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COMPARISON OF NON-BIOSPECIFIC EFFECTS IN IMMUNOAFFINITY CHROMATOGRAPHY USING CYANOGEN BROMIDE AND BIFUNCTIONAL OXIRANE AS IMMOBILISING AGENTS

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SUMMARY

Polypeptide antigen, glucagon, antibodies to glucagon and non-immune globulins were immobilised on agarose using CNBr and a bifunctional oxirane. Irrespective of the ligand immobilised, positively charged groups introduced to conjugates by CNBr caused electrostatic interactions with impurities and soluble biospecific ligands.

Solvents required for elution of bound antibodies and antigens were more strongly deforming when immunoaffinity conjugates were prepared with CNBr than with the oxirane. This is attributed to compound affinity resulting from reinforcement of biospecific by non-biospecific interactions. Strongly deforming solvents were still required for oxirane conjugates, however, when antibodies had high affinity for antigen.

INTRODUCTION

Cyanogen bromide[1] has been widely used to couple biospecific ligands which possess primary amino groups to agarose for immunoaffinity chromatography[2]. This method of immobilisation, however, introduces cationic groups attributed to isoureas with a pK_a value of about 10.4 (refs. 3 and 4). Nishikawa and Bailon[4] and Wilchek and Miron[5] indicate that non-biospecific electrostatic interactions involving the positive charge on the support may interfere with bioaffinity chromatography. In order to eliminate such interference, coupling methods which result in uncharged agarose derivatives have been devised and include the use of dihydrazides[5] and bifunctional oxiranes[6]. In this report CNBr is compared with bisoxiranes as immobilising agent for immunoaffinity chromatography with particular reference to (i) the binding of impurities to columns and (ii) the compound effect of non-biospecific and biospecific interactions between materials being purified and the immunoaffinity columns. The hormone glucagon and antibodies to the polypeptide are used for experimental models. Differences between the coupling methods are illustrated both with immobilised antigen in the purification of antibody, and in the converse situation. Non-immune globulins coupled to Sepharose are used as controls for antibody-Sepharose conjugates.

MATERIALS AND METHODS

Antisera from five rabbits immunised with porcine pancreatic glucagon[7] were provided by K. D. Buchanan (Department of Medicine, Queen's University, Belfast, Northern Ireland) and are coded 59a, 59b, 59c, 70, 71, 100, and 118. Antisera 59a, 59b, and 59c were taken from the same rabbit 5, 12, and 24 months, respectively, from the beginning of the immunisation period. Preparation of ^{125}I-labelled glucagon is described by Murphy *et al.*[7]. Conjugates of CNBr-activated Sepharose with glucagon, with immunoglobulin G containing antibodies (antibody-Sepharose conjugates) and with immunoglobulins G from non-immunised rabbits, were also prepared as described previously[7,8]. Corresponding conjugates were prepared using a bisoxirane, 1,4-butanediol diglycidyl ether, according to the method of Sundberg and Porath[6]. Glucagon-Sepharose conjugates (1 g wet weight) were dehydrated using acetone, dried, and hydrolysed[9] in constantly boiling HCl (5 ml) prior to amino acid analyses[10] using an automatic analyser (Locarte, London, Great Britain).

Chromatographic details including examination of column effluents are already described[8]. The following solvents were used to irrigate columns:

Ia,b,c,d: 0.04 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.4, containing 0.15, 0.5, 1.0, and 2.0 M NaCl, respectively.

IIa,b,c,d,e: 0.15 M NaCl adjusted to pH 8, 9, 10, 10.5, and 11, respectively, with aqueous ammonia (sp. gr. 0.88).

III: 0.1 M acetic acid adjusted to pH 2.2 with formic acid.

IV: 4 M guanidine hydrochloride.

Columns were equilibrated with buffer Ia prior to application of samples.

RESULTS AND DISCUSSION

When serum from non-immunised rabbits was applied at 5° to glucagon-Sepharose conjugates prepared using CNBr (Fig. Ia), most of the protein which bound
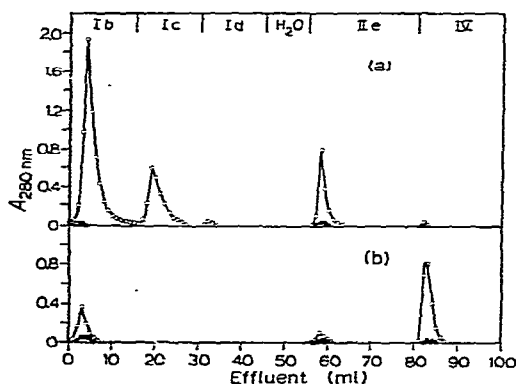

Fig. 1. Elution of proteins bound non-biospecifically to glucagon-Sepharose columns. Conjugates were prepared using the oxirane (●) and CNBr (○). Serum (2 ml) from non-immunised rabbits was applied to columns equilibrated with solvent Ia and unbound materials were washed through with two column volumes of solvent Ia. The absorbance of the effluent fractions was measured at 280 nm during subsequent irrigation of the columns. The above chromatographic procedures were carried out at 5° (a) and 20° (b).

non-biospecifically could be eluted at pH 7.4 by increasing the concentration of NaCl in solvent I. The remainder of the protein bound was eluted at pH 11 using solvent IIe. Less protein was bound to the same conjugates at 20° (Fig. 1b). At the higher temperature, most of the non-biospecifically bound protein could be eluted by solvent IV but not by solvent IIe. These results indicate that electrostatic interactions contribute significantly to the binding of proteins to the columns at 5° whereas at 20° it is likely that hydrophobic interactions are responsible. In contrast to the results obtained with CNBr as coupling agent, the binding of proteins to conjugates prepared using the oxirane was negligible at either temperature (Figs. 1a and b). When serum was applied at 5° to antibody-Sepharose conjugates, the elution profiles of proteins bound non-biospecifically were identical to those shown in Fig. 1a for antigen-Sepharose conjugates. The above results suggest that non-biospecific binding to the conjugates prepared using CNBr is due to the method of coupling more than to the ligand immobilised.

The chromatographic properties of ^{125}I-labelled glucagon on columns of non-immune globulins coupled to Sepharose (Fig. 2) are a further indication that non-biospecific binding between materials applied and columns is stronger when CNBr rather than the oxirane is used for immobilisation. The binding of a small proportion ($<5\%$) of the hormone applied to conjugates prepared using the oxirane and the retention of the hormone until the columns are irrigated with solvent IId may be due to electrostatic interactions between glucagon and the immobilised globulins.
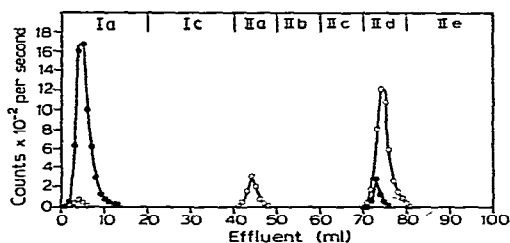

Fig. 2. Chromatography of ^{125}I-labelled glucagon on immobilised immunoglobulin G from non-immunised rabbits. The globulin-Sepharose conjugates were prepared using the oxirane (●) and CNBr (○).

The observations reported here concerning non-biospecific adsorption displayed by conjugates prepared using CNBr are in agreement with elution profiles reported previously[8] for proteins bound non-biospecifically to immunoaffinity columns. In general, the results confirm the suggestion[3,4] that a positively charged group, with a pK_a about 10.4, is introduced to Sepharose when CNBr is the coupling agent. This pK_a value could explain why dilute alkaline solutions, solvents IId and e, are more efficient than concentrated solutions of NaCl, solvents Ic and d, for the elution of some of the non-biospecifically bound materials (Figs. 1a and 2). Another cationic group, however, seems to cause binding of materials which can be eluted by solvent IIa (Fig. 2; see also Figs. 2 and 3 of a previous report[8]).

When antiserum 59b is applied to glucagon-Sepharose conjugates prepared using CNBr, solvent IV was required for elution of antibodies whereas solvent IIe was effective when the oxirane was used (Fig. 3a). The stronger binding of the anti-
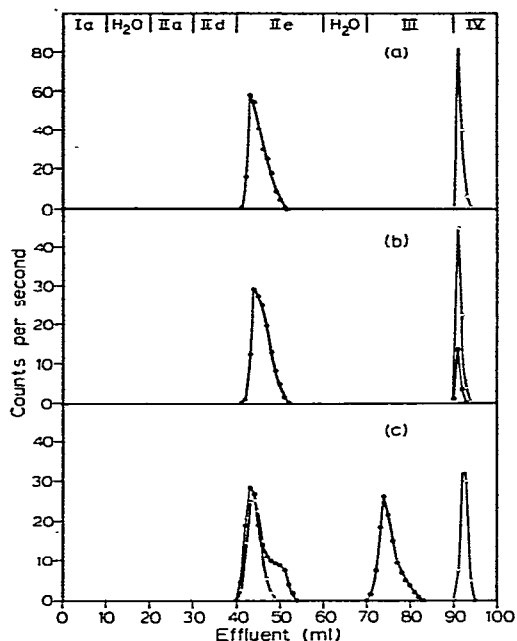
Fig. 3. Chromatography of antibodies on immobilised glucagon. Antisera 59b (a), 70 (b), and 118 (c) were applied to conjugates prepared using the oxirane (●) and CNBr (○). Antibodies in effluent fractions were detected by incubation with ^{125}I-labelled glucagon (30 pg) under conditions of radioimmunoassay as described previously[7,8].

bodies on the conjugates prepared using CNBr is attributed to compound affinity as explained by O'Carra[11] for small-ligand affinity chromatography: Biospecific interactions are mainly responsible for the binding of antibodies to conjugates prepared using the oxirane and are disrupted by solvent IIe (Fig. 3a). The same solvent is sufficient for disruption of non-biospecific interactions on conjugates prepared using CNBr (Figs. 1a and 2; see also a previous report[8]). When biospecific interactions are reinforced, however, by non-specific interactions, a more chaotropic solvent is required (Fig. 3a). The chromatographic consequences of compound affinity are also evident when antiserum 70 is applied to both types of conjugate (Fig. 3b). Most of the constituent antibodies are eluted by solvent IIe in place of IV when the oxirane rather than CNBr is used for immobilisation. A small proportion of the antibodies in this serum appears to have a high affinity for glucagon as indicated by the need for solvent IV for their elution from conjugates prepared with oxirane. Antiserum 118 also contains antibodies of mixed affinities for immobilised glucagon (Fig. 3c). A fraction containing low-affinity antibodies is readily eluted from both types of conjugate by solvent IIe. A second fraction containing higher affinity antibodies is retained on conjugates prepared with CNBr until solvent IV is used. Most of these antibodies, however, appear to be eluted from conjugates prepared using the oxirane by solvent III and the remainder as a shoulder which trails the first peak eluted by solvent IIe.

    The possibility that the differences in the chromatographic properties of the two types of conjugate (Fig. 3) are due to a variation in the site(s) of attachment of

the glucagon to the Sepharose, rather than to compound affinity, was considered. The pH 9.5 required for conjugating the hormone to oxirane-activated Sepharose is more alkaline than that used for CNBr-activated Sepharose (pH 8.2). Attachment of glucagon through the ε-amino group (pK_a in polypeptides = 10.2)[12] of Lys 12 to oxirane-activated Sepharose could perturb the steric complimentarity to the antibody-binding site, thereby reducing the strength of binding and the need for strongly chaotropic solvents. This occurs when chemical modifications are introduced to glucagon-Sepharose conjugates[8]. The hydrolysates of both types of conjugate, however, contained no histidine and had the same molar ratio (0.5) of lysine to arginine as has native glucagon. Thus, the hormone seems to be attached to the solid phase through the N-terminal histidyl residue irrespective of the method of immobilisation.

Variations in elution profiles observed when ^{125}I-labelled glucagon is chromatographed on antibody-Sepharose prepared using the oxirane and CNBr (Fig. 4) are also attributed to compound affinity. Most of the immobilised antibodies from antiserum 59a appear to have a low affinity for glucagon (Fig. 4a). The ^{125}I-labelled hormone applied is eluted mainly by solvents IId and e from conjugates prepared using the oxirane and CNBr, respectively. The remainder of the antigen applied appears to be bound through strong biospecific interactions with antibodies of high affinity since it is retained on both conjugates until the columns are irrigated with
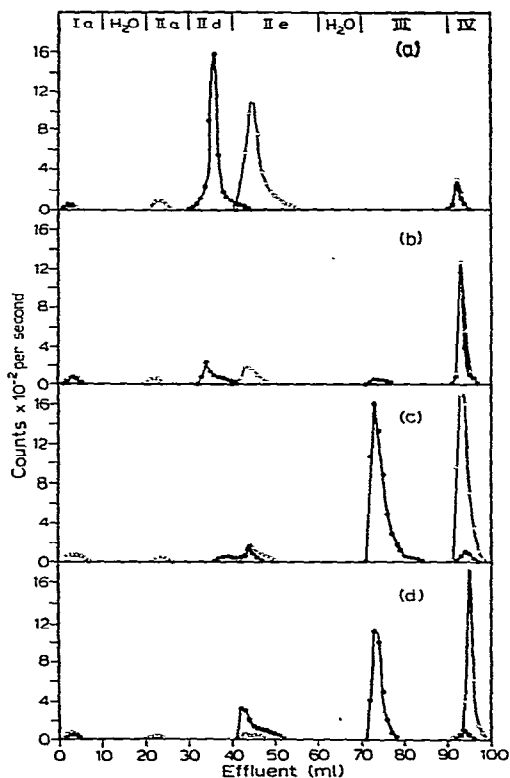

Fig. 4. Chromatography of ^{125}I-labelled glucagon on antibody-Sepharose conjugates. The immunoglobulins G isolated from antisera 59a (a), 59c (b), 71 (c), and 100 (d) were immobilised with the oxirane (●) and CNBr (○).

solvent IV. In contrast to the results obtained with antiserum 59a, only a small proportion of the immobilised antibodies from antiserum 59b have affinities which are low enough to permit detection of chromatographic differences between the methods of immobilisation (Fig. 4b). Less than 10 % of the antigen applied is eluted by solvent IId from conjugates prepared using the oxirane and by solvent IIe when CNBr is used. Most of the glucagon is bound to high-affinity antibodies and is not eluted from either conjugate until solvent IV is used. These results are in agreement with the general tendency for the affinities of antibodies to increase during the course of immunisation[2]. When globulins from antisera 71 and 100 are immobilised (Figs. 4c and d) most of the ^{125}I-labelled glucagon applied is retained on conjugates prepared using CNBr until columns are irrigated with solvent IV. In the absence of non-biospecific interactions when the oxirane is used for immobilisation, most of the antigen applied is eluted by solvent III.

The chromatographic profiles shown in Figs. 3 and 4 indicate that the elimination of compound affinity, by changing from CNBr to the oxirane as immobilising agent, may reduce the need for strongly deforming solvents. In some cases, however, the strength of biospecific interactions alone may be so high that strongly chaotropic irrigants such as solvents III and IV are still required for elution (Figs. 3c, 4b, c, and d). Chemical modifications could then be introduced to the ligands immobilised using the oxirane in order to permit the use of mild eluting solvents as shown already for antibody and antigen immobilised using CNBr[8]. In a preliminary experiment, glucagon-Sepharose prepared using the oxirane was treated with tetranitromethane. When antiserum 59b is applied to the modified conjugate, antibodies are eluted by solvent IId in place of IIe from the control column.

Avoidance of compound affinity may not be sufficient reason to use the oxirane rather than CNBr as immobilising agent. Results obtained here and in previous studies on immunoaffinity chromatography[8] show that clear separation between materials bound non-biospecifically and antibodies or antigen being purified is possible with conjugates prepared using CNBr. Good separations are also possible even when it is necessary to weaken the strength of biospecific interactions by chemical modification of the immobilised ligand[8]. Furthermore, lower efficiency of coupling when the oxirane rather than CNBr is used to immobilise proteins on Sepharose may be a disadvantage in the case of scarce or expensive antibodies or antigens. It is necessary to present about tenfold higher concentrations of glucagon to the oxirane- than to CNBr-activated Sepharose in order to achieve similar levels of hormone in the conjugates. While the efficiency of coupling of proteins to oxirane-activated Sepharose can be improved by increasing the temperature to 50°, many polypeptide materials would be irreversibly denatured by this treatment. The apparent advantage of being able to achieve higher levels of immobilised ligand in conjugates prepared using CNBr, however, could be negated by their propensity to bind materials non-biospecifically. Extensive non-biospecific binding of proteins could result in the occlusion of biospecific sites, thereby preventing full chromatographic exploitation of the conjugates. This problem might be expected when materials to be purified are present in low concentrations in crude extracts of tissue or in sera. The main advantage of using oxiranes as immobilising agents for immunoaffinity chromatography may be the absence of extensive non-biospecific binding of impurities during the application of samples to columns.

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