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Binding of Human Fibroblast Interferon to Concanavalin A-Agarose. Involvement of Carbohydrate Recognition and Hydrophobic Interaction†

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ABSTRACT: Human fibroblast interferon binds to a concanavalin A-agarose (Con A-Sepharose) equilibrated with methyl α-D-mannopyranoside, or levan; in contrast, it is only partially retarded on a similar column equilibrated with ethylene glycol. Interferon does not bind, however, to a lectin column equilibrated with both methyl α-D-mannopyranoside and ethylene glycol. Thus, a hydrophobic interaction between fibroblast interferon and the immobilized lectin seems to account for a large portion of the binding forces involved. Other hydrophobic solutes, such as dioxane, 1,2-propanediol, and tetraethylammonium chloride, were found equally or more efficient than ethylene glycol in displacing interferon from the lectin column. The elution pattern of interferon from a concanavalin A-agarose (Con A-Sepharose) column, at a constant ethylene glycol concentration and with an increasing mannoside concentration, reveals the existence of four distinct interferon components. The selective adsorption to, and elution from, a concanavalin A-agarose (Con A-Sepharose) column resulted in about a 3000-fold purification of human fibroblast interferon and complete recovery of activity. The specific activity of the partially purified interferon preparation is about 5×10^7 units per mg of protein. The chromatographic behavior of human leukocyte interferon is remarkable in that it does not bind to concanavalin A-agarose at all indicating the absence of carbohydrate moieties recognizable by the lectin, or if present, their masked status. When concanavalin A was coupled to an agarose matrix (cyanogen bromide activated) at pH 8.0 and 6.0 human fibroblast interferon bound to both lectin-agarose adsorbents and could be recovered with methyl α-D-mannopyranoside. Concanavalin A, immobilized directly on agarose matrix at pH 8.0 and 6.0, thus displays only carbohydrate recognition toward interferon. By contrast, unless a hydrophobic solute was included in the solvent containing methyl mannoside, human fibroblast interferon could not be recovered from concanavalin A-agarose coupled at pH 9.0. When concanavalin A was immobilized via molecular arms, in tetrameric as well as dimeric forms, the binding of interferon again occurred exclusively through carbohydrate recognition. Thus, the hydrophobic interaction can be eliminated by appropriate immobilization of the lectin, and then adsorbed glycoproteins, as exemplified here by interferon, can be recovered readily with methyl mannoside alone.

The understanding of forces involved in lectin–glycoprotein recognition is of immediate importance for the judicious use of lectins in the studies of cell membrane topography (Noonan and Burger, 1973; Cuatrecasas, 1973; Penhoet et al., 1974) and their application as solid phase affinity chromatography adsorbents (Cuatrecasas and Tell, 1973; Bessler and Goldstein, 1973). These forces may encompass—a priori—both carbohydrate–protein (lectin) and protein–protein (glycoprotein–lectin) interactions.

The pioneering studies of Goldstein and his coworkers (1974) first established the structural requirements for a carbohydrate to be recognized by concanavalin A. The protein–protein interactions are still largely unexplored despite the growing list of reports on the use of lectins, concanavalin A in particular, as chromatographic adsorbents (Norden and O'Brien, 1974; Rush et al., 1974; Gurd and Mahler, 1974). It is apparent, however, that only successful purification procedures are reported, the recovery of a glycoprotein from the lectin column being taken as the immediate measure of achievement. The failure to recover a glycoprotein with a simple monosaccharide is either reported as a successful immobilization (Sulkowski and Laskowski, 1974) or otherwise becomes an unheralded personal experience.

In our previous report on the binding and elution of human fibroblast interferon from a concanavalin A-agarose column it was established that bound interferon could not

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be recovered with methyl α-D-mannopyranoside alone (Davey et al., 1974). Elution with ethylene glycol resulted, however, in considerable recovery of interferon activity. Therefore, we postulated that a hydrophobic interaction takes place between interferon and the immobilized lectin. The present report analyzes further the binding and the release of human fibroblast interferon from concanavalin A-agarose, with particular emphasis on the source of hydrophobic effect. To this end, we describe the binding characteristics of concanavalin A, coupled to an agarose matrix at pH 8.0 and 6.0, toward human interferon; furthermore, we immobilized concanavalin A in its tetrameric and dimeric forms, via molecular arms, and studied the subsequent binding of human interferon. We now report that the hydrophobic interaction of human fibroblast interferon with immobilized concanavalin A (Con A-Sepharose) is probably due to its conformational distortion imposed by coupling conditions. An outgrowth of these studies is the description of concanavalin A-agarose chromatography of human interferon, as a model glycoprotein, dependent exclusively on carbohydrate recognition.

Experimental Procedure

Materials. Concanavalin A (lyophilized), Con A-Sepharose (concanavalin A-agarose), AH-Sepharose 4B (ω-aminohexyl-agarose), and Sepharose 4B, CNBr activated, were purchased from Pharmacia Fine Chemicals. Affi-Gel 10 (activated *N*-hydroxysuccinimide ester of succinylaminopropyl-agarose) was obtained from Bio-Rad Laboratories. Methyl α-D-mannopyranoside (α-MM[1]), *p*-nitrophenyl α-D-mannoside, succinic anhydrate, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were obtained from Sigma Chemical Co. Bovine serum albumin, a crystalline preparation, was obtained from Miles Laboratories. Levan, B-512, was a generous gift of Dr. I. J. Goldstein. Fluorescamine was obtained from Roche Diagnostics and ethylene glycol from Eastman Kodak Co. Dioxane was purchased from Fisher Scientific Co., propylene glycol (1,2-propanediol) from Baker Chemical Co., and tetraethylammonium chloride from Matheson Coleman and Bell. All other reagents were of analytical grade. Double glass-distilled water was used for all solutions.

Source and Assay of Interferons. Human fibroblast interferon, induced by $rI_n \cdot rC_n$, was prepared in human foreskin diploid fibroblasts, essentially according to Havell and Vilček (1972) by Dr. Judith A. O'Malley of this laboratory. Human fibroblast interferon, induced by Newcastle disease virus, was prepared from supernatant fluids of human foreskin diploid fibroblast monolayers, 24 h after inoculation with virus (multiplicity of infection, 10) (Levy and Carter, 1968). The supernatant fluid was acidified with 1.5 M perchloric acid to pH 2.0, cleared by centrifugation after 1 h at 4 °C, and then readjusted to pH 7.4 with 1 N NaOH. Human leukocyte interferon, induced by Newcastle disease virus, was prepared according to Pidot et al. (1972) by Dr. Julius Horoszewicz of this laboratory. Interferon activity was assayed on human fibroblasts by a modification of the colorimetric technique of Finter (1969) using vesicular stomatitis virus as the challenge virus (multiplicity of infection, 0.1) (Carter et al., 1972). In our assay the titer of a human reference interferon, E01, is 1400 to 1900 in comparison to the nominal value of 5000 units.

Chromatographic Procedure. Interferon preparations were dialyzed against 0.02 M sodium phosphate (pH 7.4) containing 1 M NaCl (unless otherwise stated) for 16 h at 4 °C. Interferon samples were applied on columns (0.9 × 5.5 cm) equilibrated with 0.02 M sodium phosphate (pH 7.4) containing 1 M NaCl at room temperature and the columns were developed with 0.1 M α-MM in 0.02 M sodium phosphate (pH 7.4) containing 1 M NaCl (unless otherwise stated) and denoted as E_1 and then with E_1 containing ethylene glycol (50% [v/v]) and denoted as E_2. The columns were developed at a flow rate of 30 ml per cm^2 per hour and fractions, 1.4 ml, were collected. Samples in even numbered fractions were used to measure protein; the odd numbered fractions, collected into tubes containing 0.5 ml of a 1% solution of bovine serum albumin in 0.02 M sodium phosphate (pH 7.4) and 0.15 M NaCl, were assayed for interferon activity.

Protein Determination. Protein concentration was measured by absorbance at 280 nm or by fluorometric assay (Böhlen et al., 1973), using bovine serum albumin as a standard.

Carbohydrate Determination. The concentration of methyl α-D-mannopyranoside in solvents and column effluents was measured by the phenol–H_2SO_4 method (Dubois et al., 1956).

Succinylation of Concanavalin A. Succinylated concanavalin A was prepared according to the procedure described by Gunther et al. (1973).

Couplings. Coupling of Concanavalin A to CNBr-Activated Sepharose 4B. Concanavalin A was coupled to activated agarose according to established procedures (Axen and Ernback, 1971). The Sepharose 4B, CNBr-activated (5 g), was swollen and washed with 1 mM HCl on a glass filter for 15 min at room temperature. Lyophilized concanavalin A, 200 mg, was taken up in 30 ml of 0.1 M sodium acetate (pH 6.0), stirred for 30 min at room temperature, and clarified by brief centrifugation. When the coupling at pH 6.0 was done in the presence of metals, the lectin solution contained in addition $MnCl_2$, $CaCl_2$, and $MgCl_2$, each at a final concentration of 1 mM. The coupling of concanavalin A at pH 8.0 was performed by dissolving the lectin in 0.1 M sodium bicarbonate (pH 8.0). One trial coupling of concanavalin A in 0.1 M sodium bicarbonate (pH 9.0) containing 0.5 M NaCl gave a preparation of immobilized lectin similar in binding properties to commercial Con A-Sepharose (Pharmacia) and additional couplings were not performed; commercial Con A-Sepharose was used routinely, wherever the use of concanavalin A-agarose described as coupled at pH 9.0 is indicated. The couplings at pH 6.0 and at pH 8.0 were performed with several (at least three) lots of concanavalin A and activated Sepharose 4B.

A solution of concanavalin A was combined with washed (1 mM HCl) activated agarose and the suspension was mixed in a screw-cap bottle by end-to-end rotation at room temperature for 2 h. Under these conditions the coupling went virtually to completion as indicated by absorbance at 280 nm of the filtrate, giving about 8 mg of coupled protein per ml of bed volume of lectin-agarose adsorbent. The first wash was done with 100 ml of the solvent with which concanavalin A solution was prepared. The gels were then washed three times (alternating) with 200 ml of 0.1 M sodium acetate (pH 4.5), containing 1 M NaCl and 1 mM $MnCl_2$, $CaCl_2$, and $MgCl_2$; 0.1 M sodium acetate (pH 6.0), containing 1 M NaCl; 0.05 M sodium phosphate (pH 8.0),

[1] Abbreviations used are: α-MM, methyl α-D-mannopyranoside; NDV, Newcastle disease virus; $rI_n \cdot rC_n$, poly(riboinosinic acid)·poly(ribocytidylic acid).

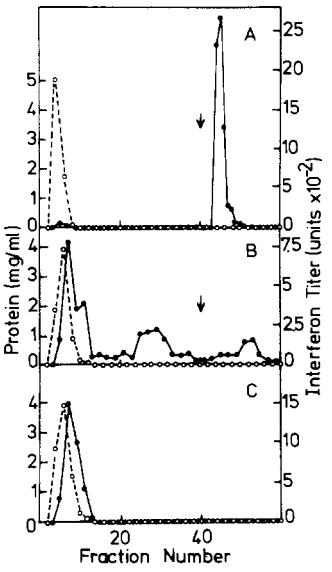

FIGURE 1: The effects of equilibration of concanavalin A-agarose columns with methyl α-D-mannopyranoside and ethylene glycol on adsorption and elution of human fibroblast interferon. An interferon preparation (7000 units and 16 mg of protein per ml), 1 ml, was applied on a column: (O - - - O) protein; (●–●) interferon. Chromatography of interferon on a lectin column equilibrated with methyl α-D-mannopyranoside (A), ethylene glycol (B), and methyl α-D-mannopyranoside and ethylene glycol (C). (A) The interferon sample, 1 ml, containing 0.1 M α-MM, was applied on a column equilibrated with 100 ml of E_1 and the column was washed with the equilibrating solvent. The breakthrough fraction contained about 98% of the applied protein and less than 1% of the interferon activity; the column was then washed with the equilibrating solvent containing ethylene glycol (50% [v/v]) as indicated (↓). All of the interferon activity (102%) was recovered. (B) The interferon sample, 1 ml, containing ethylene glycol (50% [v/v]), was applied on a column equilibrated with 100 ml of 50% ethylene glycol in 0.02 M sodium phosphate (pH 7.4) containing 1 M NaCl and the column was washed with the equilibrating solvent. The breakthrough fraction contained 98% of the applied protein and about 40% of the interferon activity. The remaining interferon activity was partially recovered (40%) by continuous elution with the equilibrating solvent and subsequent elution with the equilibrating solvent containing 0.1 M α-MM (↓). (C) The interferon sample, 1 ml, containing 0.1 M α-MM and ethylene glycol (50% [v/v]), was applied on a column equilibrated with 100 ml of E_2. The breakthrough fraction contained about 100% of the applied protein and 98% of the interferon activity.

containing 1 M NaCl. Finally, the gels were washed with 0.1 M sodium acetate (pH 6.0), containing 0.15 M NaCl and 1 mM $MnCl_2$, $CaCl_2$, and $MgCl_2$, and were allowed to age for at least 1 week at 4 °C. When used, the gels were packed into columns and equilibrated with 0.02 M sodium phosphate (pH 7.4), containing 0.15 M NaCl at room temperature.

Coupling of Concanavalin A to CH-Sepharose 4B. The coupling of the lectin at pH 6.0 was done as follows. Concanavalin A, 200 mg, was dissolved in 20 ml of water, adjusted to pH 6.0 with 0.1 N sodium hydroxide, and briefly centrifuged at room temperature. CH-Sepharose 4B, 5 g, was swollen in 0.5 l. of 0.5 M NaCl overnight, adjusted to pH 6.0 with 0.1 N sodium hydroxide, and finally washed with 0.5 l. of water. The lectin solution was then mixed with the agarose beads and the volume adjusted with water to 54 ml. Carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)], 1 M, was added dropwise (total volume, 6 ml) and the pH of the reaction mixture was maintained between 5.9 and 6.1 for the first 2 h and then the suspension was mixed in a screw-cap bottle by end-to-end rotation for an additional 22 h at room temperature with occasional adjustment of pH value. The agarose gel was then filtered on a glass filter and washed with 150 ml of each of the following: 0.02 M sodium phosphate (pH 7.4), containing 1 M NaCl; 0.05 M sodium phosphate (pH 8.0) (1 M NaCl); 0.1 M sodium acetate (pH 6.0) (1 M NaCl); 0.05 M sodium phosphate (pH 8.0) (1 M NaCl); 0.1 M sodium acetate (pH 6.0) (1 M NaCl); 0.1 M sodium acetate (pH 6.0), containing 0.15 M NaCl and 1 mM $MnCl_2$, $CaCl_2$, and $MgCl_2$. The gel was stored at 4 °C and washed before use with 1 l. of 0.1 M sodium acetate (pH 6.0) containing 0.15 M NaCl and 1 mM $MnCl_2$, $CaCl_2$, and $MgCl_2$; 200 ml of 0.1 M sodium acetate (pH 6.0) containing 0.15 M NaCl; and finally with 200 ml of 0.02 M sodium phosphate (pH 7.4) containing 0.15 M NaCl. The coupling, as indicated by absorbance of the first filtrate at 280 nm, was virtually complete.

The coupling of the lectin at pH 4.5 was done as follows. Concanavalin A, 200 mg, was dissolved in 20 ml of water, adjusted to pH 4.5 with 0.1 N hydrochloric acid, and briefly centrifuged at room temperature. CH-Sepharose 4B, 5 g, was swollen in 0.5 l. of 0.5 M NaCl, overnight, adjusted to pH 4.5 with 0.1 N sodium hydroxide, and finally washed with 0.5 l. of water. The lectin was coupled essentially as described for coupling at pH 6.0. The gel was washed also in a similar way, except for use of 0.1 M sodium acetate (pH 4.1) instead of 0.1 M sodium acetate (pH 6.0) during alternating washes with sodium phosphate (pH 8.0) (1 M NaCl).

Coupling of Succinylated Concanavalin A to AH-Sepharose 4B. Succinylated concanavalin A was coupled to AH-Sepharose 4B at pH 6.0, according to the procedure described above for the coupling of concanavalin A to CH-Sepharose 4B at pH 6.0.

Coupling of Concanavalin A to Affi-Gel 10. Concanavalin A, 200 mg, was dissolved in 25 ml of 0.1 M sodium acetate (pH 6.0) containing 0.15 M NaCl. After brief centrifugation, the solution was chilled at 4 °C for 2 h, added directly to Affi-Gel 10 in the bottle as supplied by the manufacturer, and mixed overnight at 4 °C. The washing procedure was performed at room temperature as follows: the gel was washed with 0.5 l. of 0.1 M sodium acetate (pH 6.0) containing 0.15 M NaCl and with 200 ml of 0.02 M sodium phosphate (pH 7.4) containing 1 M NaCl. The gel was stored at 4 °C and, when used, the effluent was monitored for absorbance at 260 nm to assure the completeness of deactivation.

Results

Figure 1 illustrates the binding and elution behavior of human fibroblast interferon on concanavalin A-agarose (Con A-Sepharose). All columns were preequilibrated with 0.02 M sodium phosphate buffer (pH 7.4) containing 1.0 M NaCl and in addition: 0.1 M α-MM (Figure 1A), 50% (v/v) ethylene glycol (Figure 1B), and both α-MM and ethylene glycol (Figure 1C). Interferon binds to concanavalin A-agarose in the presence of α-MM (Figure 1A). To test the binding, if any, of interferon in the presence of levan the following experiments were performed.[2] A lectin column (0.5 ml bed volume) was washed with 20 ml of levan, 1 mg/ml. An interferon preparation (3 ml) was applied on the column in 0.02 M sodium phosphate (pH 7.4)–0.15 M NaCl. Interferon activity was bound and recovered upon subsequent elution with ethylene glycol (50% [v/v]) in 0.02

[2] The experiments with levan were suggested to us by Dr. I. J. Goldstein.

M sodium phosphate (pH 7.4)–0.15 M NaCl. In a parallel experiment, the lectin column (0.5 ml) was equilibrated with 0.02 M sodium phosphate (pH 7.4)–0.15 M NaCl and the interferon sample (3 ml) was applied. A subsequent wash with levan (1 mg/ml) did not displace interferon from the column. Interferon was recovered, however, with levan (0.5 mg/ml) in 0.02 M phosphate (pH 7.4)–0.15 M NaCl containing ethylene glycol (50% [v/v]). Thus, the adsorption of interferon cannot be aborted by first saturating the lectin with levan. In addition, the elution of interferon, once adsorbed, cannot be accomplished with levan alone. Apparently, the interferon molecule has a much higher affinity for the immobilized lectin than does α-MM or levan; alternatively its binding to the lectin is not exclusively dependent on carbohydrate recognition. The interferon activity is considerably retarded, although not retained, on the column in the presence of ethylene glycol (Figure 1B). The interaction between interferon and this lectin is modified by ethylene glycol to a limited and varied extent as evidenced by the protracted elution pattern of activity and the presence of apparently discrete chromatographic fractions (Figure 1B). Thus, it was of interest to ascertain the influence of α-MM on the elution pattern of interferon adsorbed in the presence of ethylene glycol. The results are shown in Figure 1C. It is clear that the cooperative action of monosaccharide and hydrophobic solute prevented any interaction between interferon and concanavalin A-agarose. We then tried to substitute the need for both (α-MM and ethylene glycol) by using *p*-nitrophenyl α-D-mannopyranoside which is known to bind to concanavalin A by the dual forces of sugar recognition and hydrophobic bonding (Lootiens et al., 1973). We found that the addition of ethylene glycol was still necessary to recover the interferon activity (data not shown). This finding suggests that the postulated hydrophobic interaction between interferon and concanavalin A-agarose may not involve the hydrophobic binding site known to be immediately adjacent to the saccharide binding site on the lectin (Poretz and Goldstein, 1971; Bessler et al., 1973).

To probe the specificity of carbohydrate recognition, D-galactose (0.1 M) was also used rather than α-MM (0.1 M). The interferon activity adsorbed to concanavalin A-agarose (Con A-Sepharose) could not be displaced efficiently with D-galactose and ethylene glycol (50% [v/v]), even though D-mannose (0.1 M) can substitute for α-MM.

All experiments illustrated in Figure 1 were performed at high ionic strength (1 M NaCl). At 0.15 M NaCl concentration (not illustrated) the interferon activity is completely retained on the concanavalin A-agarose column and the amount of protein eluted with 0.1 M α-MM alone is similar at both concentrations of sodium chloride. Therefore, a crude preparation can be applied on the column either at 0.15 or 1.0 M NaCl; any nonspecific adsorption of proteins to the lectin column is effectively prevented at the lower salt concentration.

In order to establish the minimal concentrations of hydrophobic solutes effective in displacing interferon from the lectin column, an additional series of experiments was performed. In each of these experiments, interferon preparations were applied on concanavalin A-agarose (Con A-Sepharose), after which the columns were first equilibrated with α-MM, and then a linear gradient of a hydrophobic solute, at a constant concentration of α-MM (0.1 M), was applied. The concentration of the hydrophobic solute at the midpoint of an eluted interferon fraction is defined here, for the sake of comparison, as the minimal effective concentration. The relative effectiveness of the following solutes, in the terms just defined, was as follows: ethylene glycol (2 M), dioxane (0.8 M), propylene glycol (1.5 M), and tetraethylammonium chloride (0.2 M). The recovery of interferon activity was complete with all solutes except dioxane. The low recovery (25%) of interferon with dioxane was not caused by instability; interferon was found to be stable, in a control experiment, in the presence of dioxane (1 M) for 24 h, a time greater than the duration of column chromatography.

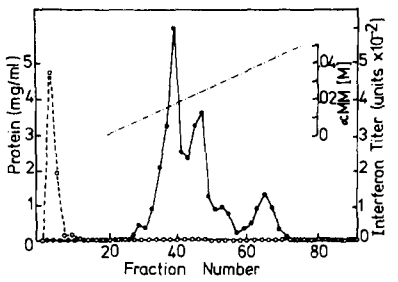

FIGURE 2: Elution of human fibroblast interferon from concanavalin A-agarose with a linear gradient of methyl α-D-mannopyranoside. An interferon preparation (8000 units and 15.8 mg of protein per ml), 1 ml, was applied on the column. The breakthrough fraction contained 96% of the applied protein. The interferon activity was recovered (99%) with a linear gradient (0 to 0.05 M) of α-MM in equilibrating solvent, containing ethylene glycol (10% [v/v]): (O - - - O) protein; (●–●) interferon; (·····) linear gradient of α-MM formed by mixing solvents; (A) 0.02 M sodium phosphate (pH 7.4) containing 1 M NaCl and 1.7 M ethylene glycol, 30 ml; and (B) 0.02 M sodium phosphate (pH 7.4) containing 1 M NaCl, 1.7 M ethylene glycol, and 0.05 M α-MM, 30 ml.

In order to establish the minimal concentration of α-MM effective in displacing fibroblast interferon, the experiment shown in Figure 2 was performed. The recovery of interferon activity was 100% within the 0.01–0.05 M range of α-MM concentration. The elution profile of interferon suggests the presence of discrete chromatographic fractions. In several experiments, using five different interferon preparations, it was established that the relative amount of activity recovered in a particular fraction could vary considerably. All preparations, however, contained several components, possibly four, as shown in Figure 2. Rechromatography of individual fractions was not undertaken.

Figure 3 represents a purification experiment of human fibroblast interferon on concanavalin A-agarose (Con A-Sepharose). A crude, unconcentrated preparation of interferon was used. The details of adsorption and elution conditions are given in the legend to Figure 3. In three separate experiments, using individually prepared interferon preparations, the specific activity of the interferon fraction recovered from the column with α-MM and ethylene glycol was no less than 5×10^7 units per mg of protein. This represents a 3000-fold purification. If the activity of interferon is corrected against the value of interferon standard, included as an internal control in the assay, then a value of 1×10^8 units per mg of protein is obtained.

Figure 4 illustrates the chromatographic behavior on concanavalin A-agarose (Con A-Sepharose) of human fibroblast and human leukocyte interferons, both induced by challenging cells with Newcastle disease virus. Human fibroblast interferon binds to the lectin column (Figure 4A), while human leukocyte interferon does not (Figure 4B). The recovery of fibroblast interferon is complete with the eluent containing 0.1 M α-MM and ethylene glycol (50% [v/v]). As both interferons were induced by the same in-

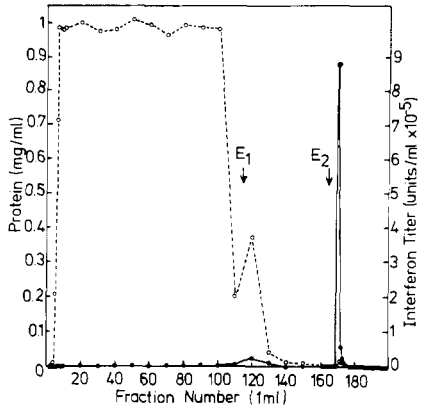

FIGURE 3: Purification of human fibroblast interferon on concanavalin A-agarose. Undialyzed interferon preparation, 100 ml, containing 11 500 units and 0.99 mg of protein per ml, was applied on a column by means of a peristaltic pump at a flow rate of 60 ml per cm^2 per h. The lectin column was equilibrated, with 0.02 M sodium phosphate (pH 7.4) containing 0.15 M NaCl. The eluent from the column was divided by a stream splitting device in a ratio of 1 to 9. The 10% portion of the eluent was collected into 1 ml of a 1% solution of bovine serum albumin, containing 0.02 M sodium phosphate and 0.15 M NaCl, and used to assay the interferon activity. The 90% portion of the eluent was used to measure the protein concentrations. Fractions from 1 to 20 and from 160 to 200 had 1 ml volume each; fractions from 20 to 160 were collected as 10-ml portions. The breakthrough fractions, 5–100, contained about 98% of the applied protein and less than 1% of the applied interferon activity. Further elution of the column was done with 0.1 M α-MM in 0.02 M sodium phosphate (pH 7.4) containing 0.15 M NaCl (E_1). This resulted in the recovery of 2% of the protein and about 10% of the interferon activity. The column was then washed with E_1 containing, in addition, ethylene glycol (50% [v/v]) and the remainder of the applied interferon activity (89%) was recovered (E_2). Fraction 171 contained 8.8×10^5 units of interferon activity (80% of the applied interferon) and 12.5 μg of protein; the specific activity is 7×10^7 units per mg (i.e., 1.8×10^8 units per mg relative to the international reference interferon which in this particular assay gave 1900 units): (O - - - O) protein; (●–●) interferon.

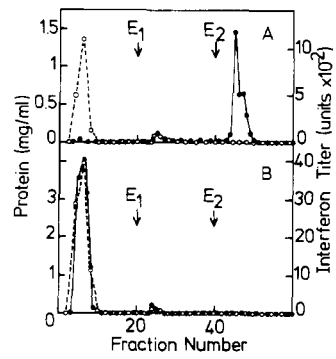

FIGURE 4: Chromatography of Newcastle disease virus induced human fibroblast and leukocyte interferons on concanavalin A-agarose. Interferon preparations were dialyzed against 0.02 M sodium phosphate (pH 7.4) containing 0.15 M NaCl, and applied on a column equilibrated with a solvent as described above for dialysis: (O - - - O) protein; (●–●) interferon. (A) Chromatography of fibroblast interferon. An interferon sample, 2 ml, containing 1400 units of interferon and 2.5 mg of protein per ml, was applied and the column was washed with equilibrating solvent. The breakthrough fraction contained about 98% of the applied protein and about 1% of the applied interferon activity. The column was then developed with 0.1 M α-MM in 0.02 M sodium phosphate (pH 7.4) containing 0.15 M NaCl (E_1) and finally with E_1 containing ethylene glycol (50% [v/v]) and denoted as E_2. Elution with E_1 resulted in displacement from the column of about 10% of the interferon activity. The remainder of the applied interferon activity (about 85%) was recovered with eluent E_2. (B) Chromatography of leukocyte interferon. An interferon sample, 5 ml, containing 3000 units of interferon activity and 3.2 mg of protein per ml, was applied and the column was washed with 0.02 M sodium phosphate (pH 7.4) containing 0.15 M NaCl. The breakthrough fraction contained about 98% of the applied protein and interferon activity. The column was then developed with 0.1 M α-MM in 0.02 M sodium phosphate (pH 7.4) containing 0.15 M NaCl (E_1). About 3% of the interferon activity was recovered with E_1. Elution of the column with ethylene glycol (50% [v/v]) in E_1 and denoted as eluent E_2 did not result in any recovery of protein or interferon activity.

ducer (virus) the difference in their interaction with concanavalin A-agarose must be related to the cell type used in induction. It may be recalled that $rI_n \cdot rC_n$-induced human fibroblast interferon displays the same binding characteristics (Davey et al., 1974) as presently reported for NDV-induced human fibroblast interferon.

All experiments described above were performed with concanavalin A-agarose (Con A-Sepharose) available from commercial sources. In view of the reports that coupling at high pH value (9.0–9.5) may result in conformational distortion of the coupled protein ligand (Cuatrecasas, 1972; Barel and Prieels, 1975), we proceeded to immobilize concanavalin A to cyanogen bromide activated agarose at pH values lower than those used (9.0–9.5) by commercial manufacturers.

Figure 5 illustrates the binding behavior of human fibroblast interferon to concanavalin A immobilized on an agarose matrix by coupling the lectin to agarose (CNBr activated) at pH 8.0 (Figure 5A) and at pH 9.0 (Figure 5B). All interferon activity was bound to the immobilized concanavalin A, regardless of the pH of the coupling reaction. The selectivity of binding of interferon was similar, if not identical, in both cases, as evidenced by the near total recovery of bulk proteins in the breakthrough fractions. The difference in binding of interferon to the lectin, immobilized either at pH 8.0 or 9.0, became conspicuous only upon subsequent elution of the columns. Namely, interferon could be recovered with 0.1 M α-MM from concanavalin A-agarose, coupled at pH 8.0. By contrast, only traces of interferon activity could be recovered with 0.1 M α-MM from concanavalin A-agarose, coupled at pH 9.0; complete recovery of interferon was accomplished only upon addition of ethylene glycol to the solvent containing 0.1 M α-MM (Figure 5B). Apparently, concanavalin A, coupled at pH 9.0, has different binding properties than when coupled at pH 8.0. To test further the possible importance of the pH value of the coupling reaction, we immobilized concanavalin A at pH 6.0. The coupling at this pH was performed in the presence and absence of metals (Mn^{2+}, Ca^{2+}, Mg^{2+}). The binding of interferon on columns equilibrated with either 0.02 M sodium phosphate (pH 7.4) (1 M NaCl) or with 0.1 M α-MM in 0.02 M sodium phosphate (pH 7.4) (1 M NaCl) was then studied. Results are illustrated in part in Figure 6. As expected, interferon was bound and could be eluted (90%) with 0.1 M α-MM (Figure 6A, E_1) from concanavalin A-agarose coupled at pH 6.0. The fraction of interferon eluted with 0.1 M α-MM containing ethylene glycol (Figure 6A, E_2), and amounting to 9% of applied activity, could be increased two- to threefold (not shown) if the coupling of concanavalin A to agarose was performed in the presence of metals. Moreover, all interferon activity could be recovered in the breakthrough fraction when concanavalin A-agarose, coupled at pH 6.0, was preequilibrated with 0.1 M α-MM (Figure 6B). This was not the case, for example, when concanavalin A-agarose, coupled at pH 9.0, was preequilibrated with 0.1 M α-MM (Figure 1). The influence of the metals on the binding properties of immobilized concanav-

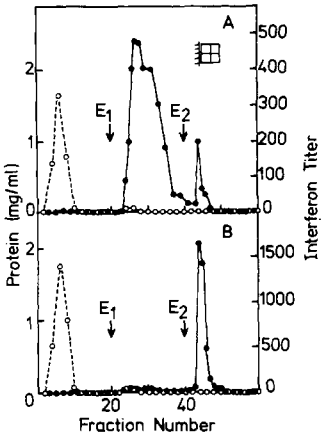

FIGURE 5: The chromatography of human fibroblast interferon on concanavalin A coupled to agarose at pH 8.0 and at pH 9.0 (Con A-Sepharose 4B, commercial). An interferon sample, 5 ml, containing 900 units of interferon activity and 2.4 mg of protein per ml, was applied on a column: (O - - - O) protein; (●–●) interferon. (A) Elution pattern of interferon from concanavalin A-agarose (coupled at pH 8.0). The breakthrough fraction contained 96% of the applied protein. Elution of the column with E_1 yielded 93% of the applied interferon activity and traces of protein. Further elution of the column with E_2 resulted in recovery of the remainder of interferon activity (7%). (B) Elution pattern of interferon from concanavalin A-agarose (coupled at pH 9.0). The breakthrough fraction contained 94% of the applied protein. Elution of the column with E_1 yielded traces of protein and about 3% of the applied interferon activity. Further elution of the column with E_2 resulted in recovery of 98% of the applied interferon activity.

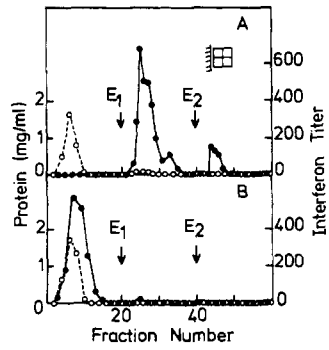

FIGURE 6: The chromatography of human fibroblast interferon on concanavalin A-agarose (coupled at pH 6.0). An interferon sample, 5 ml, containing 650 units of interferon and 2.4 mg of protein per ml, was applied on a column: (O - - - O) protein; (● - ●) interferon. (A) Elution pattern of interferon from concanavalin A-agarose (coupled at pH 6.0). The breakthrough fraction contained 98% of the applied protein. Elution of the column with E_1 resulted in recovery of 90% of applied interferon activity. Elution of the column with E_2 resulted in recovery of an additional 9% of interferon activity. (B) Chromatography of interferon on concanavalin A-agarose (coupled at pH 6.0) preequilibrated with methyl α-D-mannopyranoside. The interferon sample was applied on the lectin column equilibrated with 100 ml of E_1 and the column was washed with E_1. The breakthrough fraction contained 99% of the applied protein and 100% of the applied interferon activity. Elution of the column with E_2 resulted in no further recovery of interferon activity or protein.

alin A may deserve a separate detailed study. However, it was the omission of metals from the coupling reaction which led to elimination of the hydrophobic binding properties of this immobilized lectin. In a subsequent study we found that the presence, or absence, of metals was without any noticeable effects on the chromatographic binding properties of concanavalin A immobilized via molecular arms. Our experimental effort was therefore directed toward other means of immobilizing concanavalin A and the investigation of its binding properties. To this end, concanavalin A was immobilized on an agarose matrix via a molecular arm by coupling both to Affi-Gel 10 and CH-Sepharose 4B. Both couplings were done at pH 6.0 and, therefore, concanavalin A was presumably immobilized to a significant extent in its tetrameric form. Figure 7 illustrates the binding characteristics of both adsorbents. Interferon was partially adsorbed to a concanavalin A-Affi-Gel 10 column (Figure 7A). The adsorbed portion of activity could be recovered with 0.1 M α-MM, and, therefore, presumably was bound to the immobilized lectin only due to carbohydrate recognition. The presence of interferon in the breakthrough fraction was not due to overcharging the column as this fraction would also not bind to a freshly prepared column. The interferon molecule displayed only residual recognition of concanavalin A immobilized on CH-Sepharose 4B (Figure 7B). When mouse kidney β-glucuronidase was applied to columns prepared from the same batch of immobilized lectin, there was a selective adsorption and elution of activity with α-MM.[3] In several additional couplings using different batches of concanavalin A and CH-Sepharose 4B, we arrived at the same conclusion: namely, the inability of concanavalin A-CH-Sepharose 4B, coupled at pH 6.0, to bind interferon.

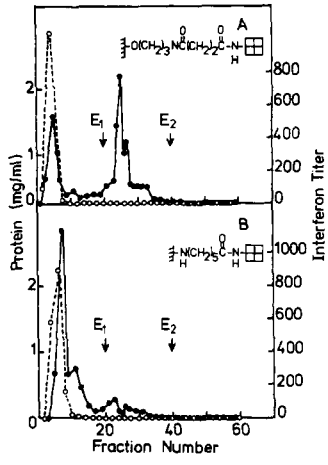

FIGURE 7: Chromatography of human fibroblast interferon on tetrameric concanavalin A coupled to agarose through a molecular arm. An interferon sample, 5 ml, containing 1100 units of interferon activity and 2.3 mg of protein per ml, was applied on a column: (O - - - O) protein; (● - ●) interferon. (A) Elution pattern of interferon from concanavalin A immobilized on Affi-Gel 10. The breakthrough fraction contained 95% of the protein and 51% of the interferon activity. Elution of the column with E_1 yielded the remaining interferon activity (50%) and traces of protein. Further elution of the column with E_2 resulted in no further recovery of interferon or protein. (B) Elution pattern of interferon from concanavalin A immobilized on CH-Sepharose. The breakthrough fraction contained 98% of the protein and about 94% of the interferon activity. The remaining interferon activity (3%) was eluted with E_1. Further elution of the column with E_2 resulted in no further recovery of interferon or protein.

The "breakthrough" fraction of interferon (tubes 1–10) (Figure 7A) and the fraction eluted with α-MM (tubes 20–30), termed the "E_1" fraction (Figure 7A), were pooled individually, dialyzed, and rechromatographed on fresh concanavalin A-Affi-Gel 10 columns (not illustrated). The chromatographic behavior of both fractions, breakthrough and E_1 fraction, remained unchanged, and, therefore, their

[3] The chromatographic behavior of mouse kidney β-glucuronidase was kindly tested by Dr. Ross A. Davey.

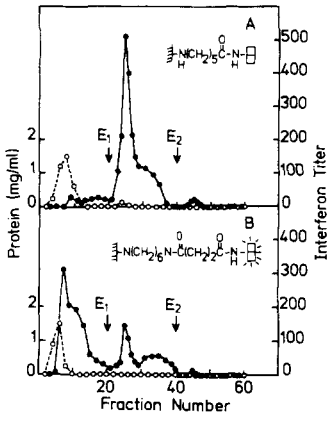

FIGURE 8: Chromatography of human fibroblast interferon on dimeric concanavalin A coupled to agarose through a molecular arm. An interferon sample, 5 ml, containing 600 units of interferon and 2.2 mg of protein per ml, was applied on a column: (O - - - O) protein; (● - ●) interferon. (A) Elution pattern of interferon from concanavalin A immobilized on CH-Sepharose. The breakthrough fraction contained 98% of the applied protein and about 8% of the interferon activity. Elution of the column with E_1 yielded 92% of the interferon activity and traces of protein. Further elution of the column with E_2 resulted in trace amounts (about 1%) of interferon activity B, elution pattern of interferon from succinylated concanavalin A immobilized on AH-Sepharose. The breakthrough fraction contained all of the applied protein (101%) and about 70% of interferon activity. Elution of the column with E_1 resulted in the recovery of the remaining interferon activity (28%). Further elution of the column with E_2 did not yield any protein or interferon.

disparate binding behavior must not be caused by a nonspecific leakage of interferon activity, e.g., due to limited capacity of the column used in the experiment described above (Figure 7A).

The pH dependence of the oligomeric structure of concanavalin A allows the immobilization of the lectin in either of two forms: as a tetramer or as a dimer (Nicolson, 1974). Concanavalin A exists as a dimer at pH 4.5, and it may be immobilized as such to CH-Sepharose by carbodiimide coupling. The product of coupling will be an unmodified dimer-CH-Sepharose 4B, and will be referred to as dimeric Con A-CH-Sepharose. A dimer of concanavalin A can be also prepared by its succinylation (Gunther et al., 1973). Succinylated dimers do not oligomerize to tetramers upon an increase in the pH values of the solvent; succinylated dimers of concanavalin A were immobilized on AH-Sepharose 4B and the product will be referred to as succinylated Con A-AH-Sepharose. The chromatographic behavior of human interferon on dimeric Con A-CH-Sepharose is illustrated in Figure 8A. Most of applied interferon activity (92%) was retained on the column, while most of the applied protein (98%) was recovered in the breakthrough fraction. When the column was developed with α-MM, the retained interferon activity was completely displaced from the column (Figure 8A, E_1). The chromatographic behavior of human interferon on succinylated Con A-AH-Sepharose is illustrated in Figure 8B. The breakthrough fraction contained all of the applied protein and about 70% of the interferon activity. The remaining interferon activity was recovered by developing the column with α-MM. When mouse kidney β-glucuronidase[3] was applied on a similar column, all of the activity was bound and recovered upon subsequent elution with α-MM. Thus, in both cases, as illustrated in Figure 8A and Figure 8B, interferon could be recovered completely with α-MM alone; there was no interferon activity displaced upon the addition of a hydrophobic solute to the eluent containing α-MM (Figure 8A, E_2, and Figure 8B, E_2).

Discussion

Human fibroblast interferon binds to a concanavalin A-agarose column equilibrated with α-MM (Figure 1A) or with levan. As both the monosaccharide and, in particular, the polysaccharide are expected to block saccharide binding sites on the lectin, one has to postulate the involvement of forces other than sugar recognition as responsible for the binding of interferon. In addition, ionic strength of the solvent seems to have only marginal effects on the adsorption and elution profile of interferon and, thus, electrostatic forces cannot account for its binding to the immobilized lectin. In contrast, application of interferon preparations on columns equilibrated with ethylene glycol resulted in a gross leakage of interferon activity (Figure 1B), while in the presence of both (α-MM and ethylene glycol) there was in fact no retention of interferon on the column.

Apparently, the combined action of α-MM and ethylene glycol can account for the forces involved in the binding of interferon to immobilized concanavalin A. We postulate, therefore, that the major binding forces are carbohydrate recognition and hydrophobic interaction. The failure of D-galactose to substitute for α-MM or D-mannose in the eluent provides the necessary element of specificity for the postulated carbohydrate recognition. The effect of inclusion of ethylene glycol in the eluent is assumed to result from the weakening of hydrophobic interaction between interferon and the immobilized lectin. Similar effects were observed with other hydrophobic solutes tested, such as dioxane, propylene glycol, and tetraethylammonium chloride, all known to disrupt water structure and to weaken the hydrophobic interactions (Frank and Evans, 1945; Némethy and Sheraga, 1962).

Hydrophobic solutes could induce conformational changes in the lectin, interferon, or both, resulting in a decreased affinity for carbohydrate residues. An alternative or complementary explanation is that of a direct effect on the hydrophobically interacting side chains of amino acid residues located on the surface of the lectin and their counterparts on the interferon molecules. The choice between any of these, or even other conceivable models, is not possible at present. For the purpose of evaluating the data reported in this communication, it is sufficient to observe that a hydrophobic solute, such as ethylene glycol, does not eliminate carbohydrate recognition as an operating affinity principle in chromatography on concanavalin A-agarose (Con A-Sepharose). It enables, however, displacement of a glycoprotein, as exemplified here by human fibroblast interferon, from an immobilized lectin, without the necessity of resorting to harsh deforming agents (sodium dodecyl sulfate, urea) or extreme pH values of the solvent. It is conceivable that in other cases, where the binding cannot be readily reversed, the inclusion of a mild hydrophobic solute will prove to be sufficient to displace a glycoprotein bound to a lectin.

Human fibroblast interferon was found previously to be eluted from a concanavalin A-agarose (Con A-Sepharose) column—with ethylene glycol alone—as a population of two distinct fractions, while about one-third of the total applied amount remained on the column (Davey et al., 1974). When the interferon preparation is applied on a column preequilibrated with ethylene glycol (Figure 1B), one observes again two fractions being eluted with ethylene glycol and a third one upon addition of α-MM to the solvent.

About one-fifth of the activity has not been recovered and it is not clear if it represents a separate component. When interferon preparations are applied on columns preequilibrated with both α-MM and ethylene glycol, all of the interferon activity is recovered in the breakthrough fraction; this eliminates physical entrapment as a source of apparent heterogeneity of the interferon preparation.

The data presented in Figure 2 indicate the presence of several, possibly four, components in our interferon preparations, as revealed by chromatography with a linearly increasing concentration of mannoside. The same elution pattern was observed consistently with five different interferon preparations, although the relative amount of interferon activity in a particular chromatographic fraction varied; hence, the observed heterogeneity is probably not a mere chromatographic artifact. As the columns were prepared from fresh adsorbent each time (the same batch of Con A-Sepharose) the quantitative variations in chromatographic fractions have to be traced to interferon preparations. Rechromatography of individual fractions should provide further comment on the observed heterogeneity.

Our previous communication established the glycoprotein nature of human fibroblast interferon (Davey et al., 1974). It was of interest, therefore, to test the chromatographic behavior of human leukocyte interferon on concanavalin A-agarose (Con A-Sepharose). The presently reported finding that human leukocyte interferon does not bind to concanavalin A-agarose (Figure 4) suggests the absence, or masked status, of carbohydrate moieties recognizable by this lectin. Further investigation, now in progress, of the chromatographic behavior of both fibroblast and leukocyte interferons on other immobilized lectins should add to our appreciation of the extent of difference in glycosylation of both of the interferons. The observed heterogeneity of fibroblast interferon in concanavalin A-agarose chromatography might indicate the varied extent of posttranslational modification (glycosylation) of fibroblast interferon. The observed behavior of leukocyte interferon on concanavalin A-agarose may constitute evidence that glycosylation of leukocyte interferon is significantly different from that of fibroblast interferon.

It may be in order to comment briefly on the purification of fibroblast interferon on concanavalin A-agarose (Con A-Sepharose). The data illustrated in Figure 3 indicate a purification factor of about 3000-fold and complete recovery of activity. Under the experimental conditions used the recovered activity is not subdivided into several fractions and, therefore, the procedure may be useful as a preliminary step in the purification scheme. Furthermore, the binding capacity of concanavalin A-agarose is high enough to process large volumes of crude interferon preparations. The stability of bound interferon compares favorably with that in solution, and therefore the desired amount of interferon may be accumulated on the same column at consecutive time intervals and finally eluted for further treatment. The availability of immobilized lectin from commercial sources allows the procedure to be readily adapted in different laboratories. The efficiency of the procedure, as measured by the purification factor, recovery, and its adaptability for processing large amounts of material, compares favorably with immunoadsorbent chromatography.

The potential of human fibroblast interferon for hydrophobic interactions has now been documented with high molecular ligands, such as serum albumin (Huang et al., 1974), and low molecular ligands, such as 6-aminocaproic acid (Davey et al., 1975), both immobilized on CNBr-activated agarose matrix. The existence of a hydrophobic binding site, in addition to a sugar binding site, has also been postulated for concanavalin A (Poretz and Goldstein, 1971; Yang et al., 1974). Although the exact localization of both sites (or subsites) remains a matter of controversy (Becker et al., 1975), there seems to be evidence that concanavalin A has a hydrophobic binding site adjacent to its saccharide binding site when in solution. In view of these observations, one might entertain the notion that the intrinsic hydrophobicity of both reactants, namely, human fibroblast interferon and concanavalin A, is the source of the strong hydrophobic interaction measurable when human fibroblast interferon is applied to a concanavalin A-agarose column.

We should, however, not disregard the possibility that the potential for hydrophobic interaction on the part of concanavalin A is not an intrinsic property but may be, in fact, generated by conformational distortion of the lectin molecule as a result of its mode of immobilization. If this were the case, then the mode of immobilization would provide an experimental means to correlate any expected conformational distortion with specific binding characteristics of the immobilized lectin. For instance, one could expect that the binding of the lectin under conditions favoring multipoint attachment would result in partial unfolding of the lectin molecule. The resulting exposure of the hydrophobic interior of the molecule would ultimately lead to a hydrophobic interaction with a suitable counterpart molecule—that is, one having intrinsic hydrophobicity. Alternatively, the binding of the lectin molecule under conditions favoring one-point attachment—such as low pH of the coupling reaction to CNBr-activated agarose or immobilization via molecular arms—would result in minimal, if any, conformational distortion of the lectin molecule. By comparing the binding characteristics of concanavalin A immobilized in these diverse ways, one should develop information on the origin of the hydrophobic interaction displayed by immobilized concanavalin A toward the human fibroblast interferon molecule.

The results (Figure 5) show that the pH value of the coupling reaction is of critical importance for the binding characteristics of this immobilized lectin. For example, concanavalin A coupled at pH 8.0 to CNBr-activated agarose binds human fibroblast interferon in a distinctly different manner than when coupled at pH 9.0; namely, the binding of interferon can be reversed with α-MM alone. This observation strongly indicates that the forces involved in binding are due to carbohydrate recognition alone, a conclusion further supported by further experiments (Figure 6). The concanavalin A-agarose used in these experiments was coupled at pH 6.0; as expected, interferon could be recovered with α-MM alone (Figure 6A). More succinctly, interferon did not bind to the lectin column which had been preequilibrated with α-MM (Figure 6B). This result was in distinct contrast to the binding characteristics of concanavalin A-agarose, coupled at pH 9.0. In the latter case, interferon did bind to the lectin column in spite of its preequilibration with α-MM (Figure 1).

Apparently, when concanavalin A is immobilized at pH 6.0, no gross distortion of its conformation takes place and, as a result, no potential for hydrophobic interaction with interferon is generated. It follows then that by blocking saccharide binding sites with α-MM, no additional binding forces toward interferon are functional. By contrast, when concanavalin A is immobilized at pH 9.0, a distortion of its

conformation takes place and, as a result, a capacity for hydrophobic interaction with interferon is developed. It follows then that interferon binds to immobilized concanavalin A despite blocking of its saccharide binding site by preequilibration with α-MM.

Having established that concanavalin A can be bound directly to an agarose matrix (CNBr activated) and will display exclusive carbohydrate recognition toward interferon, we then proceeded with its immobilization via molecular arms. Concanavalin A, in its tetrameric form, was immobilized on Affi-Gel 10 (Figure 7A) and on CH-Sepharose (Figure 7B). In both cases, all bound interferon activity could be recovered by developing the column with α-MM. Unexpectedly, part of the interferon activity did not bind to concanavalin A-Affi-Gel 10 (Figure 7A). The leakage, however, was apparently not due to a limited capacity of the column; this was evidenced by rechromatography of the breakthrough and bound E_1 fractions on similar columns. This result is again indicative of the heterogeneity in interferon preparations (Figures 1 and 2).

The breakthrough fraction of interferon activity (Figure 7A) was observed only in this particular chromatographic system, namely, concanavalin A being immobilized on Affi-Gel 10. The same preparation of interferon, when applied to concanavalin A-agarose immobilized either at pH 8.0 or 6.0, was totally bound (Figures 5 and 6A). Therefore, one cannot explain the lack of binding as the result of the existence of a subpopulation of interferon molecules devoid of carbohydrate moieties recognizable by this lectin. Also, we cannot exclude the possibility that there is a form of steric hindrance which prohibits binding of interferon molecules to concanavalin A immobilized on Affi-Gel 10. This may be the case, in particular, when concanavalin A is immobilized on CH-Sepharose (Figure 7B). In a control experiment (not shown), mouse kidney β-glucuronidase was bound to both concanavalin A-Affi-Gel 10 and concanavalin A-CH-Sepharose 4B columns[3] suggesting that the lack of binding of interferon may be rather an exception than the rule; further study with other glycoproteins should provide additional comments on the binding characteristics of concanavalin A immobilized in this particular way.

To characterize further the binding characteristics of concanavalin A immobilized on CH-Sepharose 4B, the coupling reaction was also performed at pH 4.5, a condition which should result in dimeric concanavalin A as the product. Figure 8A illustrates the binding properties of this dimeric Con A-CH-Sepharose 4B adsorbent. Although there was some leak of interferon activity, most of it was bound and could be completely recovered with α-MM alone. Apparently, any possible steric hindrance, as observed with the immobilized tetrameric lectin, was not observed with the immobilized dimeric lectin. The salient point of the experiment was, however, again the exclusive carbohydrate-mediated binding of the interferon molecule.

A chemically modified, stable, dimer of concanavalin A was also prepared and immobilized on AH-Sepharose 4B. We assume that its immobilization took place through succinic acid residues introduced by treatment of the tetrameric lectin with succinic acid anhydride, although this may not have been the exclusive mode of attachment. Even though there was a considerable selectivity of retention of interferon activity with respect to "bulk" proteins in the breakthrough fraction, the interaction of interferon with succinylated Con A-AH-Sepharose was only transient. Again, the portion of the retained interferon activity could be recovered with α-MM alone. Mouse kidney β-glucuronidase behaved similarly, being completely bound and then recovered by developing the column with α-MM.[3] Apparently, the accessibility of the saccharide binding site of the succinylated immobilized dimer of this lectin may vary from one glycoprotein to another.

In summary, concanavalin A can be immobilized on an agarose matrix and will display exclusively carbohydrate recognition related affinity toward human fibroblast interferon and possibly other glycoproteins. Apparently, immobilization of the lectin at a relatively low pH value to CNBr-activated agarose, or via molecular arms, does not result in partial unfolding of the coupled protein and, therefore, in potential for hydrophobic interactions.

Some glycoproteins, when avidly bound to Con A-Sepharose, may be discharged by the addition to the eluent (α-MM) of a mild hydrophobic solute (Davey et al., 1974). Now it seems plausible that by appropriate immobilization of concanavalin A, even this requirement can be eliminated. Thus, one can anticipate that "irreversibly" bound glycoproteins could be successfully chromatographed on modified concanavalin A-agarose adsorbents, their full recoveries being ensured simply with α-MM alone.

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Biological Activity of 1,25-Dihydroxyvitamin D_2 in the Chick†

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ABSTRACT: 1,25-Dihydroxyvitamin D_2 has been prepared from 25-hydroxyvitamin D_2 using rachitic chick kidney mitochondria. This metabolite was highly purified by Sephadex LH-20 chromatography and by preparative high-pressure liquid chromatography. Its purity was assessed by analytical high-pressure liquid chromatography which revealed no other 254-nm absorbing material and by mass spectrometry. The concentration of dilute solutions of 1,25-dihydroxyvitamin D_2 was determined by high-pressure liquid chromatography and deflection of the 254-nm column monitor. The 1,25-dihydroxyvitamin D_2 was then shown to be $\frac{1}{5}$ to $\frac{1}{10}$ as active as 1,25-dihydroxyvitamin D_3 in the chick while it had previously been shown to be equal in activity in the rat. Thus, discrimination against the vitamin D_2 side chain by the chick persists in the metabolically active 1,25-dihydroxyvitamin D compounds.

The discrimination between vitamin D_3 and vitamin D_2 that has been observed in chicks (Steenbock et al., 1932; Chen and Bosmann, 1964) is thought to be due to the rapid excretion of vitamin D_2 and its metabolites by the biliary route (Imrie et al., 1967). In support of this theory are the extremely low levels of 25-hydroxyvitamin D_2 (25-OH-D_2)[1] (Drescher et al., 1969) and 1,25-dihydroxyvitamin D_2 (1,25-$(OH)_2D_2$) (Jones et al., 1975) that are observed in the rachitic chick after the in vivo administration of physiological doses of radioactive vitamin D_2. Using in vitro techniques we were able to show that these reduced levels of hydroxylated vitamin D_2 metabolites are not due to a failure of the enzymic machinery to carry out the reactions (Jones et al., 1976). In this present paper we now show that 1,25-$(OH)_2D_2$ is only $\frac{1}{5}$ to $\frac{1}{10}$ as active as 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in the chick, demonstrating that the mechanism of discrimination applies also to the metabolically active form.

Materials and Methods

Chemicals. 25-OH-D_2 was prepared by the method of Suda et al. (1969) as modified by Jones et al. (1975). 1,25-$(OH)_2D_3$ was the synthetic material prepared by Semmler et al. (1972).

General Procedures. High-pressure liquid chromatography (HPLC) was performed on a Dupont 830 LC fitted with a 254-nm ultraviolet (uv) detector (Dupont Instruments, Wilmington, Del.) and a U6K injector (Waters Associates, Milford, Mass.). When two analytical columns (25 cm × 2.1 mm) containing Zorbax-SIL (Dupont) were arranged in series, a pressure of 4400 psi gave a solvent (15% 2-propanol–Skellysolve B) flow rate of 0.8 ml/min. Using a semipreparative column of Zorbax-SIL (25 cm × 7.9 mm) a flow rate of 1.8 ml/min (15% 2-propanol in Skellysolve B) was achieved with a pressure of 500 psi.

Mass spectrometric determinations were made with an AEI MS-9 mass spectrometer using a direct probe inlet at temperatures of 118–130 °C above ambient. All solvents were reagent grade and those used in the HPLC and mass spectrometry were doubly distilled before use.

Radioactive determinations were carried out with a Nu-

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[1] Abbreviations used are: 25-OH-D_2, 25-hydroxyvitamin D_2; 1,25-$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; HPLC, high-pressure liquid chromatography.