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BINDING OF DNA TO ALBUMIN AND TRANSFERRIN MODIFIED BY TREATMENT WITH WATER-SOLUBLE CARBODIIMIDES

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Abstract—*N*-Acylurea derivatives of albumin and transferrin prepared with the water-soluble carbodiimides *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide and *N*-ethyl-*N'*-(3-trimethylpropylammonium)carbodiimide iodide have been found to bind different types of DNA. The two proteins were reacted with varying amounts of carbodiimide in water at pH 5.5 for 36–60 hr at 20°, and then purified. In the case of iron-loaded transferrin, reactions with carbodiimides were in phosphate-buffered saline (pH 7.5) to prevent loss of iron from the protein. [^{3}H]*N*-Ethyl-*N'*-(3-trimethylpropylammonium)carbodiimide iodide was used for the determination of covalently attached *N*-acylurea groups in the modified proteins, and gel electrophoresis for changes in charge and possible aggregation through cross-linking. Binding of DNA to *N*-acylurea proteins was studied by means of agarose gel electrophoresis and nitrocellulose filter binding. *N*-Acylurea albumin and *N*-acylurea transferrin at low concentrations retarded the migration of λ-PstI restriction fragments, pBR322 plasmid and M13 mp8 single-standed DNA on agarose gels, while at higher concentrations of modified protein the *N*-acylurea protein–DNA complexes were unable to enter the gel. Nitrocellulose filter assays showed that binding of pBR322 DNA and calf thymus DNA to *N*-acylurea proteins is rapid and dependent on protein concentration and the ionic strength of the medium. *N*-Acylurea albumins prepared with each of the two carbodiimides gave comparable plots for DNA bound versus protein concentration. On the other hand, binding of DNA by *N*-acylurea transferrins differed according to the carbodiimide used in the synthesis. *N*-Acylurea CDI-transferrin (prepared with tertiary carbodiimide) was less effective than either of the two *N*-acylurea albumins in binding DNA. In contrast with these results, *N*-acylurea Me$^+$CDI-transferrin (prepared with quaternary carbodiimide) was far more effective in binding DNA and in this respect was similar to the *N*-acylurea albumins. On the basis of experiments in which *N*-acylurea protein–DNA complexes were treated with heparin, two types of binding could be distinguished. These were (i) a weak binding occurring in the initial stages of interaction and (ii) a tight binding which developed on further incubation of the complexes. These studies show that binding of DNA by *N*-acylurea proteins is a reversible process dependent on ionic strength; interaction appears to be electrostatic in nature, although other forms of binding might be involved. The possible use of *N*-acylurea proteins for DNA transfer into cell systems is discussed.

A well established procedure for raising antibodies to drugs such as 9-β-D-arabinofuranosylcytosine (ara-C) for the purpose of radioimmunoassay is to use as the immunogen ara-C covalently conjugated to a protein such as albumin. In order to obtain specificity for the arabinose moiety of the ara-C, the 5'-*O*-succinyl derivative of the nucleoside is coupled to lysine side chains of the albumin by means of the water-soluble carbodiimide *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (CDI)† [1, 2]. Carbodiimide-mediated coupling is a general procedure for linking many different types of hapten to carrier proteins [3] and is popular because of its simplicity of operation and efficiency under mild conditions.

During experiments concerned with the preparation of nucleoside–albumin conjugates using CDI as the coupling agent, we also reacted the protein with the carbodiimide in the absence of nucleoside to obtain the *N*-acylurea substituted protein [4–6]. This was used for certain studies during the course of which we observed that the *N*-acylurea protein was able to bind different types of DNA. This observation is interesting and has led us to investigate the mechanism of the interaction between carbodiimide-modified proteins and DNA and also to enquire whether we could modify proteins such as transferrin, asialo α_1-acid glycoprotein and others to bind DNA without altering their interaction with specific cell surface receptors. Receptor-mediated endocytosis of the protein (ligand)–DNA complexes might be possible, thus allowing selective transfer of DNA into cells [7, 8].

This work describes (i) initial experiments on the modification of bovine serum albumin and human serum transferrin by the water-soluble carbodiimides *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide HCl (CDI) and *N*-ethyl-*N'*-(3-trimethylpropylammonium)carbodiimide iodide (Me$^+$CDI), a CDI derivative which contains a positively charged quaternary nitrogen (Fig. 2), and (ii) a study of the interaction of these modified proteins with different classes of DNA.

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† *Abbreviations:* CDI, *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide; Me$^+$CDI, *N*-ethyl-*N'*-(3-trimethylpropylammonium) carbodiimide iodide; PBS, phosphate-buffered saline; SSC, saline sodium citrate.

MATERIALS AND METHODS

Materials. Bovine serum albumin, human serum transferrin, heparin, calf thymus DNA and DNase 1 (EC 3.1.21.1) were obtained from the Sigma Chemical Co., St Louis, MO. Restriction enzyme PstI (EC 3.1.23.31), pBR322 DNA, λ DNA and DNA polymerase 1 (EC 2.7.7.7) were from Boehringer–Mannheim. [^{3}H]Methyl iodide (specific radioactivity, 85 Ci/mmol), [^{3}H]dTTP (specific radioactivity, 97 Ci/mmol) and M13 mp8 single-stranded DNA were from Amersham, U.K. *N*-Ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide HCl (CDI) was from Merck. Agarose (ultra pure grade), acrylamide and bis-acrylamide were obtained from BioRad. DEAE Sephacel and Sephadex G-50 were from Pharmacia. All other reagents were of Analar grade.

Sheared calf thymus DNA. Calf thymus DNA (approximately 12 kb in size) was prepared by passing a solution of DNA (0.1 mg/ml) in 0.1 SSC (SSC, 0.15 M NaCl, 0.015 M trisodium citrate) through a 25G-Yale syringe needle six times.

[^{3}H]*calf thymus and pBR322 DNA*. These were labelled by the nick translation procedure [9] and the DNA purified by the spun-column method [10] using Sephadex G-50.

[^{3}H]calf thymus DNA, 6.5×10^6 c.p.m./μg. [^{3}H]-pBR322 DNA, 7.6×10^6 c.p.m./μg.

Protein determination. Protein was determined by the method of Lowry [11] using either bovine serum albumin or human serum transferrin as standard.

[^{3}H]N-*Ethyl*-N'-(3-*trimethylpropylammonium*)-*carbodiimide iodide*. The quaternary carbodiimide was prepared essentially according to the method of Kopczynski and Babior [12], with minor changes, from the hydrochloride salt of CDI (500 mg, 2.62 mmol) and methyl iodide (0.71 g, 5 mmol) containing 250 μCi [^{3}H]methyl iodide. The hygroscopic product (520 mg, 1.76 mmol) with a specific radioactivity of 44 μCi/mmol was stored in the dark over P_2O_5 at −15°.

Modification of proteins with carbodiimides. Albumin and transferrin respectively were treated with either CDI or Me$^+$CDI at various carbodiimide to protein mole ratios which ranged from 10:1 to 2000:1. A similar reaction procedure was followed in each case. The following procedure is for a typical reaction of CDI with transferrin at a mole ratio of 500:1.

N-Ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide HCl (15 mg, 80 μmol) in 1.5 ml of water was added to an aqueous solution (1.3 ml) of transferrin (12 mg, 0.16 μmol) and the clear reaction mixture carefully adjusted to pH 5.5 by the addition of dilute HCl. The mixture was allowed to stand at room temperature (18–20°) for 48–60 hr in the dark and then dialysed exhaustively against 0.05 M NaCl containing 0.3 mM EDTA (pH 7.0) at 5°. Aliquots of the dialysed product, *N*-acylurea CDI-transferrin, were stored at −15°.

Purification of the *N*-acylurea protein conjugates can be achieved by chromatography on small columns of DEAE-cellulose (Sephacel) of dimensions 1.4 × 12 cm using 0.05 M NaCl containing 0.01 M Tris–HCl (pH 7.0) as the eluent. *N*-Acylurea proteins are not retained on the column under these conditions in contrast with the unmodified proteins.

N-*Acylurea* Me$^+$*CDI*-(Fe^{3+})*transferrin*. Transferrin (4 mg, 0.04 μmol) saturated with Fe^{3+} ion [13] in 0.6 ml of phosphate-buffered saline (PBS) (pH 7.5) was added to *N*-ethyl-*N'*-(3-trimethylpropylammonium)carbodiimide iodide (7.35 mg, 24.7 μmol) dissolved in 0.8 ml water. The reaction mixture (pH 7.5) was incubated at 20° for 36 hr in the dark. It was then dialysed exhaustively against 0.1 M NaCl at 5° and stored at −15°.

In an alternative procedure, iron-free transferrin was reacted with *N*-ethyl-*N'*-(3-trimethylpropylammonium)carbodiimide iodide in PBS (pH 7.5) under identical conditions to those described above for the iron-saturated protein. It was dialysed against 0.1 M NaCl and then treated with ferric citrate before storage. In each of these alternative procedures the reaction mixtures represent a carbodiimide to protein mole ratio of 500:1.

Analysis for iron was by the α-dipyridyl method [14] and spectra (λ_{max} at 465 nm).

Number of N-*acylurea groups attached to proteins*. For this determination, transferrin or albumin was reacted with [^{3}H]*N*-ethyl-*N'*-(3-trimethylpropylammonium)carbodiimide iodide exactly as described above for the various *N*-acylurea protein preparations. Reaction was either in water at pH 5.5 or PBS at pH 7.5. For radioactive counting, aliquots of the modified transferrins were precipitated with an equal volume of cold 10% (w/v) TCA, filtered on Whatman GF/C filters, washed with 25 ml of cold 5% (w/v) TCA, dried and counted in scintillation fluid (Beckman HP/b). In the case of the modified albumin preparations, precipitation was with cold 60% (w/v) TCA and subsequent washing with cold 30% (w/v) TCA; all other aspects of treatment were the same as for the transferrins.

Agarose gel electrophoresis of N-*acylurea protein–DNA complexes*. *N*-Acylurea proteins were incubated for 20 min at 18–20° with one of the following: λ-PstI restriction fragments, pBR322 DNA or M13 mp8 DNA. Each reaction mixture contained in a final volume of 10 μl: λ-PstI (0.48 μg DNA) or pBR322 (0.33 μg DNA) or M13 mp8 (0.28 μg DNA), a variable concentration of *N*-acylurea protein, 6.6 mM Tris–HCl (pH 7.0), 3 mM sodium phosphate, 100 mM NaCl, 2 mM $MgCl_2$ and 0.1 mM EDTA. The incubated samples (*N*-acylurea protein–DNA complexes), as well as DNA controls, were mixed with a stop solution containing sucrose, urea, bromophenol blue and EDTA, then run in 1.2% agarose gels with a buffer containing 36 mM Tris–HCl, 30 mM sodium phosphate and 10 mM EDTA (final pH 7.5). Gels were stained with ethidium bromide (1.5 μg/ml).

Agarose gel electrophoresis of proteins. Proteins and *N*-acylurea proteins were mixed with stop solution and run in 1.2% agarose gels as described above. Gels were stained with Coomassie Brilliant Blue in methanol–acetic acid–water (5:1:5, v/v/v).

SDS–polyacrylamide gel electrophoresis. Proteins and *N*-acylurea proteins were analysed by SDS–polyacrylamide gel electrophoresis according to Laemmli [15].

Nitrocellulose filter binding assay. Binding assays

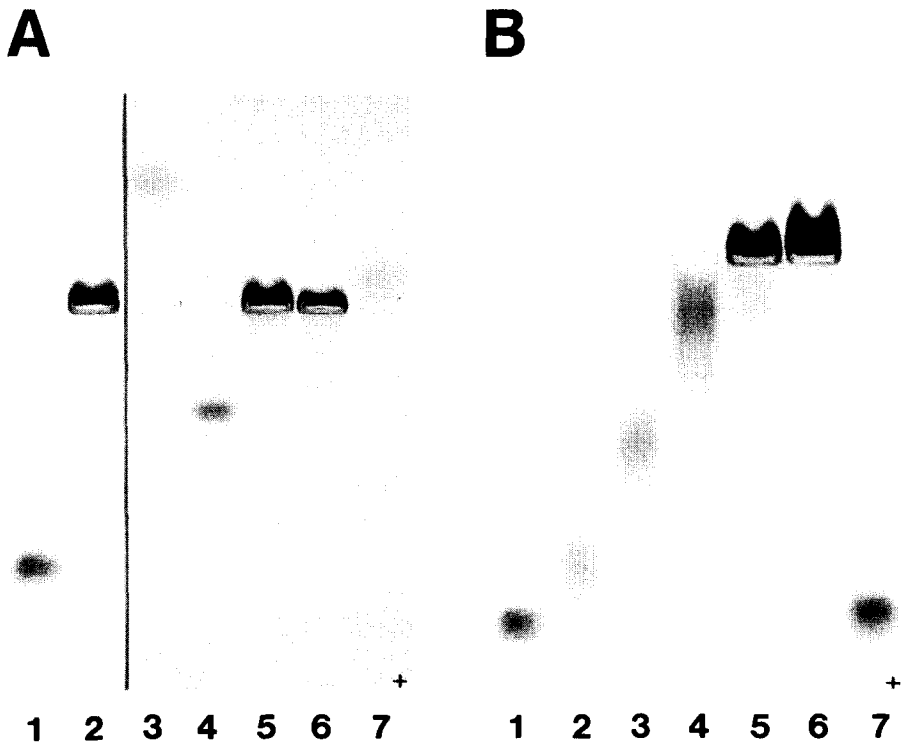

Fig. 1. Agarose gel electrophoresis of proteins and proteins modified with *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide. Protein load, 10–15 μg per well. (A) *N*-Acylurea proteins prepared at a carbodiimide to protein mole ratio of 500:1. 1, albumin; 2, *N*-acylurea CDI-albumin; 3, cytochrome C; 4, transferrin; 5 and 6, different amounts of *N*-acylurea CDI-transferrin and 7, RNase A. (B) *N*-Acylurea albumins prepared at a range of carbodiimide to protein mole ratios. 1, albumin; 2–6, *N*-acylurea albumins prepared at ratios of 10:1, 50:1, 100:1, 250:1 and 500:1 respectively; 7, albumin.

were carried out in the following manner: Each reaction mixture contained the following in a final volume of 200 μl: [^{3}H]calf thymus DNA (0.01 μg, 6.5×10^4 c.p.m.) or [^{3}H]pBR322 DNA (0.01 μg, 7.6×10^4 c.p.m.), 10 mM Tris–HCl (pH 7.5), 0.05 M NaCl, 5 mM EDTA and protein as indicated in the figures. Reaction mixtures were incubated at 18–20° for 30 min, then carefully filtered through presoaked nitrocellulose filters (Millipore Type HA, 0.45 μm) and washed with 2.0 ml of buffer containing 10 mM Tris–HCl (pH 7.5), 0.05 M NaCl and 5 mM EDTA. Filters were dried, then counted in scintillation fluid.

In certain binding experiments incubations were carried out in a buffer of higher ionic strength: 10–50 mM Tris–HCl, 0.05–1.0 M NaCl and 5 mM EDTA. Washing of the nitrocellulose filters was then executed with the buffer used for the incubation.

RESULTS

N-Acylurea proteins prepared with N-*ethyl*-N'-(3-*dimethylaminopropyl)carbodiimide*

Albumin and transferrin were treated with the water-soluble carbodiimide *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide HCl at a carbodiimide to protein mole ratio of 500:1 under dilute reaction conditions at pH 5.5. These conditions are similar to those used for the coupling of small ligands to albu-

Table 1. Reaction of [^{3}H]*N*-ethyl-*N'*-(3-trimethylpropylammonium)carbodiimide iodide with albumin and transferrin

Protein	Carbodiimide (mole) / Protein (mole) ratio	pH of reaction	Moles *N*-acylurea attached per mole protein	Moles (side chain carboxyl groups) per mole protein
Albumin	500:1	5.5	27	97
Albumin	150:1	5.5	13	97
Transferrin	500:1	5.5	20	85
Transferrin	150:1	5.5	4	85
(Fe^{3+})Transferrin*	500:1	7.5	17	85
(Fe^{3+})Transferrin†	500:1	7.5	23	85

* Transferrin was saturated with iron before carbodiimide treatment.
† Transferrin was first modified by the carbodiimide at pH 7.5 and then iron-loaded.

min for the preparation of antigens [1]. Agarose gel electrophoresis of the carbodiimide-modified proteins at pH 7.5 showed that they had undergone changes as regards electrophoretic mobility, becoming more basic (Fig. 1A). For studies on the interaction of DNA with *N*-acylurea proteins it was considered important to have a series of *N*-acylurea proteins synthesized at varying mole ratios of carbodiimide to protein. A range of *N*-acylurea albumins was prepared, therefore, which varied in the initial carbodiimide to protein mole ratio from 10:1 to 2000:1. The result of agarose gel electrophoresis at pH 7.5 of certain of these preparations is shown in Fig. 1B. It is seen that as the carbodiimide to protein ratio increases there is a progressive decrease in the mobility of the modified proteins to the positive pole of the gel, representing a decrease in electronegativity of the proteins.

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis indicated very little carbodiimide-induced polymerization of either albumin or transferrin under the reaction conditions described, even at high carbodiimide to protein ratios (results not shown).

N-*Acylurea proteins prepared with* N-*ethyl*-N′-(3-*trimethylpropylammonium*)*carbodiimide iodide*

Proteins were also treated with [^{3}H]*N*-ethyl-*N*′-(3-trimethylpropylammonium)carbodiimide iodide for the purpose of (i) introducing into the protein concerned the *N*-acylurea grouping containing a positively charged quaternary nitrogen function (Fig. 2) and (ii) allowing determination of the number of *N*-acylurea groups covalently attached to the protein after carbodiimide modification [16, 17]. Table 1 shows the number of *N*-acylurea groups covalently attached to albumin and transferrin respectively following reaction with the [^{3}H]-labelled quaternary carbodiimide at two different initial carbodiimide to protein mole ratios, 150:1 and 500:1. The values for both albumin and transferrin show that the higher the carbodiimide to protein ratio during reaction, the greater the number of *N*-acylurea groups attached to the protein product. In the case of iron-loaded transferrin treated with the carbodiimide at a 500:1 ratio (pH 7.5), the number of *N*-acylurea groups which become covalently attached is slightly different according to whether the transferrin was iron-loaded before or after carbodiimide modification. Analysis had shown that the transferrin pre-loaded with iron suffered no loss of Fe^{3+} ion on carbodiimide modification, and that the iron-free transferrin treated with Fe^{3+} ion after carbodiimide modification was able to bind the ion successfully.

Agarose gel electrophoresis of N-*acylurea protein–DNA complexes*

Results presented in Fig. 3A show the interaction and retardation of migration of λ-PstI DNA fragments by *N*-acylurea CDI-transferrin. Results presented in Fig. 3B show that *N*-acylurea CDI-derivatives of transferrin and albumin also interact with and retard the migration of pBR322 DNA. At higher concentrations of modified protein the *N*-acylurea protein–DNA complexes are unable to enter the gel.

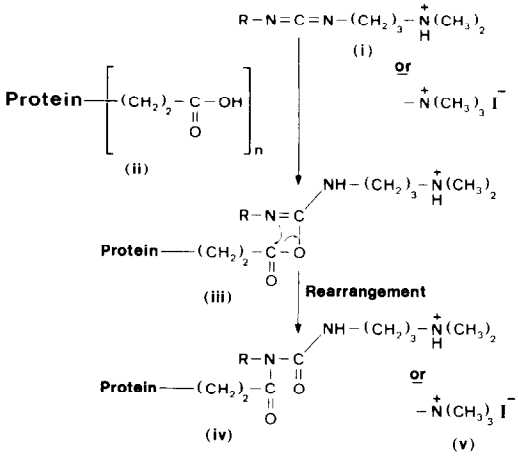

Fig. 2. Chemical structures of carbodiimide (i), protein (ii), *O*-acylurea protein (iii) and *N*-acylurea protein (iv). The quaternary carbodiimide is shown by (v). R, $-C_2H_5$.

Albumin, transferrin and the basic protein cytochrome C are without effect on DNA migration.

When *N*-acylurea proteins were run in gels with M13 mp8 single-stranded DNA, similar positive interactions were observed (results not shown).

Binding of DNA to N-*acylurea proteins using the nitrocellulose filtration method of assay*

The binding of DNA to *N*-acylurea proteins was also investigated by filtration of protein–DNA complexes through nitrocellulose filters [18–22]. The ionic strength of the incubation and washing solution was carefully controlled as the salt concentration was found to be important in the binding reactions. In these experiments we used a buffer containing 0.01 M Tris–HCl (pH 7.5), 0.05 M NaCl and 5 mM EDTA.

Figure 4 shows the binding of pBR322 DNA and calf thymus DNA to *N*-acylurea CDI-albumin at varying concentrations of protein. The two different types of DNA show similar binding characteristics up to a value of 0.05 μg protein. Thereafter, the level of calf thymus DNA binding remains constant with increasing protein concentration. Maximum binding of pBR322 DNA occurs at a value of 0.1 μg protein. Figure 4 also shows the binding of both types of DNA to *N*-acylurea Me^+CDI-albumin. Both pBR322 DNA and calf thymus DNA exhibit maximum binding with this *N*-acylurea protein at 0.02–0.05 μg protein.

Experiments on the binding of DNA by different *N*-acylurea transferrins was also carried out, and the results are shown in Fig. 5. In contrast with the binding of DNA by *N*-acylurea CDI-albumin (Fig. 4), *N*-acylurea CDI-transferrin was less efficient, requiring approximately ten times more protein (1 μg) for maximal binding. On the other hand, *N*-acylurea Me^+CDI-transferrin was able to bind both pBR322 DNA and calf thymus DNA maximally in the range 0.02–0.05 μg protein, and is therefore considerably more effective than *N*-acylurea CDI-transferrin in binding DNA. It is of interest to note that *N*-acylurea Me^+CDI-(Fe^{3+})transferrin showed

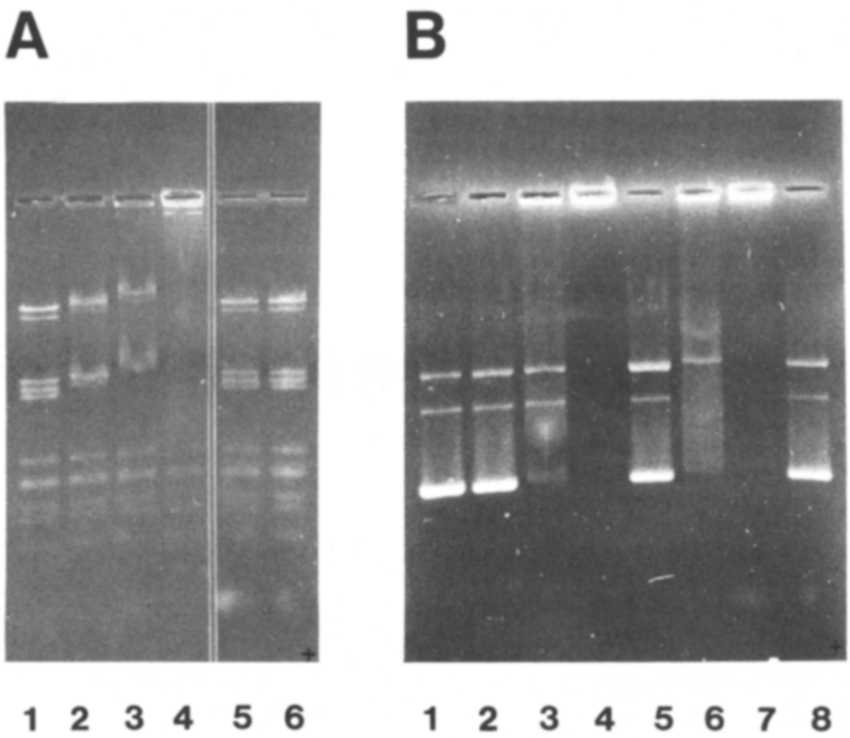

Fig. 3. Agarose gel electrophoresis of *N*-acylurea protein–DNA complexes. (*N*-Acylurea proteins prepared at a carbodiimide to protein mole ratio of 500:1.) (A) Interaction of *N*-acylurea CDI-transferrin with λ-PstI DNA restriction fragments. 1, λ-PstI DNA plus transferrin (4 μg); 2–4, λ-PstI DNA plus *N*-acylurea CDI-transferrin (2, 4 and 8 μg respectively); 5 and 6, λ-PstI DNA plus cytochrome C (4 and 8 μg respectively). λ-PstI DNA, 0.48 μg per well. (B) Interaction of *N*-acylurea CDI-proteins with pBR322 DNA. 1, pBR322 marker; 2, pBR322 plus albumin (4 μg); 3 and 4, pBR322 plus *N*-acylurea CDI-albumin (4 and 8 μg respectively); 5, pBR322 plus transferrin (4 μg); 6 and 7, pBR322 plus *N*-acylurea CDI-transferrin (4 and 8 μg respectively); 8, pBR322 plus cytochrome C (4 μg). pBR322 DNA, 0.33 μg per well.

similar binding characteristics to iron-free *N*-acylurea Me^{+}CDI-transferrin (Fig. 5).

Studies on the rate of binding of DNA to *N*-acylurea proteins was carried out at 5° and 20° using the nitrocellulose filter method of assay. At both temperatures the reaction was very fast, with binding occurring in less than 1 sec (results not given). However, as is shown later, this initial rapid binding appears to be weak, changing to a tight or more stable form of binding with time.

N-Acylurea proteins used in the binding experiments described above were all prepared at a carbodiimide to protein mole ratio of 500:1. A series of *N*-acylurea CDI-albumins varying in the carbodiimide to protein mole ratio employed during synthesis from 10:1 to 2000:1 was evaluated for binding ability by nitrocellulose filter assay. The binding of pBR322 DNA and calf thymus DNA by the various *N*-acylurea CDI-albumins is shown in Fig. 6. It should be noted that these particular binding assays were carried out in a buffer of higher ionic strength than that quoted previously; the buffer contained 0.05 M Tris–HCl (pH 7.5), 0.1 M NaCl and 5 mM EDTA. Results demonstrate that *N*-acylurea albumins prepared at CDI to protein mole ratios of 250:1 and greater are able to bind DNA.

The effect of NaCl *concentration on the binding of* N*-acylurea proteins to DNA*

A strong salt dependency of the binding between different types of protein and DNA has been observed [18, 23–26] and is indicative of electrostatic interaction between basic amino acids of the protein and phosphate groups of the nucleic acid. Structurally, the *N*-acylurea proteins (Fig. 2) are modified proteins with positively charged tertiary amino or quaternary ammonium groups attached via a spacer to glutamic and aspartic acid side chains of the protein. Electrostatic interaction between these groupings and DNA phosphates appeared likely and for this reason studies were carried out on the effect of salt concentration on DNA binding.

N-Acylurea proteins and DNA were incubated at 20° in a buffer consisting of 0.01 M Tris–HCl (pH 7.5), 0.05 M NaCl and 5 mM EDTA for 30 min; the solutions were then brought to the desired NaCl concentration, left for 5 min and finally filtered through nitrocellulose filters. Results of the salt-induced dissociations are shown in Fig. 7. Concentrations of NaCl required for the half-dissociation of individual *N*-acylurea protein–DNA complexes as deduced from the curves shown are: *N*-acylurea CDI-

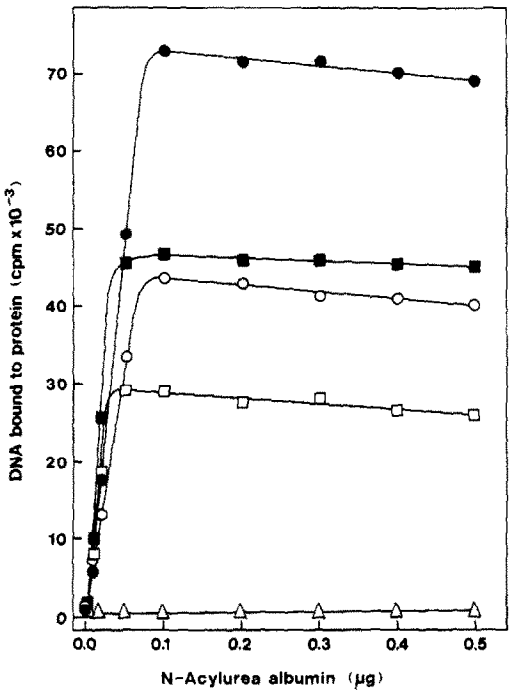

Fig. 4. Binding of DNA to *N*-acylurea albumins at varying protein concentration assayed by the nitrocellulose filtration method. DNA per reaction mixture, 0.01 μg. Modification of albumin was at a carbodiimide to protein mole ratio of 500:1. *N*-Acylurea CDI-albumin with [^{3}H]pBR322 DNA (●) and with [^{3}H]calf thymus DNA (○); *N*-acylurea Me$^+$CDI-albumin with [^{3}H]pBR322 DNA (■) and with [^{3}H]calf thymus DNA (□); albumin control with either DNA (△).

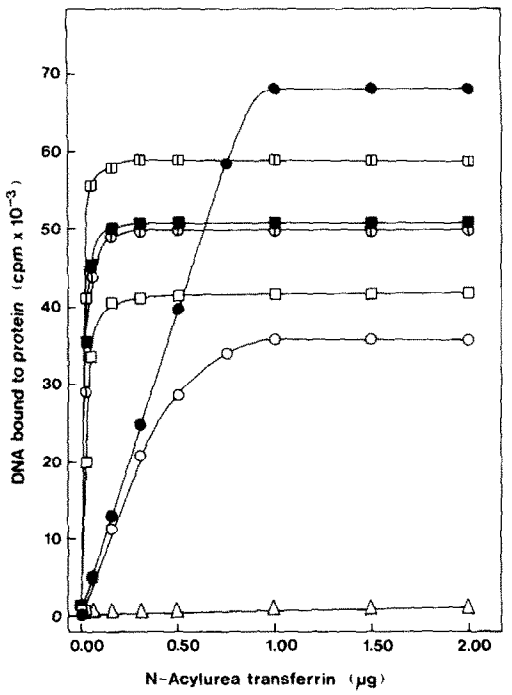

Fig. 5. Binding of DNA to *N*-acylurea transferrins at varying protein concentration assayed by the nitrocellulose filtration method. DNA per reaction mixture, 0.01 μg. Modification of the transferrin was at a carbodiimide to protein mole ratio of 500:1. *N*-Acylurea CDI-transferrin with [^{3}H]pBR322 DNA (●) and with [^{3}H]calf thymus DNA (○); *N*-acylurea Me$^+$CDI-transferrin with [^{3}H]pBR322 DNA (■) and with [^{3}H]calf thymus DNA (□); *N*-acylurea Me$^+$CDI-(Fe^{3+})transferrin with [^{3}H]pBR322 DNA (◫) and with [^{3}H]calf thymus DNA (◫); transferrin control with either DNA (△). The *N*-acylurea Me$^+$CDI-(Fe^{3+})transferrin used in these experiments was iron-loaded prior to carbodiimide modification.

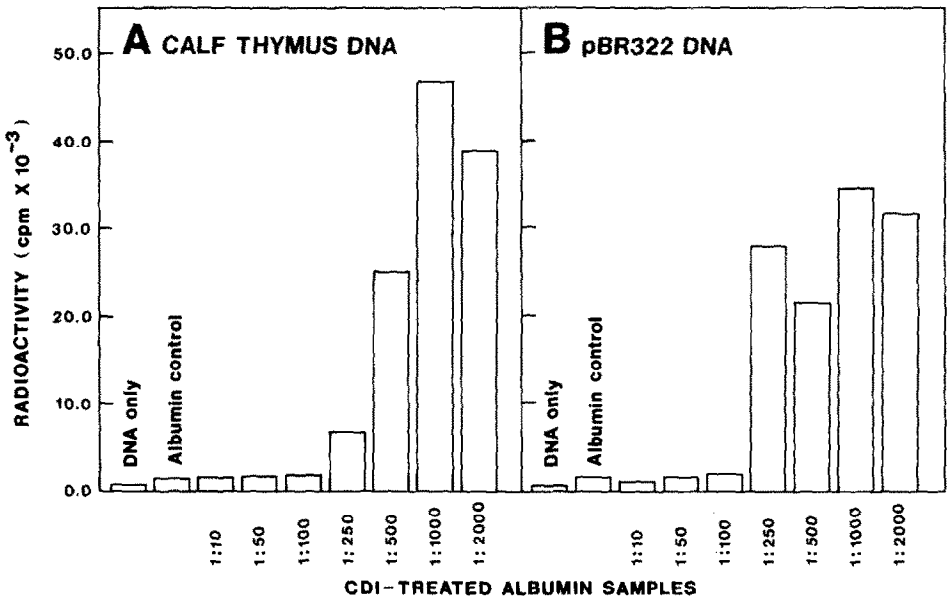

Fig. 6. Binding of DNA to *N*-acylurea albumins prepared with *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide at carbodiimide to protein mole ratios varying from 10:1 to 2000:1. All reaction mixtures contained 0.01 μg DNA and 8.8 μg protein. (A) [^{3}H]calf thymus DNA with the complete range of *N*-acylurea CDI-albumins. (B) [^{3}H]pBR322 DNA with the complete range of *N*-acylurea CDI-albumins.

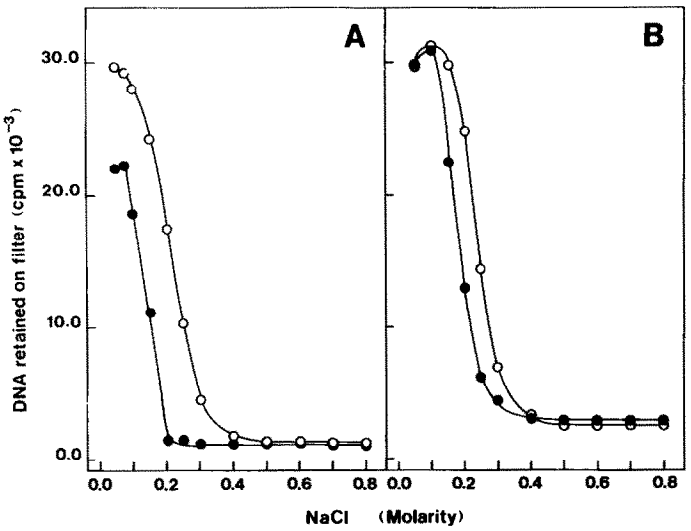

Fig. 7. The effect of NaCl concentration on the dissociation of *N*-acylurea protein–DNA complexes. *N*-Acylurea proteins were all prepared at a carbodiimide to protein mole ratio of 500:1. Protein per reaction mixture: *N*-Acylurea CDI-albumin, 0.10 μg; *N*-acylurea Me⁻CDI-albumin, 0.08 μg; *N*-acylurea CDI-transferrin, 2.0 μg; *N*-acylurea Me⁺CDI-transferrin, 0.20 μg. DNA per reaction mixture, 0.005 μg. (A) Dissociation of [^{3}H]pBR322 DNA from *N*-acylurea CDI-albumin (●) and *N*-acylurea Me⁺CDI-albumin (○). (B) Dissociation of [^{3}H]pBR322 DNA from *N*-acylurea CDI-transferrin (●) and *N*-acylurea Me⁺CDI-transferrin (○).

albumin, 0.15 M; *N*-acylurea Me⁺CDI-albumin, 0.24 M; *N*-acylurea CDI-transferrin, 0.18 M and *N*-acylurea Me⁺-CDI-transferrin, 0.26 M.

Heparin challenge experiments

Nitrocellulose filter retainable complexes are formed rapidly between *N*-acylurea proteins and DNA at both 5° and 20°. These initial complexes appear to be bound weakly, as they are dissociated if challenged by the polyanion heparin within 30 sec of formation (Fig. 8). However, formation of tightly bound complexes occurs if the binding reaction is allowed to proceed for 30 min at 20° or 37°. A heparin challenge at this time is significantly less effective in causing dissociation of the complex (Fig. 8).

Further investigations into the nature of binding

Further experiments aimed at obtaining information on the nature of the binding interactions between *N*-acylurea proteins and DNA were undertaken. Heating of *N*-acylurea CDI-albumin and *N*-acylurea CDI-transferrin at 55° for 10 min had no effect on their interaction with DNA. We were not able to extract pBR322 DNA from its complex with *N*-acylurea CDI-albumin by use of phenol in the absence of SDS. However, in the presence of 0.1% SDS at 60° for 5 min, pBR322 DNA was extracted into the aqueous phase during treatment with phenol, and recovered mainly in the supercoiled form (as shown by agarose gel electrophoresis). The results suggest that this particular *N*-acylurea protein–DNA complex is not held together by covalent bonds and that interaction does not involve the introduction of nicks into the DNA.

Competition experiments with various synthetic RNA polynucleotides produced inhibition of binding of pBR322 DNA to *N*-acylurea CDI-transferrin to differing degrees. Poly(G) produced the strongest inhibition, followed by poly(A), poly(U) and poly(C). Poly(m^{7}G) and poly(I) also inhibited binding but to a lesser degree than poly(G). The mononucleotides dGMP and dAMP were without effect on binding.

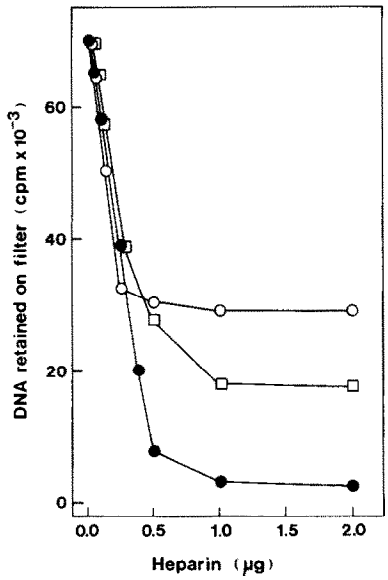

Fig. 8. The effect of heparin on the binding of DNA to *N*-acylurea transferrin. [^{3}H]pBR322 DNA (0.01 μg) and *N*-acylurea CDI-transferrin (500:1 mole ratio preparation; 4.0 μg) were incubated together for different times at either 20° or 37°. Each sample was then challenged with heparin for 30 sec and immediately processed by nitrocellulose filtration. Incubation for 0.5 min at 20° (●); incubation for 30 min at 20° (□); incubation for 30 min at 37° (○).

DISCUSSION

The work presented in this paper describes the modification of albumin and transferrin with water-soluble carbodiimides to give *N*-acylurea proteins which are able to bind various types of DNA, including λ-PstI restriction fragments, pBR322 plasmid, calf thymus DNA and single-stranded M13 mp8 DNA.

Binding reactions were investigated by two methods. Initial experiments made use of agarose gel electrophoresis to show that DNA migration was retarded in the presence of *N*-acylurea proteins, and that at higher concentrations of modified protein, *N*-acylurea protein–DNA complexes were not able to enter the gel. Further experimental work was based on the binding of *N*-acylurea protein–DNA complexes to nitrocellulose membrane filters. Using this type of assay we were (i) able to study the effect of *N*-acylurea protein concentration on its binding to calf thymus and pBR322 DNA, (ii) measure the rate of formation of complexes, which is rapid, (iii) show that *N*-acylurea protein–DNA interactions are sensitive to salt concentration, and (iv) distinguish two modes of interaction between *N*-acylurea proteins and DNA.

Additional experiments involving phenol extraction of *N*-acylurea protein–DNA complexes in the presence of SDS showed that pBR322 could be recovered in an intact supercoiled form. This information, together with the results of salt dissociations and heparin competition experiments, suggests that binding is electrostatic in nature, although other forms of binding might also be involved.

Results presented in Fig. 7 highlight the dependence of the apparent association constant (K_{obs}) for the *N*-acylurea albumin-pBR322 and *N*-acylurea transferrin-pBR322 interactions on monovalent ion concentration [M^+]. The reduction of K_{obs} with increasing [M^+] suggests a strong electrostatic component in the free energy of the binding interaction.

Assuming B-conformation of DNA in solution [25], the geometry of known DNA binding proteins allows for considerable contact with the duplex as determined by model building, revealing a large non-electrostatic component in the DNA complex. Thus Cro, lambda repressor and CAP are believed to interact with DNA of standard B-geometry through hydrogen bonding of base pairs in the major groove with specific side chains in α-helical regions located therein [26]. Van der Waals interactions as well as the expected electrostatic attractions are apparent [26]. The non-electrostatic component of the ligand–nucleic acid complexes formed between carbodiimide-modified albumin or transferrin and DNA is believed to be minor, since the lack of detectable complex formation between unmodified albumin and transferrin with either pBR322 or calf thymus DNA indicates poor molecular contact. The concept of relative surface exposure of nucleic acids, which has not been developed as extensively as with proteins, has been investigated by Alden and Kim [27] to identify the most accessible atoms or regions of nucleic acids available for intermolecular interactions. These studies reveal that the accessible area in B-DNA is rather polar, with phosphate oxygens accounting for 45% of this surface. Their peripheral location further supports the notion that the charge–charge interactions observed in our studies between carbodiimide-modified proteins and DNA are accounted for in large measure by ion pairs formed between DNA phosphate and the nitrogens of the *N*-acylurea groups attached to the proteins. This may not be the case with single-stranded M13 mp8 since the accessible surface of single-stranded DNA is known to be larger and considerably more hydrophobic due to greater base exposure [27]. Indeed, model building suggests that ion pairs of the type mentioned may be stabilized by specific hydrogen bonding between *N*-acylureas and the bases.

Melancon and co-workers [23] have carried out detailed studies on the binding of *Escherichia coli* RNA polymerase to restriction enzyme digests of T7D111 DNA. In addition to normal promoter complexes, these authors observed both fast-forming heparin-sensitive complexes and polymerase–DNA complexes insensitive to challenge with heparin but formed at a slower rate. It is of interest to note that in this respect our synthetic *N*-acylurea proteins form complexes with DNA which have similarities to the non-promoter complexes found in the bacterial system (Fig. 8).

The preparation of nucleic acid–protein conjugates for use as immunogens has been outlined by Stollar [28]. In one method, methylated serum albumin which is positively charged is used to prepare insoluble complexes with DNA. In an alternative method, a nucleotide or oligonucleotide is reacted with a water-soluble carbodiimide and then added to a protein for coupling; the nucleotide or oligonucleotide appears to become linked to the protein through a covalent phosphoramidate bond. Interestingly, Stollar notes that in certain cases an excess of carbodiimide modifies the protein carboxyl groups such that the resultant protein forms a precipitate with DNA.

Cheng *et al.* [7] have recently suggested that the introduction of DNA into cells with genetic defects may be achieved by linking the DNA to a protein which can be bound by specific cell surface receptors. Internalization via receptor-mediated endocytosis might thus allow entry of the DNA into a specific cell type. These workers have devised a method which involves chemical modification of both protein and DNA components, followed by the covalent linking of the two moieties through disulphide exchange reactions. The finding that *N*-acylurea albumin was able to bind DNA non-covalently and reversibly suggested that carbodiimide-modification of transferrin and other polypeptides which react with cell surface receptors might give *N*-acylurea substituted molecules that are able to bind DNA and still interact successfully with their receptors, thereby affording an alternative approach to this possible method of DNA (gene) transfer [7, 8]. Reaction of iron-free and iron-loaded transferrin with carbodiimides was therefore undertaken. It is notable that the resultant *N*-acylurea transferrins, both iron-free and iron-loaded, bind DNA to a similar degree (Fig. 5).

Work on the interaction of *N*-acylurea protein–DNA complexes with cells is in progress.

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