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EFFECTS OF POLY(ETHYLENE GLYCOL)-BOUND ALCOHOLS AND AMINES ON THE PARTITION OF ALBUMINS AND THYLAKOID MEMBRANES IN AN AQUEOUS TWO-PHASE SYSTEM

GÖTE JOHANSSON
Department of Biochemistry, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
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SUMMARY

A number of alcohols and some amines have been bound to carboxymethyl-poly(ethylene glycol), obtained by oxidation of poly(ethylene glycol) with permanganate. These polymer derivatives influence to various degrees the partition of serum albumin and chloroplast thylakoid membranes between the phases of a liquid–liquid two-phase system composed of water, dextran and poly(ethylene glycol). Higher fatty alcohols and certain steroids are especially active in increasing the affinity of the biological material for the poly(ethylene glycol)-rich upper phase. In a few cases the polymer-bound group caused exclusion of the membrane fragments from the upper phase and the interface. The possible use of the polymer derivatives for selective adjustment of partition of biological material within aqueous two-phase systems is discussed.

INTRODUCTION

The partition of particles as well as proteins between the phases (and the interface) of aqueous two-phase systems can drastically, and sometimes selectively, be changed by introducing polymer-bound chemical groups of low concentration in one of the phases[1–4]. The systems are composed of water and two polymers, usually dextran and poly(ethylene glycol) (PEG). The former polymer is concentrated in the lower phase, the latter in the upper phase[1,2]. Binding of a substance, with affinity for biological material, to one of the polymers affects the partition of the material. It will be accumulated in the phase containing the polymer that carries the bound group. The most used polymer derivatives are PEGs carrying fatty acid groups (as esters), charged groups or triazine dyes[1–10]. The biological material studied includes serum proteins[1], enzymes, nucleic acids, membrane fragments, cell organelles, and cells[1,2].

One of the technical problems in the application of this technique for purification, known as affinity partitioning[5], is the covalent binding of the affinity groups to PEG[11]. In the present work molecules carrying hydroxyl or amino groups have been bound to PEG via ester or amide linkages by first introducing terminal carboxylic groups on the polymer. The effect of the polymer derivatives on partition of albumins and thylakoid membranes from spinach chloroplasts have been studied.

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MATERIALS AND METHODS

Materials

Dextran with M_r = 500 000 was obtained from Pharmacia (Uppsala, Sweden), poly(ethylene glycol) with M_r = 6000–7500 from Union Carbide (New York, U.S.A.), bovine serum albumin, fraction V, and hen ovalbumin, grade V, from Sigma (St. Louis, MO, U.S.A.). Membrane fragments of the thylakoid system of chloroplasts isolated from spinach, *Spinacea oleracea*, were prepared according to Andersson *et al.*[12], using a Yeda press.

Carboxymethyl-PEG

PEG (1.6 kg), dissolved in 4 l of water, was mixed at room temperature with a solution of 50 g of potassium permanganate in 1.5 l of water. The solution was stirred mechanically until the permanganate was consumed (2.5 h), and pH was adjusted to 7.0 by addition of 2 *M* sulphuric acid. The mixture was then heated to 45°C and the manganese dioxide was removed by suction filtration. This procedure was facilitated by addition of cellulose powder. The colourless filtrate was adjusted to pH 2.5 with dilute sulphuric acid and evaporated in vacuum at 75°C using a rotary thin-film evaporator. The remaining polymer was dissolved in 5 l of hot toluene, the solid salt was removed by filtration, and the solvent was evaporated as above. The solid polymer was brittle and white with a yellow tint. It contained 0.14 mmol carboxylic groups per gram, and the yield was 90%. The molecular weight, estimated (as described below) with viscometry, was 5600.

Esters

Carboxymethyl-PEG (10 g) was dissolved in 20 ml of dry pyridine on a water-bath at 50°C. Then 1 g of toluene-*p*-sulphonyl chloride (recrystallized from petroleum ether) was added and, after 5 min on the water-bath, 6 mmol or less (see Table I) of the alcohol to be esterified were also added. After 90 min on the bath 200 ml of toluene were added, and the mixture was kept at the same temperature for 1 h. The precipitate formed was removed by filtration, with cellulose powder used as a filter aid. The polymer precipitated at 3°C and was recovered by suction filtration. It was washed on the filter with 55 ml of toluene. The product was recrystallized from 150 ml of absolute ethanol at 3°C, and the polymer was washed on the filter with 50 ml of ethanol in the cold. After drying in the air the polymer was ready for use. The yield was 65–75%.

Amides

These were prepared in the same way as the esters, except that amines were used instead of alcohols.

Degree of substitution

The degree of substitution was determined by passing 0.5 g of polymer dissolved in 10 ml of water through a column (10 × 1 cm I.D.) of Dowex 50W × 4 in acid form, followed by titration with 0.2 *M* sodium hydroxide to pH 7.7 (glass electrode). The consumption of alkali was compared with that of carboxymethyl-PEG, which had gone through the total synthesis and purification steps but in absence of

TABLE I

ANALYTICAL DATA OF CARBOXYMETHYL-PEG AND ITS DERIVATIVES

Polymer-bound substance	*Amount of substance used for esterification of 10 g polymer (mmol)*	*Degree of substitution (%)*	*Viscosity of 50 g/l polymer solution in 100 mM potassium sulphate relative to this solvent*
–	–	0	1.94
Ethanol	6	20	2.06
Octan-1-ol	6	5	2.07
Dodecan-1-ol	6	9	2.06
Tetradecan-1-ol	6	21	2.17
Benzyl alcohol	1	20	2.08
3-Phenylpropan-1-ol	1	24	2.19
5-Phenylpentan-1-ol	1	22	2.18
10-Phenyldecan-1-ol	1	27	2.10
Phytol	1	24	2.21
Retinol	0.85	0.9	2.18
Decane-1,10-diol	1	22	2.09
Dodecane-1,12-diol	1	24	2.08
Cholesterol	1	23	2.18
Deoxycholic acid	1	N.D.*	2.22
11α-Hydroxymethyltestosterone	0.5	N.D.	2.20
5α-Androstan-17α-methyl-3β,17β-diol	0.7	N.D.	2.13
Dihydrotestosterone	0.7	N.D.	2.36
Pyridoxal	0.5	N.D.	2.17
Pyridoxamine	0.5	N.D.	2.14
Cephalin	1	N.D.	2.20
AMP	0.5	N.D.	2.21
Folic acid	0.5	1.2	2.13
Riboflavin	0.6	0.17	2.31
FMN	0.5	0.15	2.11
FAD	0.012	0.01	2.36
Benzylamine	1	23	2.13
4-Phenylbutylamine	1	26	2.17
p-Anisidine	1	N.D.	2.13

* N.D. = not determined.

alcohol. The amounts of bound folic acid (345 nm), riboflavin, FMN, and FAD (all 443 nm) were determined photometrically with a Unicam SP800 B spectrophotometer.

Viscosity

The viscosity was determined with a Ubbelohde viscometer with a flow-time of 29 s for water. Solutions containing 50 g of polymer per litre and 0.1 *M* potassium sulphate were used.

Determination of molecular weight

The viscosities of aqueous solutions of the polymers containing 0.1 *M* sodium chloride were measured at four polymer concentrations, and the limit viscosity was determined by extrapolation to infinite dilution. The molecular weight was calculated according to Ring *et al.*[13].

Partition

Biphasic systems containing 7% (w/w) dextran, 5% (w/w) PEG including ester or amide of carboxymethyl-PEG, 100 m*M* sodium acetate, and 5 m*M* sodium phosphate buffer in water were used for the partition of proteins (1 or 2 g/l) at pH 7.0. Membranes (1.6 g/l, wet weight) were partitioned in systems containing 7% (w/w) dextran, 7% (w/w) PEG including derivatives, 100 m*M* potassium sulphate, and 9 m*M* potassium phosphate buffer at pH 7.4. The temperature in both cases was 20°C. The concentration of material in the system and in the phases (after equilibration) was determined by absorbance measurements at 285 nm (proteins) or 680 nm (membranes) after dilution with water (six times) and with corresponding blanks from a system without biological material using a Beckman Acta CIII spectrophotometer. Preparation of the systems and the analysis of the phases have been described in detail elsewhere[1,2]. In the case of membranes the absorbance of total material (samples from mixed system) was also determined at 550 nm. The partition of coloured polymer derivatives and the free substances was determined in the same way at 443 nm (riboflavin, FMN, FAD) or 345 nm (folic acid).

RESULTS

Synthesis

Oxidation of PEG decreased the molecular weight from 6900 to 5600. This shows that, besides oxidation of the terminal hydroxymethyl groups, an oxidative cleavage of the polymer chain occurs. The subsequent esterification step, in which the polymer is recrystallized twice, increases the molecular weight to 5700, as determined for the ethyl ester. The analytical data of the polymer esters are given in Table I. Polymers bearing hydrophobic groups show an increased viscosity of its water solutions, which points to a certain polymer–polymer interaction. The derivatives show high absorbance at wavelengths below 275 nm owing to impurities that cannot be removed by crystallization. The esters can also be obtaining by using the salt form of carboxymethyl-PEG but the degree of substitution is then reduced to one tenth. The lower molecular weight has a relatively low effect on the composition of the two phases as can be seen in the phase diagram (Fig. 1). The partition coefficients of some of the esters are given in Table II.

Partition of proteins

By replacing a part of PEG for certain esterified carboxymethyl-PEG the affinity of serum albumin for the upper phase increases. The effect increases with the percentage of bound groups and is noticeable for octyl but more pronounced for the dodecyl and myristyl groups (Fig. 2). The small changes in partition that can be seen for the ethyl, benzyl and octyl esters are due to changes in the composition of the phases as result of the successive decrease in the mean molecular weight of PEG. The

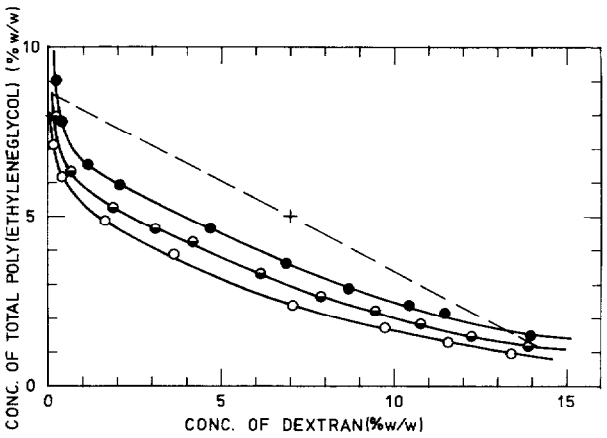

Fig. 1. Phase diagram at 20°C for aqueous solution of dextran with PEG, ○; carboxymethyl-PEG and PEG in the weight ratio 1:1, ◒; carboxymethyl-PEG, ●. The systems also contained 100 mmol/kg sodium acetate and 5 mmol/kg sodium phosphate buffer at pH 7.0. The dashed line is the tie-line for the system containing 7% dextran and 5% PEG, of which 50% was in the carboxymethyl form.

same result is obtained with unsubstituted carboxymethyl-PEG. The partition of ovalbumin is only weakly affected by these esters (Table III). Other ester-bound long-chained alcohols (*e.g.* 10-phenyldecanol and phytol) also show a strong effect on the partition of serum albumin, as well as cephalin and deoxycholic acid. Surprisingly, neither androstane-type steroids nor riboflavin-containing groups or folic acid have any effect on the partition.

Partition of fragments from chloroplasts

The membranes can be both in the two phases and at the interface between them[12]. If the amount of the ester or higher fatty alcohols is increased, the membranes move from the lower phase and the interface, over the interface and to the upper phase. The partition at a high concentration of ester (50% of total PEG) is given in Table II.

TABLE II

PARTITION COEFFICIENTS, *K*, OF FREE AND POLYMER-BOUND COLOURED SUBSTANCES AND OF PEG

Values for systems containing 7% (w/w) dextran, 3.5% (w/w) PEG, 3.5% (w/w) carboxymethyl-PEG derivative, 100 mmol/kg potassium sulphate and 9 mmol/kg sodium phosphate buffer at pH 7.4.

Substance	*K*	
	Free	*Polymer bound*
Riboflavin	1.08	6.0
FMN	0.73	6.9
FAD	0.67	11
Folic acid	1.05	2.4
PEG	11	

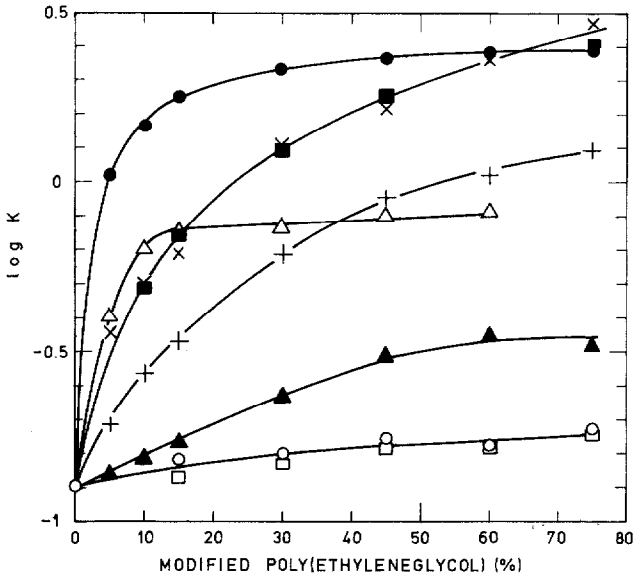

Fig. 2. The logarithmic partition coefficient, log K, at 20°C of bovine serum albumin (1 g/kg) in systems containing 7% (w/w) dextran, 5% (w/w) PEG together with its derivatives, 100 mmol/kg sodium acetate and 5 mmol/kg sodium phosphate buffer, with the pH adjusted to 7.0. The ordinate gives the percentage of total PEG in the form of derivatives of this polymer. Carboxymethyl-PEG, ○; and its esters with ethanol, □; dodecan-1-ol, ■; tetradecan-1-ol, ●; dodecane-1,12-diol, △; phytol, ▲; deoxycholic acid, +; and cephalin, ×.

The partition of membranes is affected by the same groups that influence the partition of serum albumin. Some of the groups show a positive effect on the partition, taking the material to upper phase, or, if it less effective, to the interface. A few of the bound groups (5-phenylpentyl, androstane-17α-methyl-3β,17β-diol, dihydrotestosterone, FAD and 4-phenylbutylamine), on the other hand, increase the affinities of the membranes for the lower phase. By comparing the apparent absorbance of the fragments at 550 and 680 nm it can be determined whether the membranes have been further disintegrated during the partitioning[15]. This is the case with the esters of long-chain alcohols, phytol, cholesterol, deoxycholic acid and cephalin (Table III), where the ratio A_{550}/A_{680} is lower than for the original suspension.

DISCUSSION

The relatively mild oxidation of PEG converts a relatively large number of the terminal hydroxymethyl groups into carboxylic groups without lowering the molecular weight of the polymer to any great extent. The introduction of carboxylic groups makes it possible to bind alcohols and amines to the polymer. These groups of substances can easily be bound to the acid group on the polymer with toluene-*p*-sulphonyl chloride[14]. Cyclohexylcarbodiimide is incapable of forming esters between alcohols and carboxymethyl-PEG (data not shown).

The esters are restricted mainly to the upper phase of the two-phase system

TABLE III

PARTITION OF BOVINE SERUM ALBUMIN, OVALBUMIN AND CHLOROPLAST MEMBRANES

The composition of the systems is given under Materials and methods in the text. Temperature, 20°C; K = partition coefficient; A_{550}/A_{680} was measured on dilute samples from mixed systems.

Polymer-bound substance	*Serum albumin (2 g/kg)* log K*	*Ovalbumin (2 g/kg)* log K*	*Chloroplast membranes***			
			Per cent in/at			$\frac{A_{550}}{A_{680}}$
			Upper phase	*Inter-face*	*Lower phase*	
–	−0.83	−0.50	2	60	38	0.30
Ethanol	−0.75	−0.43	3	62	35	0.30
Octan-1-ol	−0.44	−0.40	26	35	39	0.30
Dodecan-1-ol	+0.09	−0.46	91	7	2	0.22
Tetradecan-1-ol	+0.26	−0.42	100	0	0	0.19
Benzyl alcohol	−0.76	−0.48	4	68	28	0.31
3-Phenylpropan-1-ol	−0.76	−0.50	3	62	35	0.31
5-Phenylpentan-1-ol	−0.83	−0.51	4	18	78	0.29
10-Phenyldecan-1-ol	+0.03	−0.43	85	12	3	0.25
Phytol	−0.01	−0.42	100	0	0	0.18
Retinol	−0.86	−0.53	35	26	39	0.29
Decan-1,10-diol	−0.61	−0.46	20	71	9	0.31
Dodecan-1,12-diol	−0.43	−0.49	48	50	2	0.30
Cholesterol	−0.74	−0.49	91	4	5	0.20
Deoxycholic acid	−0.10	−0.51	86	1	13	0.25
11α-Hydroxymethyltestosterone	−0.83	−0.57	2	62	36	0.30
5α-Androstan-17α-methyl-3β,17β-diol	−0.74	−0.53	4	24	72	0.30
Dihydrotestosterone	−0.83	−0.52	1	25	74	0.30
Pyridoxal	−0.80	−0.47	3	60	37	0.30
Pyridoxamine	−0.81	−0.51	6	46	49	0.31
Cephalin	+0.07	−0.42	100	0	0	0.24
AMP	−0.87	−0.56	4	61	35	0.31
Folic acid	−0.91	−0.49	4	63	33	0.32
Riboflavin	−0.70	−0.44	1	75	24	0.31
FMN	−0.79	−0.45	2	63	35	0.31
FAD	−0.61	−0.37	0	25	75	0.27
Benzylamine	−0.80	−0.49	3	52	45	0.32
4-Phenylbutylamine	−0.84	−0.53	5	30	65	0.31
p-Anisidine	−0.82	−0.48	3	56	41	0.31

* System contained 100 mmol/kg sodium acetate; 50% of PEG consisted of the polymer derivative.
** System contained 100 mmol/kg potassium sulphate; 25% of PEG consisted of the polymer derivative.

because they partition as the PEG, which is the dominant part of the molecule. Despite the very low concentration of the bound groups, of the order of 1 mM, the effect on the partition of biological materials can be drastic. This is especially so for the membranes, and is due to the large number of binding sites for the group on each particle.

It is remarkable that a number of space-filling groups such as androstane-type steroids and coenzymes do not have a positive effect on the partition of either serum

albumin or the membranes. Comparing the simple carbon chains, it is found that there is a weak effect with the eight-carbon octyl group whereas chains of twelve carbon atoms or more act strongly. The largest distances over the androstanes and coenzymes (except FAD) are equal to straight chains of eight carbon atoms or less. The reduced affinity of the bound groups for membranes and albumin could therefore be due to the nearness of the PEG chain. The need for a spacer to obtain the interaction has been discussed previously[16]. The deoxycholic acid and cholesterol molecules, which correspond to chains of eleven and thirteen carbon atoms, exceed the critical length and show strong interaction with the membranes. This is in good correlation with the effect of esters of PEG with fatty acids[17]. It was shown that the chain length had to be at least twelve carbon atoms for there to be interaction with the thylakoid membrane system. The inactive behaviour of FAD (corresponding to 23 carbon atoms) may depend on where the molecule is linked to the polymer. If the linkage is in the middle of the molecule at one of the ribose rings the distance from this point to farthest part of the group would be too short for interaction. For interaction to occur between the group and the biological material it seems necessary to have a spacer of at least eight carbon atoms length between the polymer and the group.

The effect of some polymer-bound groups to decrease the affinity for the upper phase must depend on the change in its liquid structure. This phenomenon has also been found for esters of PEG with lower fatty acids[16].

The clear tendency of the active polymer derivatives to fragment membranes further may be counteracted by finding groups that interact with specific sites on the membranes without dipping into the inner parts of the membrane structure.

The affinity partition can be used for preperative as well as analytical purposes. (1) By binding a group (to the PEG) with specific affinity for a substance to be isolated, the latter can be extracted from a mixture if only the other constituents stay in the lower phase or at the interface. (2) By studying the effect of various ligands on the partition of a given substance, the type and relative amounts of binding sites could be estimated.

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