90 or AM 300 machine. The spectra were obtained in deuterochloroform or D_2O solutions, using TMS, benzene, chloroform, or dioxane as internal references.

The IR spectra were recorded on a Nicolet FT IR-80 machine in D_2O solutions in CaF_2 sample cells, or $CDCl_3$ solutions in NaCl cells.

UV absorptions were measured on a Varian Techtron 635 UV-VIS spectrophotometer.

The activity of the ^{14}C labeled polymers was measured on a LKB Wallac 1215 Rackbeta II liquid scintillation counter.

10.2.2 The reverse dye partition test

Preparation of the dye reagent [88]: A 0.008% aqueous solution of Patent Blue VF (Aldrich, indicator grade) was

shaken with an equal volume of 0.01% dodecylammonium hydrochloride in chloroform. The chloroform layer was used as the "dye reagent".

The dye reagent was shaken with an equal volume of the aqueous sample solution, and the optical density of the chloroform layer was measured at 630 nm.

10.2.3 Titration of the polymers

Titrations of functionally terminated polymers were carried out with 0.1 M sodium hydroxide solutions of either ca. 300 mg polymer / 25 ml boiled deionized water or ca. 80-100 mg polymer / 1 ml water. The solutions were initially adjusted to pH 2.3 and titrated to pH 10.8 using calomel electrodes. The volumes of base used were corrected by subtracting the volumes of base used in the titration of blanks. A typical titration curve is shown in Figure 15 .

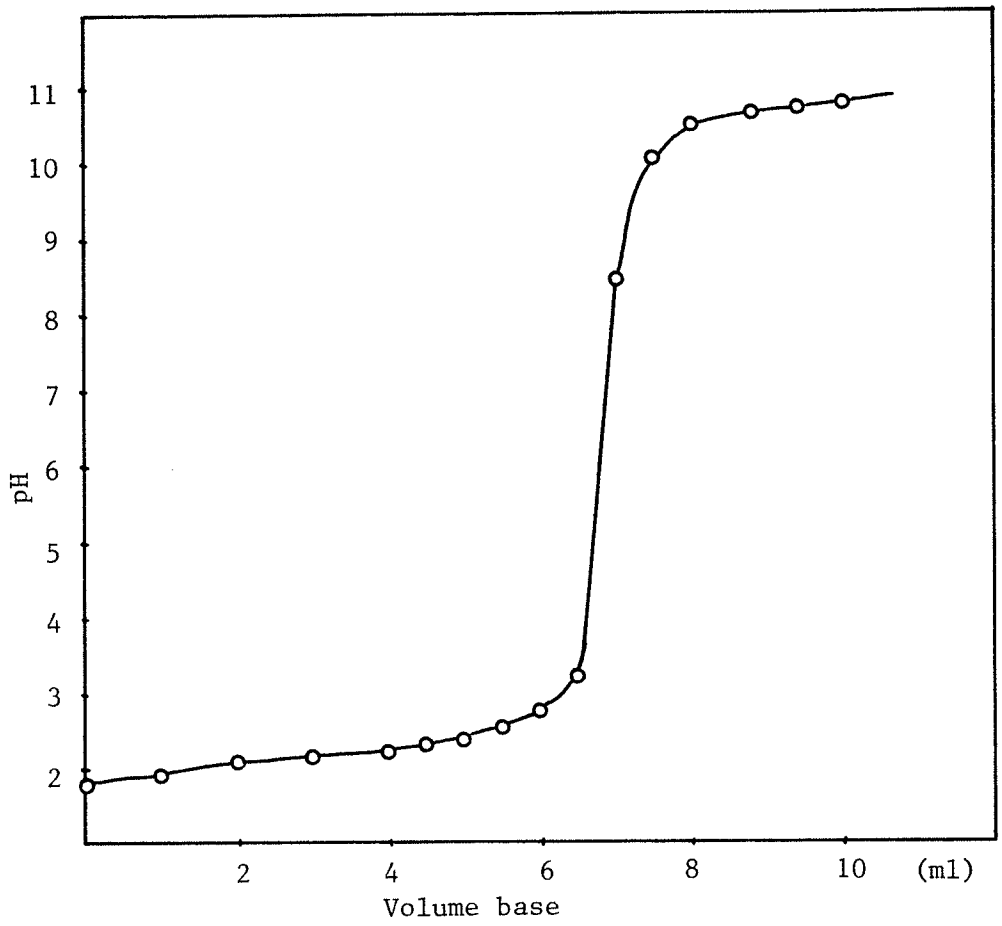


Figure 15: Titration of PVA-CO_2H with sodium hydroxide

10.2.4 Electrophoresis

Electrophoresis of carboxyl terminated PVA was carried out for 20-30 min. at 300 V on cellulose acetate strips in tris(hydroxymethyl)aminomethane (Tris) or phosphate buffers of pH 8.8 or in phosphate buffer of pH 12.

10.2.5 Sanger's test [94]

Into 10 ml volumetric flasks were added 3 ml methanol, 1 ml 0.1 M K_2HPO_4 in deionized water, 1 ml dinitrofluorobenzene (DNFB) solution in methanol (1.0 mg/ml), the required amount of a polymer solution or as a reference 1 ml hexamethylene diamine solution (34.8 mg/100 ml H_2O). The volume was made up to 10 ml with deionized water. The flasks were heated for 15 min. at 50°C, then cooled and one drop of 6 M HCl was added. The absorbance at 425 nm was measured against a blank. The extinction coefficient for DNP-lysine is 50.45 [118].

10.2.6 The fluorescamine test [95]

Preparation of the fluorescamine solution:

3 mg fluorescamine was transferred to a flamed vial, and the vial was sealed with a serum cap. 10 mg freshly distilled dioxane was added with a syringe. The solution was used immediately after preparation.

Determination of amino groups:

10-100 μl of the amine solution (or H_2O for the blank) was transferred to a test tube, the volume was made up to 100 μl with deionized water, and phosphate buffer (3.5 ml, 0.05 M, pH 8) was added. Then 0.5 ml freshly prepared fluorescamine solution was added while the test tube was being shaken on a Vortex mixer. The relative fluorescence was measured within 5-30 min after the reaction had occurred. Excitation wavelength = 390 nm, emission wavelegnth = 475 nm.

10.3 COLOUR SPOT TEST FOR PVA

One drop of PVA solution was added to a solution of one drop of saturated boric acid and one drop of a KI_3 solution (50 mg I_2 and 500 mg KI in 50 ml distilled water) on a spot plate. The presence of PVA was indicated by the appearance of a deep blue colour.

10.4 SYNTHESES

10.4.1 General

The vinyl acetate (Aldrich) used in the polymerizations was distilled prior to use.

When dry solvents were used the drying procedures described here were followed.

a. Diethyl ether:

The solvent was distilled over $LiAlH_4$ and was stored over sodium wire.

b. Dioxane:

The solvent was dried with KOH pellets, and was distilled over $LiAlH_4$ under reduced pressure.

c. DMF:

The solvent was dried with KOH pellets, and was distilled over CaO under reduced pressure.

d. HMPA:

The solvent was refluxed over CaO under reduced pressure in a N_2 atmosphere for one hour, and was then distilled under reduced pressure.

e. Methanol:

The solvent was refluxed over Mg turnings, until no more heat evolved, and was distilled.

f. THF:

The solvent (250 ml), predried over KOH pellets, was refluxed over sodium metal under nitrogen for 0.5 hr. About 2 g benzophenone was added, and the solution was refluxed under nitrogen until it turned dark blue. A required amount of THF was distilled immediately before use.

g. Pyridine:

The solvent was distilled, first over p-toluenesulfonyl chloride, and then over CaH_2.

10.4.2 Hydrolysis of PVAc to PVA

The following procedure was used in all hydrolyses of PVAc to PVA.

The PVAc (ca. 2.5 g) was dissolved in 150 ml hot ethanol. Water (ca. 80 ml) was added, until further addition would have precipitated the polymer. The solution was kept at 60°C, and 1 M KOH was added dropwise at such a rate that the pH was maintained between 10 and 11. Ethanol was added in small portions during the hydrolysis to keep the polymer in solution. When the hydrolysis was completed the solution was stirred for 15 min. at 60°C to ensure complete reaction. The solution was dialysed (Spectrapor 1 or 2) for three to four days against running distilled water, and was lyophilized.

10.4.3 Preparation of PVAc-CO_2H via direct polymerization

10.4.3.1 $KMnO_4$/oxalic acid as initiator system (5.1.1)

A solution of $KMnO_4$ (0.05 g, $3.2 \cdot 10^{-4}$ moles, 0.01%) and oxalic acid (0.5 g, $5.6 \cdot 10^{-3}$ moles, 0.1%) in water (500 ml) was deoxygenated for 30 min with N_2. Vinyl acetate (7.5 ml, 0.08 moles, 1.5%) was added, and the solution was again

purged with N_2 for 10 min. The solution was left stirring overnight at room temperature. The big lumps of polymer were collected, dissolved in ethanol and reprecipitated with water. The procedure was repeated twice to remove the initiator. The polymer was then dissolved in CCl_4 and the solution evaporated, finally under high vacuum, to yield dry polymer in 2.2 g yield (31.4%). M_n =243,000, (DP=2800).

10.4.3.2 Fe^{2+}/H_2O_2 as initiator system (5.1.2)

A solution of vinyl acetate (4 ml, 0.043 moles) and $FeSO_4$ (2.2 mg, $1.45 \cdot 10^{-5}$ moles) in water (40 ml) was deoxygenated with N_2 for 30 min. in a sealed flask. H_2O_2 (30%, 0.1 ml, $1 \cdot 10^{-3}$ moles) was added with a syringe under vigorous stirring. The solution turned cloudy at once. It was heated to 60°C on a wax bath, and was left stirring overnight. A sticky yellow polymer precipitated. The solution was decanted off, and the polymer was dissolved in toluene. Pentane was added to precipitate the polymer, which retained the same yellow appearence. It was dissolved and reprecipitated from ethanol/H_2O , twice, was dissolved in CCl_4 and the solution was evaporated under high vacuum. The yield was 1.8 g colourless, glassy polyvinyl acetate (48%). M_n = 42,200 (DP=490).

10.4.4 Preparation of PVA-CO_2H via chain transfer

10.4.4.1 Chain transfer to polymer (5.2.1.)

Vinyl acetate (20 ml, 0.22 moles) and dibenzoyl peroxide (43 mg, $1.76 \cdot 10^{-4}$ moles) were added to a dry flask equipped with a magnetic stirring bar. The flask was sealed with a serum cap and the solution was purged with dry N_2 for 30 min. The flask was then immersed in a 60°C wax bath, and the solution was stirred for 0.5 hr. The polymerization was stopped by addition of hydroquinone (10 mg, $9.1 \cdot 10^{-5}$ moles) dissolved in a small amount of vinyl acetate. The solution was evaporated, the polymer dissolved in CCl_4 and the solution was evaporated again, the procedure repeated twice, to remove most of the monomer. The last traces of monomer were removed under high vacuum, to yield 2.26 g dry , brittle polymer (14%).

Hydrolysis, followed by dialysis for four days and lyophilization gave 1.22 g white fluffy polymer (91%).
M_n= 39,000 (DP = 890).

10.4.4.2 Chain transfer to methyl propionate (5.2.3.1)

Vinyl acetate (20 ml,0.22 moles), methyl propionate (20 ml, 0.21 moles) and dibenzoyl peroxide (240 mg, 0.001 moles) were added to a dry flask equipped with a magnetic stirring bar. The flask was sealed with a serum cap and was purged

with dry N_2 for 20 min. The flask was immersed in a 60°C wax bath, and the solution was stirred at that temperature for 24 hrs. The solution was evaporated under reduced pressure. The polymer was dissolved in CCl_4 and the solution was evaporated, repeating the procedure thrice. The polymer was then dried under high vacuum.

The dry polymer was hydrolysed in ethanol/water at 60°C with 1 M KOH. The polymer solution was then dialysed for four days against running distilled water, and lyophilized. Yield 5.97 g white polymer (63%). M_n = 3600 (DP=82).
IR (D_2O/DCl): ca 1740 cm^{-1} (C=O)
NMR (D_2O; ^{13}C): δ = 178 (C=O)

10.4.4.3 Preparation of ^{14}C-carbonyl labeled PVA-CO_2H (5.2.3.2)

a. Preparation of ^{14}C-carbonyl labeled methyl propionate

^{14}C-carbonyl labeled sodium propionate solution (0.5 ml of an ethanolic solution, $2.2 \cdot 10^{12}$ Bq/mole (60.2 Ci/mole), New England Nuclear; 0.08 mg, $8.3 \cdot 10^{-7}$ moles) was evaporated to dryness in vacuo. p-Toluenesulfonic acid (10 mg, $5.3 \cdot 10^{-5}$ moles) and unlabeled methyl propionate (5 ml, $5.2 \cdot 10^{-2}$ moles) were added to the dry salt. The solution was stirred at 30°C for five days. Sodium bicarbonate (9 mg, $1.05 \cdot 10^{-4}$ moles) was added, and the methyl propionate was sublimed under reduced pressure into a dry flask where the subsequent polymerization was carried out. The average

total activity of the methyl propionate was $1.49 \cdot 10^{7}$ Bq/mole ($0.404 \cdot 10^{-3}$ Ci/mole).

b. Polymerization of vinyl acetate in ^{14}C-carbonyl labeled methyl propionate

Vinyl acetate (5 ml, 0.054 moles) and dibenzoyl peroxide (60 mg, $2.5 \cdot 10^{-4}$ moles) were added to ^{14}C-carbonyl labeled methyl propionate (5 ml, 0.052 moles). The flask was sealed with a serum cap and the solution was purged with dry N_2 for 20 min. The reaction mixture was then stirred at 60°C for 24 hrs. Excess vinyl acetate and methyl propionate were removed under high vacuum, and the polymer was purified by precipitation from benzene with pentane five times. The polymer was finally lyophilized from benzene. The procedure was repeated until a constant activity was obtained for the PVAc. Yield 3.7 g (74%).

The average activity of the PVAc was $2.56 \cdot 10^{7}$ Bq/mole ($0.692 \cdot 10^{-3}$ Ci/mole).

The polymer was hydrolysed in ethanol/water at 60°C with 1 M KOH. The polymer solution was then dialysed against running distilled water for five days and lyophilized.

The activity of the PVA was $2.25 \cdot 10^{7}$ Bq/mole ($0.609 \cdot 10^{-3}$ Ci/mole).

10.4.4.4 Chain transfer to acetonitrile (5.2.3.3.)

Vinyl acetate (21 ml, 0.232 moles), acetonitrile (122 ml, 2.32 moles, freshly distilled), and dibenzoyl peroxide (100 mg, $4.13 \cdot 10^{-4}$ moles) were added to a dry flask, fitted with a magnetic stirring bar. The flask was sealed with a serum cap, and the solution was purged with dry N_2 for 20 min. The solution was then stirred at 60°C for 72 hrs. The excess monomer and solvent were evaporated under reduced pressure. CCl_4 was added, and the solution was evaporated. This procedure was twice. Finally the polymer was dried under high vacuum. The dry polymer was dissolved in t-butyl alcohol and freeze-dried to yield 6.66 g crystalline PVAc (33%).

IR ($CDCl_3$) : 2249 cm^{-1} ($C \equiv N$).

The polymer (0.32 g) was hydrolysed in ethanol/water at 60°C, and the solution was dialysed and lyophilized. Yield 0.10 g (63%). M_n = 4600 (DP=104).

1.1 titrable groups/PVA.

IR (D_2O) : A very weak peak at 1740 cm^{-1} (C=O).

10.4.4.5 Chain transfer to 11-bromoundecanoic acid (5.2.3.4)

Vinyl acetate (15 g, 0.18 moles), 11-bromoundecanoic acid (15 g, 0.058 moles; Aldrich, technical grade, recrystallized twice from ethanol) and dibenzoyl peroxide (15 mg, $6.2 \cdot 10^{-5}$ moles) were placed in a dry flask equipped with a magnetic

stirring bar. The flask was sealed with a serum cap. The mixture was heated until the 11-bromoundecanoic acid formed a homogeneous solution with the vinyl acetate. The solution was purged with dry N_2 for 30 min, and was then stirred at 60°C for four days. The solution was evaporated under reduced pressure and the solid was dissolved in hot ethanol. The 11-bromoundecanoic acid crystallized upon cooling and was filtered off. This procedure was repeated twice.

The polymer was hydrolysed in ethanol/water at 60°C with 1 M KOH. During the hydrolysis a white solid precipitated. The solid was filtered off, and the solution was dialysed against running distilled water for four days. During the dialysis more solid precipitated and was filtered off. A KI_3/boric acid spot test on the filtrate showed that no polymer was present.

10.4.5 Preparation of PVA-CO_2H via PVAc-OH

10.4.5.1 Preparation of PVAc-OH via chain transfer to methanol (5.2.2.1)

a. Polymerization

Vinyl acetate (20 ml, 0.22 moles), dry methanol (80 ml, 2.0 moles) and dibenzoyl peroxide (240 mg, $1 \cdot 10^{-3}$ moles) were placed in a dry flask equipped with a magnetic stirring bar. The flask was sealed with a serum cap. The solution

was purged with dry N_2 for 30 min. and stirred at 60°C for 24 hrs. The excess monomer and solvent were removed by evaporation under reduced pressure. CCl_4 was added and the solution was evaporated, with this procedure being repeated twice. The polymer was then dried under high vacuum to yield 17.5 g colourless, brittle polymer (92%). M_n = 5300. NMR ($CDCl_3$/TMS): δ =1.8 (m, $-CH_2-$), 2.05 ($-OCH_3$), 4.08 (t, $-CH_2-(CO)-OAc$), 4.9 (m, -CH-).

b. Silylation of the terminal hydroxyl group of PVAc

In order to determine the presence of the terminal hydroxyl group of PVAc-OH by NMR, silylation of the polymer was carried out. The procedure of Sweeley et al. [126] was followed.

Hydroxyl terminated polyvinyl acetate (0.46 g, $6.0 \cdot 10^{-5}$ moles, M_n =7600) was dissolved in dry pyridine in a flamed vial fitted with a serum cap. Hexamethyldisilazane (0.2 ml, $9.5 \cdot 10^{-4}$ moles) and trimethylchlorosilane (0.1 ml, $7.88 \cdot 10^{-4}$ moles) were added. A white solid started to precipitate at once. The reaction mixture was stirred for 22 hrs at room temperature. The mixture was then filtered through a fine sintered glass filter. The polymer was precipitated from the filtrate by addition of pentane and purified by dissolving it in benzene and reprecipitating with pentane five times. Finally the polymer was lyophilized from benzene. The yield was 0.40 g polymer (88%).

NMR($CDCl_3$/TMS): δ = 0.015 ($(CH_3)_3$-Si), 3.5 (t, $-CH_2$-O-Si), 4.05 (t, $-CH_2$-(CO)-OAc).
Peak intensities: ca. 2 (0.015 ppm), ca. 1 (4.05 ppm), trace (3.5 ppm).

10.4.5.2 Jones' oxidation of PVAc-CH_2-OH [127]

PVAc-OH (5.4 g, $7.2 \cdot 10^{-4}$ moles, M_n = 7600) was dissolved in acetone (35 ml, previously distilled from $KMnO_4$). An excess of a solution of 10% CrO_3 in 5% H_2SO_4 was added, and the reaction mixture was left stirring overnight. The excess oxidizing agent was destroyed by addition of 1 ml isopropyl alcohol. The inorganic salts were filtered off, and the polymer was precipitated with water. It was then dissolved and reprecipitated from ethanol/water four times.

The polymer was hydrolysed in ethanol/water at 60 °C with 1 M KOH. After dialysis for four days against running distilled water the polymer was lyophilized. The yield was 0.14 g (2.6%). The low yield may have resulted from leakage through the dialysis tubing.

10.4.5.3 Oxidation of PVAc-CH_2-OH with CrO_3 in pyridine

The oxidizing agent was prepared according to Cornforth et al. [128] by dissolving CrO_3 (5 g, $3.3 \cdot 10^{-2}$ moles) in H_2O (3 ml). This solution was added dropwise to 30 ml ice cold

pyridine (distilled before use). PVAc-OH (0.36 g, $4 \cdot 10^{-5}$ moles; M_n =7600) was dissolved in pyridine (2 ml), and 5 ml of the CrO_3/pyridine solution was added. The reaction mixture was stirred at room temperature for three days.

The polymer was precipitated from the solution by addition of water. It was dissolved and reprecipitated from ethanol/water until all the colour was removed. The polymer was then taken up in CCl_4, the solution evaporated, and finally the polymer was dried under high vacuum for 2 hrs.

The polymer was hydrolysed in ethanol/water at 60 °C with 1 M KOH. The solution was then dialysed and lyophilized. Yield about 10%.

No carbonyl peak was found in the IR spectrum of the product.

10.4.6 Preparation of amino terminated PVA

10.4.6.1 Reduction of nitrile terminated PVAc (5.2.4.1)

Nitrile terminated PVAc, prepared as described in 10.4.4.4 (3.3 g, $3.7 \cdot 10^{-4}$ moles) was dissolved in glacial acetic acid (25 ml). Pd(C) (150 mg; 10%) was added. The suspension was stirred under H_2 (1 atm.) at room temperature for 20 hrs. The catalyst was removed by filtering twice

through a double layer of filter paper (Whatman # 1). The solution was evaporated under reduced pressure, CCl_4 was added and evaporated twice, and the polymer was finally dried under high vacuum.

The polymer was hydrolysed in ethanol/water with 1 M KOH, dialyzed for five days against running distilled water, and finally lyophilized. Yield 0.348 g (21%).
Sanger's test for amino groups gave a negative result.

10.4.6.2 Chain transfer to n-butyl acetamide (5.2.4.2)

a. Preparation of n-butyl acetamide

Acetic anhydride (142 ml, 1.5 moles; distilled prior to use) and NaOH (80 g, 2 moles) in water (200 ml) were simultaneously added dropwise to a cold solution of n-butylamine (100 ml, 1 mole) at such a rate that the pH was kept at 11. When all the acetic anhydride was used, the solution was stirred until the pH had stabilized at 10. The solution was extracted with diethyl ether, the ether layer was dried with $MgSO_4$, and finally the solvent was removed under reduced pressure. The amide was purified by vacuum distillation (b.p. 75°C at 0.25 mmHg). The yield was 93.5 g (81%).
The correct structure was confirmed by NMR.

b. Polymerization of vinyl acetate in n-butyl acetamide

Vinyl acetate (21 ml, 0.232 moles), n-butyl acetamide (67 g, 0.58 moles), and dibenzoyl peroxide (35 mg, $1.4 \cdot 10^{-4}$ moles) were added to a dry flask equipped with a magnetic stirring bar. The flask was sealed with a serum cap, and the solution was purged with N_2 for 30 min. The solution was stirred at 60°C for 22 hrs. Water was added to the reaction mixture. The polymer precipitated and the H_2O/amide solution was decanted off. The polymer was washed with water.

The polymer was hydrolysed in ethanol/water at 60°C with 1 M KOH. The volume of the solution was reduced on a rotary evaporator prior to dialysis for four days against running distilled water. Lyophilization gave 3.01 g polymer (30%). M_n =5500 (DP=125).
No amine groups could be detected by Sanger's or fluorescamine tests.

c. Telomerization of vinyl acetate in n-butyl acetamide

Vinyl acetate (4 g, 0.05 moles), n-butyl acetamide (161 g, 1.4 moles) and dibenzoyl peroxide (0.79 g, $3 \cdot 10^{-3}$ moles) were added to a dry flask, which was sealed with a serum cap. The solution was purged with dry N_2 for 20 min., and was then stirred at 60°C for four days. The reaction mixture was distilled under high vacuum to remove excess vinyl acetate and n-butyl acetamide. Water (150 ml) was added to the residue. The precipitated high molecular weight frac-

tion was collected, and hydrolysed with 1 M KOH. The solution was dialysed against running distilled water for two days, and was lyophilized. Yield 43 mg pure polymer.
No amino groups could be detected by Sanger's test.

The milky white water solution containing the low molecular weight fractions of the PVAc was extracted with chloroform. The organic solution was dried with $MgSO_4$, and the chloroform was evaporated. The remaining liquid was distilled under high vacuum to give n-butyl acetamide and a residue of 3 ml (3 g) yellow highly viscous liquid.
NMR of residue ($CDCl_3$/TMS): δ = 2.0 (s, amide (CO)-CH_3), 2.05 (s, vinyl acetate (CO)-CH_3).

10.4.7 Preparation of reference polymers

In order to obtain noncarboxylated and carboxylated PVA to be used as reference polymers in the analyses of PVA-CO_2H both PVA-OH and succinylated PVA were prepared.

PVA-OH was obtained by hydrolysis of PVAc-OH prepared as in 10.4.5.1.

Succinylated polyvinyl alcohol was prepared as follows:

Polyvinyl alcohol (Aldrich, 75% hydrolysed, M_n =3000) (5g, $1.7 \cdot 10^{-3}$ moles) was dissolved under reflux in pyridine (100 ml). Succinic anhydride (2.5g, $2.5 \cdot 10^{-2}$ moles) was added to the cooled solution. The solution was refluxed for 2 hrs and then left stirring at room temperature over night.

The polymer was precipitated with petroleum ether (bp.35-58 °C). The solution was decanted off, and the precipitated polymer was washed three times with petroleum ether. After the remaining solvent had been evaporated under reduced pressure the polymer was dissolved in H_2O/NaOH by heating. Acidification of the solution reprecipitated the polymer. This procedure was repeated twice. Finally the polymer was dissolved in a basic aqueous solution, dialyzed for four days and lyophilized. The yield was 3.79g (59%).
IR(D_2O): 1730-1770 cm^{-1} (C=O).
Carboxyl content by titration: 7.6 CO_2H/PVA.

10.4.8 Coupling reactions

10.4.8.1 Preparation of γ-methyl-γ-butyrolactone (6.2.1)

The lactone was prepared following the procedure of Hassner et al. [134].

$NaBH_4$ (0.65 g, 0.017 moles) was added to a solution of ethyl levulinate (5 g, 0.035 moles) in isopropyl alcohol (20 ml). The solution was stirred for one hour. Hydrochloric acid (6 M) was added dropwise to destroy the excess hydride. CCl_4 was added and the solution was dried with $MgSO_4$, and filtered. The lactone was isolated by distillation under reduced pressure, b.p. 135-137°C/ca. 100 mmHg. Finally the lactone was purified by column chromatography on silica, with $CHCl_3$ as eluent.

NMR ($CDCl_3$/TMS): δ = 1.43 (3H,d, $-CH_3$), 2.2-2.7 (4H,m, $-(CH_2)_2-$), 4.7 (1H,m, -CH-).

10.4.8.2 Lactone investigation

Determination of the rate constants of the hydrolysis of γ-butyro lactone:

γ-Butyrolactone (Aldrich) (1 ml) in 40 ml H_2O was titrated with a 1.31 M NaOH solution at 24.5±0.5°C and at 0°C in such a way as to keep the pH constant at 10.0±0.5. The pseudo first order rate constants $k_{25}=1.7\cdot10^{-4}$ s^{-1} and $k_0=3.0\cdot10^{-5}$ s^{-1} were obtained from a plot of $\ln(V_\infty-V_t/V_\infty)$ vs. t (Figure 16). The experimental data are summarized in Table 13.

10.4.8.3 Coupling of γ-butyrolactone to benzylamine (6.2.2)

γ-Butyrolactone (0.60 ml, $7.8\cdot10^{-3}$ moles) was added to a solution of benzylamine (0.65 ml, $5.9\cdot10^{-3}$ moles) in phosphate buffer (10 ml, 0.1 M, pH 10). The solution was stirred at room temperature overnight, and was then extracted with chloroform. The organic layer was dried over $MgSO_4$, and the solvent was removed under vacuum. The residual yellow liquid was dissolved in a minimum amount of chloroform. CCl_4 was added dropwise, until the amide precipitated. The crystals were collected by filtration. Yield 0.14 g (11%). M.p. 61 - 65°C.

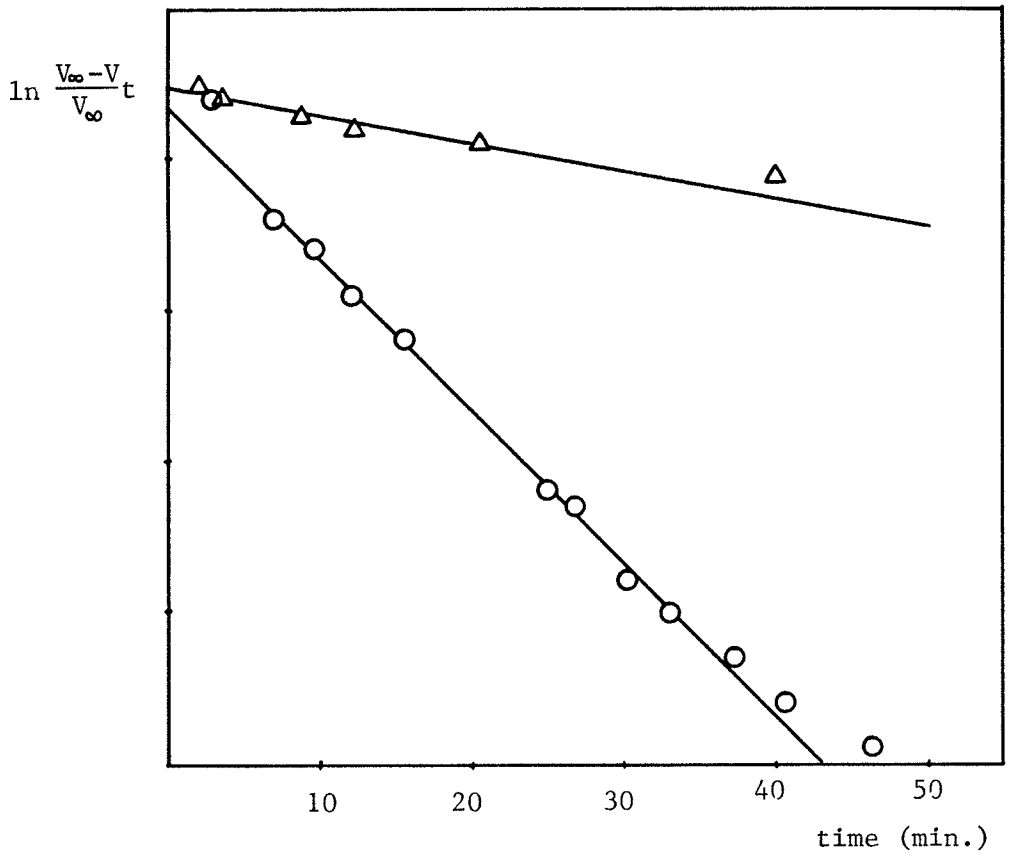


Figure 16: Rate constants for titration of γ-butyrolactone

The pseudo first order rate constants for titration of γ-butyrolactone with sodium hydroxide were determined at 24.5 °C (o) and 0 °C (Δ).

TABLE 13

Titration of γ-butyrolactone

V_t (ml NaOH)	t (s)	$-\ln(V_\infty - V_t / V_\infty)$
24.5 °C:		
0.6	155	0.06
1.3	430	0.14
1.5	575	0.16
1.7	720	0.19
2.0	930	0.22
2.7	1500	0.32
2.8	1605	0.33
3.1	1810	0.38
3.3	1990	0.40
3.5	2245	0.43
3.7	2455	0.46
3.9	2785	0.49
4.1	2950	0.53
0 °C:		
0.5	125	0.05
0.6	210	0.06
0.7	520	0.07
0.8	740	0.08
0.9	1215	0.09
1.0	2400	0.11
1.2	5040	0.13

Concentration of NaOH = 1.13 M
V_∞ for both titrations = 10 ml

NMR ($CDCl_3$/TMS): δ = 1.8-1.9 (2H,m, $-CH_2-C\underline{H}_2-CH_2-$), 2.39 (2Ht, $-CH_2-CO-$), 3.69 (2H, t, $-C\underline{H}_2-OH$), 4.42 (2H,d, $C_6H_5-C\underline{H}_2-$), 7.31 (5H,s, $-C_6H_5$).

MS: 193 m/e (M^+), 106 m/e ($^+NH-CH_2-C_6H_5$), 91 m/e ($^+CH_2-C_6H_5$)

10.4.8.4 Microscale coupling reactions of model lactones to amines (6.2.2)

The lactone (γ-butyrolactone or γ-methyl-γ-butyrolactone) (2 drops or 6 μl) were dissolved in 0.25 ml of either DMF, borate buffer (0.05 M, pH 8.2), or phosphate buffer (0.1 or 1 M, pH 10) directly in a NMR tube. The amine (phenylamine or hexamethylenediamine) (2 drops or 6 μl) were added. The solution was left standing for three days, or was heated on a steambath for 0.5 hrs. The NMR spectra were recorded to determine qualitatively the presence of amide product as indicated by signals at 4.4 ppm (d,$-CH_2-\emptyset$) and 3 ppm ($-CH_2-N$).

10.4.9 Preparation of α-DNP-lysine (6.1)

α-DNP-lysine was prepared by benzoylation of the ε -position of the copper(II) complex protected lysine [133], and then dinitrophenylation of the α position according to Sanger [94].

A solution of DL-lysine monohydrochloride (4.67 g, 0.0256 moles; Sigma), basic copper carbonate (7.5 g, $6.1 \cdot 10^{-2}$ moles) and water (50 ml) was refluxed for one hour. The mixture was filtered while hot, and the precipitate was washed with water. Sodium hydroxide solution (21 ml, 2.78 M) was added to the filtrate, and benzoyl chloride (3.5 ml, 0.029 moles) was added dropwise while the solution was kept at 0°C. The ε-benzoyllysine-copper complex which precipitated, was collected by filtration and washed with water.

The ε-benzoyl lysine-copper complex was suspended in hydrochloric acid solution (60 ml, 2 M) and hydrogen sulfide was bubbled through the suspension at 40°C. The excess H_2S was trapped in an acidic copper sulfate solution. N_2 was finally bubbled through the suspension to remove all unreacted H_2S. The suspension was filtered, and the filtrate evaporated under vacuum. The crystals obtained were dissolved in warm methanol/water (15 ml/50 ml), and the solution was neutralized with ammonium hydroxide. The precipitated amino acid was collected by filtration and washed with a small amount of acetone. The yield of ε-benzoyl lysine was 5.86 g (91%).

The ε-benzoyl lysine (5.56 g, 0.023 moles) and sodium bicarbonate (7.7 g, 0.1 moles) were dissolved in water (100 ml) by heating. A solution of dinitrofluorobenzene (5.4 ml, 0.043 moles) in ethanol (200 ml) was added and the solution was stirred at room temperature for 2 hrs. The ethanol was evaporated under reduced pressure. Water was added until the crystals dissolved. The solution was extracted several times with diethyl ether (3 x 50 ml) until the excess dinitrofluorobenzene was removed. The aqueous layer was acidified, and an amorphous solid precipitated. The solid was recrystallized from ethanol. Yield 5.66 g ε-benzoyl-*α*-DNP-lysine (58%).

The ε-benzoyl group was removed by refluxing the blocked amino acid in glacial acetic acid (50 ml) and hydrochloric

acid (12 M, 50 ml) for 3 days. The solution was evaporated to dryness, the residue dissolved in H_2O (30 ml) and the undissolved material filtered off. The filtrate was neutralized with pyridine, and the crystals were collected, washed with water and a small amount of ethanol, and dried under vacuum. Yield 2.55 g α-DNP-lysine (60%).
The correct structure was confirmed by NMR.

10.4.9.1 Attempted direct coupling of PVA-CO_2H to α-DNP-lysine (6.2.2)

Since the terminal carboxyl group of PVA may exist in a lactone form, a direct coupling of the polymer to α-DNP-lysine was attempted.

A solution of carboxyl terminated PVA (ca. 100 mg) in water (10 ml) was treated as required (acidified to pH 3 or boiled 0.5 hr on a steambath at pH 1). α-DNP-lysine (33 mg, $1 \cdot 10^{-4}$ moles) was suspended in borate or phosphate buffer (10 ml, pH 10). The solutions were combined and left stirring overnight at room temperature. The solution was then concentrated on the rotary evaporator, and gel chromatographed on Sephadex G 25 eluted with distilled water. The uncoloured polymer emerged from the column with the void volume, while the yellow amine remained in the column. No amine could be detected in the polymer fraction (UV absorption at 425 nm) and no polymer could be detected in the amine fraction (KI_3/boric acid spot test).

The same reaction was also carried out with both the PVA-CO_2H and the amine dissolved in DMF, again with negative results.

10.4.10 Coupling reactions of acids to amines with DCC (6.3.1.)

10.4.10.1 Reaction of 4-hydroxybutyric acid with benzylamine

γ-Butyrolactone (1 ml, 0.013 moles) was heated in phosphate buffer (100 ml, 1 M, pH 10) to ensure opening of the lactone ring. The solution was cooled to room temperature, and DCC (2.68 g, 0.013 moles) in dioxane (5 ml) was added. After stirring for 10 min., benzylamine (1.42 ml, 0.013 moles) was added, and the stirring was continued overnight. The solution was then extracted with $CHCl_3$, the organic layer dried over $MgSO_4$, and the solvent evaporated. The residue, 4.2 g, contained a mixture of the reactants, DCU, and the amide, as detected by TLC (silica, $CHCl_3$:methanol 4:1).

10.4.10.2 Reaction of phenylacetic acid with benzylamine

DCC (2.06 g, 0.01 moles) was added to a solution of phenylacetic acid (1.36 g, 0.01 moles) in DMF (20 ml, freshly distilled). The solution was stirred for 45 min. Benzylamine (1.1 ml, 0.01 moles) in a small amount of DMF was added, and the reaction mixture was stirred at room temperature

for 15 hrs. The DCU was removed by filtration, and the filtrate was evaporated into dryness under high vacuum. The yellow solid was taken up in chloroform, the solution was washed with 5% $NaHCO_3$ and dilute hydrochloric acid, dried with Na_2SO_4, and evaporated to dryness. The residual white solid was recrystallized from CCl_4. Yield 0.70 g (31%). NMR ($CDCl_3$/TMS): δ = 3.56 (2H,s, $-CH_2-(CO)-$), 4.35 (2H,d, $-\underline{CH}_2-NH-$), 7.19 (5H,s, arom. H), 7.23 (5H,s, arom. H).

10.4.10.3 Attempted coupling of PVA-CO_2H to *a*-DNP-lysine (6.3.1)

PVA-CO_2H (0.20 g, $5.6 \cdot 10^{-5}$ moles, M_n =3600) was dissolved in DMF (50 ml) by refluxing the solution for 10 min. The solution was cooled to room temperature, DCC (21 mg, $1 \cdot 10^{-4}$ moles) was added, and the solution was stirred for one hour at room temperature. *a*-DNP-lysine (33 mg, $1 \cdot 10^{-4}$ moles) in borate buffer (150 ml, 1 M, pH 10) was added, and the solution was stirred at room temperature overnight. The DCU was filtered off, and the filtrate was concentrated by rotary evaporation. The concentrated polymer solution was gel chromatographed on Sephadex G 25 with distilled water as eluent. The colourless polymer eluted with the void volume, while the yellow *a*-DNP-lysine stayed on the column. No polymer could be detected in the fractions containing *a*-DNP-lysine (KI_3/boric acid spot test).

10.4.11 Coupling of ester activated PVA-CO_2H to ε-DNP-lysine

10.4.11.1 Preparation of N-hydroxysuccinimide activated PVA-CO_2H (6.4.2)

PVA-CO_2H (0.270 g, $7.5 \cdot 10^{-5}$ moles, M_n =3600) was dissolved in dry DMF (10 ml) by refluxing the mixture for 10 min. The solution was cooled on ice, causing some of the PVA to reprecipitate as a very fine dispersion. N-Hydroxysuccinimide (0.25 g, $2.2 \cdot 10^{-3}$ moles) and DCC (0.45 g, $2.2 \cdot 10^{-3}$ moles) in 1 ml DMF were added, and the solution was stirred at 4°C for 15 hrs. An attempt was made to remove the DCU by filtration through sintered glass, but it was unsuccessful. The polymer was precipitated with cold, dry diethyl ether, and was collected, together with the DCU, by filtration. The precipitate was washed with cold ether, and was partially dried in a vacuum desiccator. Yield 0.45 g. The polymer was used in the subsequent coupling step without further purification.

10.4.11.2 Coupling of the activated PVA-CO_2H to ε-DNP-lysine

ε-DNP-lysine (32 mg, $7.5 \cdot 10^{-5}$ moles) was dissolved in borate buffer (5 ml, 0.1 M, pH 8.2) in a vial by heating (water bath). The solution was cooled on ice, and the N-hydroxysuccinimide activated PVA-CO_2H obtained as described in 10.4.11.1 was added. The vial was stoppered and shaken vig-

orously. The mixture was stirred for 18 hrs at 4°C. A portion of the opaque solution (1.5 ml) was gel chromatographed on Sephadex G 25.

Elution with distilled water yielded a pale yellow PVA solution, leaving most of the amine behind on the column. The polymer solution was dialyzed for five days against running distilled water, and was lyophilized.
The polymer analysed for 0.06 amino groups per polymer chain (UV absorption at 425 nm).

10.4.12 Functionally terminated PVA with spacer arms

10.4.12.1 Preparation of undecanoic acid terminated PVA (7.1.)

A carboxyl terminated PVA with a ten carbon spacer arm was prepared by reaction of PVAc-OH with the methyl ester of 11-bromoundecanoic acid with sodium hydride as anion generator.

The methyl ester of 11-bromoundecanoic acid was prepared in 3% HCl/methanol following standard procedures [153].

PVAc-OH (1.45 g, $1.9 \cdot 10^{-4}$ moles, M_n =7600) was dissolved in dry benzene (50 ml). Half of the benzene was distilled off to azeotropically remove any water. The solution was transferred to a dry 3-necked flask equipped with a condenser protected with a drying tube, a serum cap through which

dry N_2 was flushed throughout the experiment, and a glass stopper. Methyl 11-bromoundecanoate (0.08 ml, $2.8 \cdot 10^{-4}$ moles) and sodium hydride as an oil dispersion (30mg, $6.25 \cdot 10^{-4}$ moles, 50% dispersion) were added. The solution was stirred under N_2 for one hour. Most of the benzene was evaporated under reduced pressure. The polymer was then precipitated from the concentrated solution with pentane. CCl_4 was added and the solution was evaporated to dryness. The procedure was repeated twice.

The polymer was hydrolysed with 1 M KOH in ethanol/water, dialysed for five days against running distilled water , and lyophilized. Yield 0.58 g polymer.

IR(D_2O/DCl): 1730 cm^{-1} (C=O).

^{13}C NMR(D_2O): δ = 178 (C=O).

Carboxyl content by titration: (0.7-0.9)±0.3 CO_2H/PVA.

10.4.12.2 Coupling of PVA-$(CH_2)_{10}$-CO_2H to ε-DNP-lysine

a. Preparation of the N-hydroxysuccinimide ester of the polymer (7.2.1)

Undecanoic acid terminated PVA(0.50 g, $1.25 \cdot 10^{-4}$ moles, M_n =4000) was dissolved in dry DMF (10 ml) by heating. The solution was cooled to 4°C. N-Hydroxysuccinimide (0.36 g, $3.13 \cdot 10^{-3}$ moles), and DCC (0.66 g, $3.2 \cdot 10^{-3}$ moles) in dry DMF (1.5 ml) were added under nitrogen. The reaction mixture was stirred for 15 hrs. at 4°C. The polymer was precipi-

tated under nitrogen with cold, dry diethyl ether. The precipitate was collected by filtration, washed with cold diethyl ether under the inert atmosphere, and dried in a desiccator under vacuum. The polymer was used without further purification in the subsequent coupling step.

b. Coupling of the activated undecanoic acid terminated PVA to ε-DNP-lysine (7.7.2)

ε-DNP-lysine (16.3 mg, $3.9 \cdot 10^{-5}$ moles) was dissolved in borate buffer (10 ml, 0.1 M, pH 8.2) in a vial by heating. The solution was cooled to 4°C, and added to the activated PVA, obtained as described above in a vial. The vial was sealed and shaken to dissolve the polymer. The reaction mixture was stirred at 4°C for 15 hrs. The solution was then gel chromatographed on Sephadex G 25 with water as eluent. Two yellow bands were formed on the column, the first one containing the polymer. The polymer fraction was dialysed for five days, and was lyophilized. The yellow polymer (20%) obtained analysed for 0.26 DNP groups per polymer chain (UV absorption at 425 nm).

10.4.12.3 Coupling of PVA-$(CH_2)_{10}$-CO_2H to ovalbumin (7.2.3)

The succinimide ester of undecanoic acid terminated PVA was prepared as described in 10.4.12.2.

Egg ovalbumin (31.5 mg, $7.05 \cdot 10^{-7}$ moles; Fluka) was dissolved in cold borate buffer (2.5 ml, 0.1 M, pH 8.2). The succinimide ester of undecanioc acid terminated PVA ($1.34 \cdot 10^{-4}$ moles) was added. The reaction mixture was stirred at 4°C for 15 hrs, and was then desalted by dialysis against running distilled water for five days.
The solution was chromatographed through a DEAE cellulose ion exchange column. The unreacted PVA was eluted with phosphate buffer (0.008 M, pH 7.7), and the protein eluted with acetate buffer (0.05 M, pH 4). No polymer could be detected in the protein fraction (NMR).

10.4.13 Preparation of carboxyl terminated PEG (8.1.)

10.4.13.1 Oxidation of PEG-CH_2-OH with pyridinium : dichromate (8.1 #1)

The reaction was carried out according to the oxidation procedure of Corey [139].

Preparation of the pyridinium:dichromate complex:

CrO_3 (3 g, 0.03 moles) was dissolved in 3 ml H_2O by heating. The solution was cooled, and pyridine (2.4 ml, moles) was added dropwise. Acetone (12 ml) was added, and the solution was kept on an ice/salt bath until no more crystals formed. The crystals were collected, washed with acetone, and dried in a vacuum desiccator.

Oxidation:

Monomethoxy PEG (0.297 g, $5.9 \cdot 10^{-5}$ moles, Hoechst, M_n = 2400) was dissolved in DMF (2 ml) by heating (steam bath). The dry pyridinium:dichromate complex (77 mg, $2.6 \cdot 10^{-4}$ moles) was added to the cooled solution. The solution was stirred overnight at room temperature. Cold diethyl ether was added to the solution to precipitate the polymer, which, together with the inorganic salts, was collected by filtration. The precipitate was dissolved in water, the solution was filtered to remove black insoluble specks, and was finally dialyzed for 6 days. Yield 0.18 g.
No carbonyl carbon could be detected by ^{13}C NMR.

10.4.13.2 Oxidation of PEG-OH with MnO_2/H_2O_2 (8.1.#2)

The oxidation was carried out following the procedure of Boccu and coworkers [67].

Preparation of activated MnO_2[154]:

A solution of $MgSO_4 \cdot H_2O$ (8.41 g, 0.06 moles) in H_2O (15 ml) and a solution of NaOH (11.7 ml, 40%) were added simultaneously with stirring to a hot solution of $KMnO_4$ (9.60 g, 0.06 moles). The stirring was continued for 1 hr. The precipitate was collected by centrifugation and washed with water until the washings were colourless. The precipitate was dried at 120°C.

Oxidation:

Monomethoxy PEG (3.32 g, $1.36 \cdot 10^{-3}$ moles; Hoechst, M_n = 2400), activated MnO_2 (3.32 g) and dry methylene chloride (30 ml) were mixed and stirred overnight at room temperature. The inorganic salts were removed by vacuum filtration, the brown filtrate was decolorized with active charcoal, and evaporated. Yield 3.16 g polymer.

The polymer was dissolved in 3% H_2O_2 (60 ml), and was left stirring overnight. A portion of the solution (30 ml) was ion exchange chromatographed (Dowex 1·8-50). The polymer did not adsorb to the column, but was eluted with water. The eluate was changed to hydrochloric acid (0.02 M), but no more polymer was collected. The polymer fractions were lyophilized. The polymer solution was dialysed for 3 days, and lyophilized. Yield 0.83 g .
No carbonyl peak could be detected by ^{13}C NMR.

10.4.13.3 Reaction of PEG-OH with ethyl-α-bromoacetate

a. Reaction with sodium hydride as catalyst (8.1.#3)

Monomethoxy PEG (5 g, $2.05 \cdot 10^{-3}$ moles, M_n =2400) and dry THF (20 ml) were added to a dry, three necked flask equipped with a condenser protected with a drying tube, a serum cap, through which dry N_2 was flushed during the experiment, and with a glass stopper. The solution was heated to dissolve all of the polymer, and then cooled. Sodium hydride as an 50% oil suspension (0.48 g, 0.01 moles) and then

ethyl-α-bromoacetate (1.3 ml, 0.01 moles) were added to the solution at room temperature. The solution was stirred for 15 hrs. Ethanol was added dropwise to destroy excess sodium hydride. The polymer was then precipitated by addition of cold petroleum ether, and the solvent was decanted off. The precipitate was dissolved in methylene chloride, a few drops of water was added, and the solution was dried with $MgSO_4$. After filtration the solvent was evaporated under reduced pressure. The polymer was redissolved in a minimum amount of methylene chloride, and then reprecipitated with ice cold petroleum ether. The procedure was repeated five times. Finally the polymer was collected by filtration and washed with petroleum ether. Yield 3.59 g.

NMR ($CDCl_3$/TMS): δ =1.3 (t, ester $-CH_3$), 4.10 (s,$-O-CH_2-CO-$), 4.5 (q, ester $-CH_2-$), 3.38 (s, $-OCH_3$), relative intensity of $-OCH_3$ to $-O-CH_2-CO$ = ca. 15 : 1.

The ester terminated polymer (3.24 g) was hydrolysed in water at 50°C with 1 M KOH. The base was added dropwise to maintain the pH around 10. After the hydrolysis the solution was neutralized (HCl) and gel chromatographed on Sephadex G 25. The fractions containing polymer were combined and lyophilized. Yield 3.1 g.

NMR($CDCl_3$/TMS): δ = 3.95 (s,$-O-CH_2-CO-$)

Carboxyl content by titration: 0.1 CO_2H/PEG.

b. Reaction with Na/naphthalene catalyst (8.1.#4)

Preparation of Na/naphthalene [140]:

Naphthalene (91.538 g, $1.2 \cdot 10^{-2}$ moles, recrystallized) was dissolved in dry THF (20 ml) in a dry flask. Sodium metal (0.21 g, $8.7 \cdot 10^{-3}$ moles) was added in small pieces, under nitrogen. The flask was sealed with a serum cap, flushed with N_2, and the solution was stirred until all of the metal had dissolved. The reagent was stored in the flask until needed.

Monomethoxy PEG (3 g, $1.23 \cdot 10^{-3}$ moles; Hoechst, M_n =2400) was dissolved by heating in dry THF (15 ml) in a dry three necked flask equipped with a condenser protected with a drying tube, a serum cap through which dry N_2 was flushed through the experiment, and with a glass stopper. Na/naphthalene reagent (ca 5 ml) was added dropwise with a syringe to the cool solution until the colour of the complex remained in the solution. Ethyl-α-bromoacetate (0.78 ml, $6.15 \cdot 10^{-3}$ moles) was added, and the solution stirred for 3.5 hrs. The solvent was evaporated, and the polymer purified by precipitation from benzene/petroleum ether, five times. Finally the polymer was dried under high vacuum. Yield 3.14 g.

NMR($CDCl_3$/TMS): δ =1.30 (t, ester $-CH_3$),
4.10 (s,$-O-CH_2-CO-$), 4.25 (q, ester $-CH_2-$),
3.38 (s,$-OCH_3$), relative intensity of $-OCH_3$ to $-CH_2-CO-$
= ca 3 : 2.

The ester terminated polymer (1.70 g) was hydrolysed in water at 50°C with 1 M KOH. The solution was neutralized

and was gel chromatographed on Sephadex G 25. The polymer fractions were combined and lyophilized. Yield 1.50 g.

^{1}H NMR($CDCl_3$/TMS): δ =3.95 (s,-O-CH_2-CO-).

^{13}C NMR($CDCl_3$/TMS): δ = 174 (C=O).

Carboxyl content by titration: 0.7 CO_2H/PEG.

10.4.14 Polymerization of ethylene oxide onto alcohols

10.4.14.1 Polymerization with sodium hydride as catalyst (8.2.1)

PVAc-OH (0.244 g, $3.31 \cdot 10^{-5}$ moles; M_n =7600) was dissolved in dry THF (5 ml) in a vial. A 50% oil dispersion of sodium hydride (8 mg, $1.61 \cdot 10^{-4}$ moles) was added under nitrogen, and the vial was sealed with a serum cap. Dry ethylene oxide (0.20 g, $4.55 \cdot 10^{-3}$ moles) was condensed under nitrogen into a dry vial cooled on dry ice/ethanol. The polymer solution was transferred to the condensed ethylene oxide, and the vial was sealed. The solution was stirred at room temperature for 18 hrs. The solvent was then removed under reduced pressure. The polymer was dissolved in a small amount of benzene, 2 drops of water were added, and the solution was dried with $MgSO_4$. After filtration the solution was evaporated to dryness under reduced pressure. Yield 0.13 g polymer.

The NMR spectrum showed no peak characteristic of polymerized ethylene oxide.

10.4.14.2 Polymerization with Na/naphthalene as catalyst (8.2.1)

a. Reaction with benzyl alcohol:

Benzyl alcohol (0.5 ml, $4.6 \cdot 10^{-3}$ moles) and dry THF (3 ml) were added to a dry vial, and the vial was sealed with a serum cap. Na/naphthalene reagent ((see 10.4.13.3.b) ca. 12 ml) was added dropwise with a syringe until the colour of the complex remained in the solution. Dry ethylene oxide (2 g, $4.6 \cdot 10^{-2}$ moles) was condensed under nitrogen into a vial cooled on dry ice/ethanol. The vial was sealed with a serum cap, and the polymer solution was transferred with a syringe into the condensed ethylene oxide. The solution was stirred at room temperature for 19 hrs. The solvent was evaporated, and the viscous residue was dissolved in a small amount of benzene. Water (2 drops) was added and the solution was dried with $MgSO_4$ and filtered. The solvent was evaporated, and the residual naphthalene was sublimated off under high vacuum, together with the unmodified alcohol. Yield 0.21 g yellow oily liquid.

TLC (hexane:ethyl acetate, 1:1) R_f =0.0, 0.07, 0.19, 0.30 and 0.9. R_f for benzylamine = 0.56, for naphthalene = 0.9.

NMR ($CDCl_3$/TMS): δ = 3.49 (s, $-(CH_2)_2-O-$), 4.40 (s,$-CH_2-\emptyset$), 7.20 (s, arom. H).

Reaction in the presence of HMPA

The experiment was carried out as above except for the addition of HMPA (1.4 ml) to the alcoholate solution, and distillation of the condensed ethylene oxide into this solution. After removal of excess naphthalene by sublimation under high vacuum only benzyl alcohol and HMPA could be detected by NMR.

b. Reaction with PVAc-OH

PVAc-OH (0.20 g, $2.6 \cdot 10^{-5}$ moles; M_n =7600) was dissolved in dry THF (3 ml) in a vial. The vial was sealed with a serum cap and flushed with dry N_2. Na/naphthalene reagent (ca 5 ml, see 10.4.13.3.b) was added with a syringe until the colour of the complex remained in the solution. Ethylene oxide (0.80 g, $1.82 \cdot 10^{-2}$ moles) was condensed under nitrogen into a vial cooled on dry ice/ethanol. The polymer solution was transferred to the condensed ethylene oxide. The vial was sealed, and the solution was stirred at room temperature for 19 hrs. The solvent was removed under reduced pressure. The polymer was dissolved in a small amount of benzene, 2 drops of water were added, and the solution was dried with $MgSO_4$. After filtration the solution was evaporated to dryness. Yield 0.107 g.

No peaks characteristic of polymerized ethylene oxide could be detected by NMR.

10.4.14.3 Polymerization with triethylamine as catalyst (8.2.2)

a. Reaction with benzyl alcohol

Benzyl alcohol (0.5 ml, $4.6 \cdot 10^{-3}$ moles), triethylamine (1 ml) and dry THF (5 ml) were added to a dry pressure flask under nitrogen. Dry ethylene oxide (3 ml $6.0 \cdot 10^{-2}$ moles) was condensed into a calibrated test tube cooled on dry ice/ethanol. The test tube was stoppered with a rubber stopper through which a thin polyethylene tubing was drawn to reach the bottom of the test tube. The condensed ethylene oxide was forced into the pressure flask by slightly heating the test tube. The pressure flask was sealed, and the solution was stirred at 55-60°C for 3 days. After the reaction the solvent and triethylamine were removed by evaporation under high vacuum. Yield 3.01 g crude product, containing alcohol and ethylene glycol in a ratio 1:15 and triethylammonium salt as a contaminant.
NMR($CDCl_3$/TMS): δ = 3.65 (s, $-(CH_2)_2-O-$), 4.6 (s,$-CH_2-\emptyset$), 7.30 (s, arom. H), relative intensity of arom H = 5, for $-(CH_2)_2-O-$ = 60.

0.37 g of the crude product dissolved in methanol/water (1:1) was chromatographed on a Dowex 50W-X8 cation exchange column. Elution with methanol/water yielded 0.24 g benzyl alcohol-PEG in a ratio 1:4.

b. Reaction with PVAc-OH

PVAc-OH (0.667 g, $8.8 \cdot 10^{-5}$ moles; M_n =7600) was dissolved in dry THF (6 ml) under nitrogen in a dry pressure flask. Triethylamine (1 ml) was added. Dry ethylene oxide (1 ml, $2.0 \cdot 10^{-2}$ moles) was condensed into a calibrated test tube, and was forced into the pressure flask following the procedure in 10.4.14.3.a The solution was stirred at room temperature for 3 days. A sample was taken and evaporated to dryness under reduced pressure. CCl_4 was added and the solution was evaporated. This procedure was repeated twice. NMR($CDCl_3$/TMS): δ =3.55 ($-(CH_2)_2-O-$).

Ethylene oxide (1 ml, $2.0 \cdot 10^{-2}$ moles) was added to the solution, and the stirring continued at 55-60°C for 3 days. During this time the solution had turned dark, and the polymer formed a black precipitate which was insoluble in methylene chloride. The product was neither isolated nor analysed.

10.4.14.4 Polymerization with BF_3-etherate as catalyst (8.2.3)

The procedure of Geckeler and Mutter [34] for polymerizing ethylene oxide onto Cinerubin A was followed.

PVAc-OH (0.74 g, $9.7 \cdot 10^{-5}$ moles; M_n =7600) was dissolved in dry dioxane (3 ml). Ethylene oxide (0.2 g, $4.5 \cdot 10^{-3}$ moles) was condensed into dry, ice-cooled dioxane in a vial. The polymer solution was added to the frozen ethylene oxide solution, and finally BF_3-etherate (3 μl) was added. The

vial was sealed and the solution was stirred at room temperature for 3 days. The solution was evaporated to dryness under reduced pressure. The polymer was dissolved in CCl_4 and the solution was evaporated. This procedure was repeated twice. Finally the polymer was dried under high vacuum. Yield 1.07 g.

NMR ($CDCl_3$/TMS): δ =3.6 (-(CH_2-O-).

Ratio PVAc to PEG ca 1:1.

Part of the polymer was dissolved in a minimum amount of ethanol and was reprecipitated with water. The procedure was repeated six times. Finally the polymer was dissolved in benzene, the water removed by azeotropic distillation, and the polymer lyophilized from the benzene.

NMR ($CDCl_3$/TMS): δ = 3.6 (-(CH_2-O-), very low intensity.

10.4.15 Preparation of PVAc/PVA-PEG graft copolymer (8.3.)

Entry # 1 (Table 10):

A mixture of monomethoxy PEG (1.0 g, $4.1 \cdot 10^{-4}$ moles; M_n = 2400, Hoechst), dibenzoyl peroxide (0.10 g, $4.1 \cdot 10^{-4}$ moles) and vinyl acetate (5 ml, 0.05 moles) in a flask sealed with a serum cap was purged with dry N_2 for 30 min. The flask was immersed in a 60°C wax bath, which caused the PEG to dissolve. The solution was stirred for 40 min. The excess vinyl acetate was removed under reduced pressure. The polymer was dissolved in CCl_4 and the solvent was evaporated,

with the procedure being repeated three times. Yield 3.17 g.

NMR (D_2O/HOD): δ = 3.6 (-$(CH_2)_2$-O-), relative intensity = 4, 4.8 (-CH-), relative intensity 8.4.

Preparation of the PVAc-PEG-CO_2H graft polymer (entry # 4 (Table 10)) was carried out in the same way, using PEG-CO_2H prepared as in 10.4.13.3.b.

The hydrolysis was carried out in ethanol/water at 60°C as described in 10.4.13.3.a using 1 M KOH.

Purification of the graft copolymers:

Several attempts were made to isolate the graft from the homopolymers. The most successful method to remove PEG from the graft was by trituration of the hydrolysed graft with methylene chloride. All other methods resulted in a fractionation of the graft into fractions containing different ratios of PVAc/PVA to PEG and, in the precipitation procedures, with the loss of a considerable amount of material.

a. Precipitation from ethanol/water (entries # 1 and # 2 (Table 10)):

The graft polymer (initial ratio PVAc:PEG = 8.4:1) was dissolved in the minimum amount of ethanol, and was reprecipitated with water, repeating the procedure three times. The polymer was collected by centrifugation and was air dried.

NMR (D_2O/HOD): δ = 3.6 ($-(CH_2)_2-O-$), relative intensity = 4, 4.8 (-CH-), relative intensity = 14.

b. Extraction of PVA-PEG-OH graft with methylene chloride/ water (entry # 3 (Table 10)):

PVA-PEG-OH graft copolymer (0.78 g, PVA:PEG = 1.4:1) was extracted with methylene chloride/water. A thick emulsion formed. After centrifugation the aqueous layer was removed and evaporated. Yield 0.11 g polymer.
NMR (D_2O/HOD): δ =3.55 ($-(CH_2)_2-O-$), relative intensity = 4, 3.85 (-CH-), relative intensity = 7.1.
TLC: 1. (alumina,methanol as eluent) R_f = 0.7 ; for PEG-OH R_f = 1.0 ; for PVA-OH R_f = 0.0 ; for a mixture of PVA + PEG R_f = 0.0 ,1.0 .
2. (silica, methanol as eluent) R_f = 0.0 ; for PEG-OH R_f =0.3 ; for PVA-OH R_f = 0.0 .

Three additional consecutive extractions of the methylene chloride layer with water gave polymer with a decreasing ratio of PVA to PEG:

2nd extraction, yield 0.11 g, PVA:PEG (NMR) = 8:1
3rd " , " 0.13 g, " " = 6.8:1
4th " , " 0.12 g, " " = 2.8:1

The methylene chloride layer (9 mg) contained only PEG. Emulsion layer (0.13 g): PVA:PEG = 1:12 (NMR)

Redistribution of the third H_2O extract into methylene chloride/ water:

methylene chloride layer: no polymer present.

water layer: PVA:PEG = 6.8:1 (NMR).

c. Column chromatography of the PVAc-PEG-OH graft on silica (entry # 3 (Table 10)):

PVAc-PEG-OH graft copolymer (0.37 g, PVAc:PEG = 1.4:1) dissolved in methylene chloride was applied to a silica column and was eluted with methylene chloride:t-butyl alcohol (6:4). 0.23 g of the polymer was collected.

NMR ($CDCl_3$/TMS): δ = 3.66 ($-(CH_2)_2-O-$), relative intensity 4, 4.9 (-CH-), relative intensity 7.6.

The amount of t-butyl alcohol in the elution solvent was increased gradually, until pure alcohol was used, but no more polymer was eluted. The solvent was changed to pure methanol, and the rest of the polymer eluted in consecutive fractions:

1st fraction, 56.0 mg, PVAc:PEG = 1:2.9

2nd " , 40.8 mg, " = 1:5.6

3rd+4th " , 46.0 mg, only PEG present

d. Trituration of PVA-PEG-CO_2H graft (entry # 4 (Table 10)):

PVA-PEG-CO_2H graft copolymer (0.103 g, PVA:PEG = 1.7:1) was triturated with five consecutive portions of methylene chloride (3 ml). The methylene chloride solutions were evaporated, weighed and analysed by NMR.

A total of 24.6 mg PEG was obtained in the first four triturations, after which no more polymer dissolved in the methylene chloride. The residue (58.4 mg) contained PVA:PEG in a ratio 9:1 .

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