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A Simple Method for Coupling Proteins to Insoluble Polysaccharides

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Summary. Proteins have been coupled to insoluble polysaccharides following periodate oxidation to produce reactive aldehyde groups which will react with side chain amino groups of proteins. The coupling is stabilized and unreacted aldehydes blocked, by reduction with sodium borohydride.

INTRODUCTION

The covalent coupling of protein to insoluble matrices has found considerable application in immunology and enzymology. Probably the most widely used technique at the present time is the cyanogen bromide method described by Axen, Porath and Ernback (1967), which was a considerable advance from the practical point of view on previous methods.

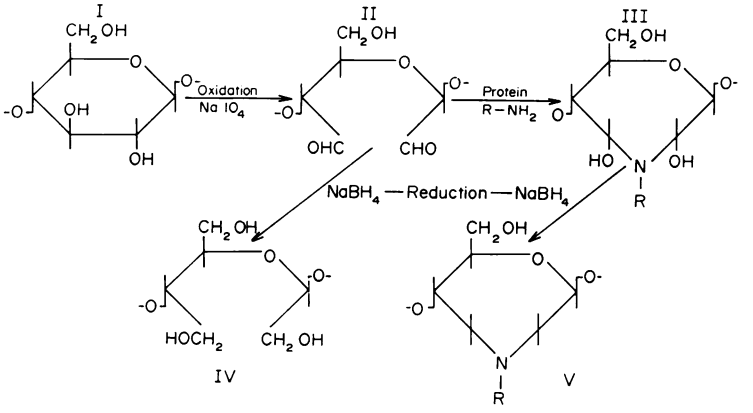

FIG. 1. Sequence of reactions used to couple protein to polysaccharides.

This paper describes a simple method in which the polysaccharide is 'activated' by oxidation with periodate. The general course of the periodate oxidation reaction of polysaccharides (Guthrie, 1961) is shown, using as an example the β-D-glucopyranose residue (I) which is a common structural feature of numerous polysaccharides (Fig. 1). Aldehydes (II) generated by the oxidation reaction provide a reactive functional group for the attachment of proteins. Under mild conditions nucleophilic attack by epsilon-NH_2 groups of lysine could be expected to give rise to carbinolamines (III). Treatment of the coupled material with sodium borohydride will reduce any remaining aldehydes, block-

ing any further coupling (IV). It will also reduce the unstable carbinolamines to the more stable alkylamines (V).

The oxidation reaction is applicable to a wide range of carbohydrates and under appropriate conditions most peptides and proteins could be coupled. The use of this reaction for coupling protein and polysaccharide to red cells will be described elsewhere (Sanderson and Wilson, 1971).

MATERIALS AND METHODS

The polysaccharides cellulose CC41 (Whatman), Sephadex and Sepharose (Pharmacia) were first swollen in water for several days. Oxidation was carried out with different concentrations of sodium periodate ($NaIO_4$) for different periods of time as indicated. The oxidized polysaccharides were washed with water by centrifugation and 10 mg (dry weight) suspended in 1 ml of phosphate buffered saline (PBS) pH 8 containing 10 mg of [^{125}I]bovine serum albumin (BSA), and agitated continuously for 20 hours. Preliminary experiments with cellulose indicated that coupling required at least 24 hours, but for convenience the uptake after 20 hours was measured. Reduction was carried out with a freshly prepared solution of 1 per cent sodium borohydride ($NaBH_4$). The particles were then washed twice in PBS by centrifugation and resuspended in 1 ml of 0·5 M NaCl, 0·01 M acetate buffer pH 4. Radioactivity in each 10 mg sample was counted and after standing overnight part of the acetate buffer was removed and counted to determine the elution of protein. At least four replicates were tested in each group, the major source of error being the loss of adsorbant when removing the supernatant. The level of sensitivity of detecting [^{125}I]BSA eluted into the acetate buffer was estimated to be 0·005 mg; below this amount count rates were too close to background. The level for eluted [^{125}I]IgG was estimated to be 0·0002 mg.

Crystalline BSA (Armour Pharmaceutical) and human IgG (purified from serum by DEAE cellulose fractionation) were trace labelled with [^{125}I] by the chloramine-T technique (Hunter, 1967) and diluted in unlabelled protein. BSA was used at 25 counts/sec/mg and IgG at 300 counts/sec/mg. Radioactivity was determined in a Nuclear Enterprises NE 1832 γ-scintillation counter. Samples were counted for 2000 counts or 1000 seconds, counting both the gamma and X-ray emissions. Punch tape data were processed with a computer program written by Dr D. Franks to convert to counts/sec, correct for background, convert to milligrams of protein and to calculate group means and standard deviations.

RESULTS

The amounts of [^{125}I]BSA taken up by the polysaccharides are shown in Table 1. Cellulose was not visibly changed by periodate oxidation, although it tended to aggregate when coupled to protein. Ten milligrams of cellulose oxidized in 1 ml of 0·1 M $NaIO_4$ for 24 hours adsorbed 0·3 mg of BSA, compared with 0·05 mg taken up non-covalently by untreated cellulose. Cellulose stored at 4° for 1 month after oxidation showed no significant decrease in the amount of BSA coupled.

The periodate oxidation of Sephadex went effectively to completion within 1 hour and longer periods of oxidation (up to 20 hours) did not increase the capacity of the oxidized Sephadex to take up protein. Sephadex G-25 was resistant to damage by periodate and

10 mg of the superfine grade coupled about 0·3 mg of BSA, whereas only 0·05 mg were taken up non-covalently. Oxidation of Sephadex G-75 by 0·1 M periodate resulted in a considerable decrease in the packed volume of the Sephadex at pH 8–9. After coupling the remaining material was a yellow colour and gave high radioactivity counts indicating a considerable uptake of protein but the product formed aggregates and was unsuitable for most applications. However, 0·01 M periodate caused no visible change in Sephadex G-75, and it then coupled 2 mg of BSA/10 mg of Sephadex. Sephadex G-200 was similarly damaged by alkali following oxidation and showed considerable protein uptake after oxidation by 0·1 M periodate. After oxidation by 0·01 M periodate the G-200 remained visibly normal and coupled 1·3 mg of BSA/10 mg.

TABLE 1

THE COUPLING OF BSA TO POLYSACCHARIDES

	Concentration of $NaIO_4$ (M)	Time (hours)	BSA mg/10 mg polysaccharide	Elution (mg)
Cellulose	0·1	4	0·08 ± 0·01	—*
	0·1	24	0·29 ± 0·09	—
	Not oxidized		0·05 ± 0·01	—
Sephadex				
G-25 Superfine	0·5	1	0·42 ± 0·02	< 0·005
	0·1	1	0·13 ± 0·01	—
	0·01	1	0·08 ± 0·03	—
	Not oxidized		0·05 ± 0·01	—
G-25 fine	0·1	1	0·09 ± 0·01	< 0·005
	0·01	1	0·03 ± 0·01	< 0·005
	Not oxidized		0·02 ± 0·01	< 0·005
G-75 fine	0·01	1	1·97 ± 0·13	0·02
	Not oxidized		0·07 ± 0·01	< 0·005
G-200 fine	0·01	1	1·33 ± 0·13	0·04
	Not oxidized		0·13 ± 0·03	0·06
Sepharose 4B	0·5	1	0·15 ± 0·01	—
	0·5	18	0·20 ± 0·04	—
	0·1	1	0·13 ± 0·01	< 0·005
	Not oxidized		0·06 ± 0·02	< 0·005

* Not tested.

Sepharose 4B remained visibly unchanged by alkali after 18 hours oxidation in 0·5 M $NaIO_4$. After oxidation for 1 hour by 0·1 M $NaIO_4$ 10 mg of Sepharose took up 0·13 mg of BSA.

Effect of reduction on coupling to Sephadex G-75

Reduction was carried out by suspending the oxidized Sephadex in 1 ml of 1 per cent $NaBH_4$ for 15 minutes and the reduced material was then washed three times by centrifugation. The effect on the coupling reaction is shown in Table 2. Reducing before adding protein effectively blocks the coupling reaction. The difference between the amount of protein in the samples reduced after coupling, and those not reduced is not significant. However, the elution of BSA into the acetate buffer was significantly lower from the reduced samples ($P < 0·01$).

Non-covalent adsorption of IgG

Sephadex G-75 (fine grade) was oxidized with 0·01 M periodate and coupled with BSA. Under these conditions about 2 mg of BSA/10 mg of Sephadex would be taken up (Table 1). The Sephadex-BSA was washed twice with PBS by centrifugation.

The Sephadex-BSA was in contact with 2·2 mg of [^{125}I]IgG pH 8, for 1 hour, it was then washed twice with PBS, resuspended in acetate buffer and the radioactivity taken up by 10 mg of Sephadex-BSA counted. After standing overnight elution into the acetate buffer was determined by withdrawing part of the supernatant and counting for radioactivity (Table 3). The Sephadex-BSA adsorbed 0·09 mg of [^{125}I]IgG/10 mg, however, only 1 per cent of this was eluted into the acetate buffer. Reduction of the Sephadex-BSA with $NaBH_4$ did not significantly lower the non-covalent adsorption of IgG however the elution of [^{125}I]IgG into the acetate buffer was significantly lower ($P<0·01$).

TABLE 2

EFFECT OF REDUCTION ON COUPLING BSA TO SEPHADEX G-75

Treatment	BSA (mg/10 mg G-75)	Elution (mg)
Reduced before coupling BSA	0·14 ± 0·05	< 0·005
Not reduced	1·97 ± 0·13	0·02
Reduced after coupling BSA	1·32 ± 0·34	< 0·005

TABLE 3

NON-COVALENT ADSORPTION OF IgG TO SEPHADEX G-75 AND G-75-BSA

Adsorbant	IgG (mg/10 mg Sephadex G-75)	Elution (mg)
G-75-BSA (not reduced)	0·09 ± 0·004	0·0009
G-75-BSA (reduced)	0·07 ± 0·01	0·0002
G-75 (untreated)	0·06 ± 0·01	0·0002
G-75 (soaked in BSA)	0·02 ± 0·002	0·0008

To test whether this relatively high non-covalent adsorption was a general property of Sephadex, untreated G-75 was tested after similar contact with [^{125}I]IgG. Non-covalent adsorption of [^{125}I]IgG was of the same order as the adsorption of [^{125}I]BSA (non-oxidized controls in Table 1). Soaking G-75 in BSA significantly blocked the uptake of [^{125}I]IgG ($P<0·01$).

DISCUSSION

Periodate oxidation of polysaccharides provides a simple method for producing reactive functional groups for the attachment of proteins. The periodate is water soluble, easily standardized and readily removed by washing after reaction. Thus precise control of the reaction conditions is possible. The 'activated' polysaccharide can be stored at 4° without loss of activity for at least a month.

In unpublished experiments with cyanogen bromide we have found that Sephadex G-25 will couple 0·17 ± 0·01 mg of BSA/10 mg and Axen *et al.* (1967) showed that G-200 coupled 2 mg chymotrypsin/10 mg. These polysaccharides coupled 0·42 mg and 1·3 mg

of BSA respectively (Table 1) by the periodate technique. The results with cyanogen bromide coupling to Sepharose suggest higher uptakes of protein than that achieved with periodate. Thus Cuatrecasas (1969) coupled about 1 mg of insulin and Omenn, Ontjes and Anfinsen (1970) coupled about 2 mg of staphylococcal nuclease to 10 mg of Sepharose. This is compared to 0·2 mg of BSA by the periodate method (Table 1), but this is probably adequate for many applications.

Controls in which untreated polysaccharides were mixed with protein solution indicated that a surprising amount of protein was adsorbed. This seems not to have been adequately documented in the past, although it is clearly a general feature of these insoluble polysaccharides. However, IgG non-covalently adsorbed to Sephadex G-75 was not eluted under conditions of low pH and high ionic strength, suggesting that this method of coupling may be suitable for the preparation of immunoadsorbants for the purification of specific antibody.

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