THE PREPARATION OF HIGH MOLECULAR WEIGHT DIVALENT "ANTIGENS"[1] OF DEFINED SIZE

M. P. Cooke, B. G. Archer, and H. Krakauer

Program in Biochemistry and Biophysics and the Department of Chemistry
Washington State University, Pullman, WA. 99163

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Summary: A simple, rapid procedure for the synthesis of divalent "antigens" that consist of high molecular weight polyethylene oxide chains bearing dinitrophenyl groups at their ends and for their fractionation to near size-homogeneity is described.

There arose in the course of our thermodynamic investigation of the flexibility of the hinge of certain immunoglobulins a requirement for a linear molecule of large size whose ends bore identical antigenic determinants and were separated by specifiable average distances. This requirement was met by PEO-DNP_2. Rather wide utility can be anticipated for this material in the exploration of the properties of antibodies and of the behavior of receptors of cells of the central and efferent limbs of immune response (1) (a) because of the immunological simplicity and definition that it possesses (PEO is immunologically inert) and (b) because of the dimensional specifications that may be imposed on it.

Materials and Methods

The high molecular weight PEO, Carbowax® PEG 68,000, prepared by an anionic polymerization process, was a gift of Union Carbide Co. (The commercially available PEO, Polyox®, prepared by free-radical polymerization

(1) These compounds are designated antigens rather loosely on the basis of their size and polyvalency. Whether they are capable of stimulating the production of specific antibody has not yet been determined.

(2) Abbreviations Used: DNP - dinitrophenyl; PEO - polyethylene oxide; PEO-DNP_2 - PEO bearing a DNP group at each end; MP - methoxypolyethylene oxide, PEO one of whose terminal hydroxyls has been methylated; MP-DNP - MP whose hydroxyl has been replaced by DNP; THF - tetrahydrofuran; PBKS - 0.45M K_2SO_4, 0.01M phosphate, pH 7.8

is not suitable because it lacks -OH termini, nor is the highly branched Carbowax® 20M.) Monovalent hapten was prepared from MP, Carbowax® Methyoxy Polyethylene Glycol 750, a smaller polymer with a very narrow chain length distribution, also from Union Carbode. n-Butyllithium, 1.6N in hexane (Foote Chemical Co.) and 1-fluoro-2,4 dinitrobenzene (J. T. Baker Chemical Co.) were used as purchased. Anhydrous, O_2-free THF was obtained by distillation from $LiAlH_4$ under N_2.

The small but not negligible fraction of ends of the high polymer, PEO, that are vinyl ethers, $R\text{-}O\text{-}CH_2{=}CH_2$ (2), is readily hydrolyzed in aqueous solution by exposure to pH 2.3 for 10 min. at room temperature under N_2 to yield hydroxyl termini. After neutralization, the major portion of the water of the reaction mixture may be removed by evaporation at reduced pressure and the remainder by three cycles of dissolution in benzene and its evaporation in a rotary evaporator. The resulting material is sufficiently dry to be used directly in the subsequent steps.

In a typical preparation 2 gm of PEO or MP were dissolved in 50 ml of dry THF containing about 1 mg of 1,10-phenanthroline. The hydroxyl groups of the PEO or MP were then titrated under N_2 with n-butyllithium to just beyond the phenanthroline end point (appearance of red color) (3). An amount of fluorodinitrobenzene calculated to be in 2.5 fold excess of the n-butyllithium was added and allowed to react for 10 minutes, followed sequentially by the addition of 10 ml of 0.5M phosphate, pH 8.1 and 50 ml of H_2O. The bulk of the THF was removed on a rotary evaporator. The reaction mixture was cleared of sediment by centrifugation and by passage through filters with 0.1μ pores. The polymers were freed from residual THF, dinitrophenol, and other impurities by chromatography on Bio-Gel P6 equilibrated with PBKS in the case of $PEO\text{-}DNP_2$ and Sephadex G-10 equilibrated with H_2O in the case of MP-DNP. (Because of the apparent tight adhesion of dinitrophenol to PEO in the absence of salt, H_2O alone cannot be used in the final purification of $PEO\text{-}DNP_2$.) $PEO\text{-}DNP_2$ was concentrated

Table I. Properties of MP-DNP

$\left(\frac{\partial n}{\partial c}\right)^{(1)}_{H_2O}$	(measured)	$147.2 \pm 0.5^{(3)}$ ml/mol
$\left(\frac{\partial n}{\partial c}\right)^{(1)}_{H_2O}$	(calculated, eq'n(1))	145.5 ± 0.2 ml/mol
$\varepsilon^{M}_{298\ nm}$		$10{,}900 \pm 50\ M^{-1}\ cm^{-1}$
$K_{binding}$ [2]	(to IgG(T) antibody)	$7.3 \times 10^{6}\ M^{-1}$

(1) λ = 546 nm

(2) See reference (5)

(3) All uncertainties are standard errors

Table II. Refractive Index Increments of PEO

$\left(\frac{\partial n}{\partial c}\right)^{(1)}$	(in H_2O)	0.1340 ± 0.0006 ml/gm
$\left(\frac{\partial n}{\partial c}\right)^{(1)}$	(in PBKS)	0.1240 ± 0.0007 ml/gm
$\left(\frac{\partial n}{\partial c}\right)^{(1)}_{\mu}$	(In PBKS)	0.1043 ± 0.0012 ml/gm

(1) λ = 546 nm

by pressure filtration through Diaflo®UM-20, UM20E, UM10 or PM10 membranes and MP-DNP by evaporation.

Size-fractionation of PEO-DNP_2 was achieved by chromatography on Sephadex G-200 equilibrated with PBKS containing 1% isopropanol as an antioxidant.

Light-scattering measurements and refractometry were performed on a Brice Phoenix photometer and differential refractometer.

Results and Discussion

The molar extinction coefficient of DNP groups conjugated to PEO was

determined by means of spectrophotometric measurements on solutions of MP-DNP whose molar concentrations were measured by titration of the $-NO_2$ ring substituents with $TiCl_3$ (4) and is given in Table I. In addition, it was verified that such groups retained affinity for specific anti-DNP antibody: a fluorescence-quenching titration of equine anti-DNP IgG(T) with MP-DNP revealed an equilibrium constant for the binding of this hapten very similar to that for DNP-glycine (5).

The completeness of the reactions of this preparation was demonstrated by the agreement to within 1% (Table I) of the molar refractive index increments of MP-DNP in water, $\left(\frac{\partial n}{\partial C}\right)$, obtained by direct measurement and by calculation based on the additivity of these quantities:

$$\left(\frac{\partial n}{\partial C}\right)^{(MP-DNP)} = \left(\frac{\partial n}{\partial C}\right)^{MP} + \left(\frac{\partial n}{\partial C}\right)^{(DNP-OH)} - \left(\frac{\partial n}{\partial C}\right)^{(H+OH)} \qquad (1)$$

(The last term on the right, the correction for the loss of H_2O that accompanies the substitution of DNP on MP is obtained from a comparison of $\left(\frac{\partial n}{\partial C}\right)$ of ethanol with that of diethyl ether or of those of glucose and fructose with that of sucrose.)

The refractive index increments, on a mass concentration (gm/ml) basis for PEO in water and PBKS both at fixed composition, $\left(\frac{\partial n}{\partial c}\right)_c$, and at fixed chemical potential of the solvent components, $\left(\frac{\partial n}{\partial c}\right)_\mu$, the latter required for the light-scattering measurements (6), are given in Table II. The last quantity was obtained in the following way. A known quantity of PEO in PBKS was passed over a Bio-Gel P6 column that had been washed with the same buffer to establish equilibrium between the solvent components in the PEO solution and in PBKS. The entire volume containing PEO was collected and from the ratio of the volumes applied to the column and collected and the initial concentration, the final concentration of PEO was calculated. The ratio to this of the refractive index of the PEO solution relative to that of the PBKS is just $\left(\frac{\partial n}{\partial c}\right)_\mu$.

Table III. Molecular Properties of Fractions of PEO-DNP_2

$\bar{M}_w$ (1) (g/mol) once fractionated	twice fractionated	$\bar{M}_w/\bar{M}_n$	R_G	$\frac{R_G}{(\bar{M}_w)^{1/2}}$ (2)	$\left(\frac{\partial n}{\partial c}\right)_\mu$ (ml/g)
83400±4000		1.33±.06	148 Å	0.51	0.1054
	82400±2400	1.13±.04	122	0.42	0.1052
	65200±1800	1.06±.03	113	0.44	0.1054
	56500±1800	1.00±.04	84	0.35	0.1055
54800±1600		1.31±.03	101	0.43	0.1060
	42800±1300	0.97±.03	88	0.43	0.1059
	35400±1300	1.03±.04	-	-	0.1063
17000±1500		0.99±.11	-	-	0.1083

(1) once fractionated material was passed over a 5 x 75 cm Sephadex G-200 column equilibated with PBKS, twice fractionated material was obtained by passing a cut from once-fractionated material over a 5 x 30 cm G-200 column equilibated with PBKS.

(2) The ratio $R_G/(\bar{M}_w)^{1/2}$ should be constant (9) for polymer fractions which are nearly homogeneous in molecular weight.

Fractionated samples of PEO-DNP_2 were characterized by means of measurements of their number and weight average molecular weights, $\bar{M}_n$ and $\bar{M}_w$. The concentrations, in gm/ml, of the polymer solutions equilibrated with PBKS as above, were measured by differential refractometry and were based on $\left(\frac{\partial n}{\partial c}\right)_\mu$ for PEO-DNP_2 calculated from that of PEO by means of an equation analogous to (1). (The error introduced as a result of the application of an equation valid for systems at fixed solvent composition to those at fixed chemical potential is negligible in this case because of dominating contribution of PEO- that for the DNP ends never exceeds 3.5%. In consequence, $\left(\frac{\partial n}{\partial c}\right)_\mu$ for PEO-DNP_2 shows very little dependence on the molecular weight (Table III)).

$\bar{M}_n$ of a given sample of PEO-DNP_2 is given by the ratio of the concentrations in terms of mass (gm) and moles per liter, the latter determined spectrophotometrically on the basis of the extinction coefficient of MP-DNP. $\bar{M}_w$ and the root-mean-square radius of gyration, R_G, may be measured by light scattering photometry (7), the latter only for samples

with $\bar{M}_w$ > 40,000 and with less precision the lower the $\bar{M}_w$. (PBKS was the solvent in all measurements because at 25° it is very nearly a θ solvent for PEO, i.e. PEO behaves nearly thermodynamically ideally in it and assumes a compact form (8)). The quantities that characterize fractions of PEO-DNP_2 obtained by one passage or two over Sephadex G-200 are given in Table III. The efficiency of the fractionation, best, as expected for material of low molecular weight, is indicated by the approach of the ratio $\frac{\bar{M}_w}{\bar{M}_n}$ to 1.0. This and the fact that within experimental error the ratio does not fall below 1.0 also verify that there is neither significant branching of the polymer chains nor that a substantial number of ends have failed to react.

PEO-DNP_2 in PBKS does not undergo detectable degradation at room temperature for at least twelve days.

We have found the above procedures for the production of large quantities of this well-defined "antigen" convenient and rapid. A one gram quantity may be synthesized in one man-day. The fractionation is more lengthy but does not require much attention.

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