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Biochemical Study of KB-Cell Receptor for Adenovirus

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Three different approaches were used in an attempt to characterize the KB-cell receptor for adenovirus: affinity chromatography, immunoadsorption and cross-linking with a cleavable bifunctional reagent. The first system used an affinity gel consisting of adenovirus-fibre projection linked to Sepharose matrix by an intermediate bis(aminopropyl)amine arm, the amino groups of the fibre ligand being preserved by prior citraconylation. The second system consisted of adenovirus complete penton capsomere attached to anti-(penton base) antibody and cross-linked to polyacrylamide particles with glutaraldehyde. In this latter affinity model, the penton-fibre projection was appropriately oriented outwards, as in the virus particle. Both affinity systems permitted isolation from a KB-cell plasma-membrane extract of fibre-binding and penton-fibre-binding protein material, which inhibited adenovirus attachment. The penton–immunoadsorbent appeared more efficient and more specific than the affinity column of fibre–bis(aminopropyl)amino-Sepharose gel in specific activity of inhibition of adenovirus attachment. The third method consisted of reversibly cross-linking KB-cell receptor proteins with adenovirus particles by means of a cleavable di-imidoester and isolation of the complexes by sucrose-density-gradient centrifugation. Polypeptide analysis on sodium dodecyl sulphate/polyacrylamide gel of labelled KB-cell surface proteins, selected by the different procedures, showed that three major protein subunits of 78000, 42000 and 34000 mol.wt. were common to the three selection systems. A possible model for the structure and function of the KB-cell receptor for adenovirus is discussed.

Attachment of an animal virus at the plasma membrane constitutes the initial event between virion and host cell. As regards the physiology of a virus such as adenovirus, this step is critical because it conditions the following events, penetration and replication. As regards the biology of mammalian cells, this virus–cell interaction can offer a valuable clue to the characterization of the plasma-membrane glycoproteins among which a particular species (the receptor) is involved in virion-specific recognition (Lonberg-Holm & Philipson, 1974).

The adenovirion has an icosahedral shape and is formed of 240 capsomeres with an hexagonal symmetry, the hexons, located on the facets and the edges of the capsid, and 12 vertex capsomeres surrounded by five neighbouring hexons, called the pentons. The penton is formed of a base, anchored in the capsid, and of a fibrous projection terminated by a knob, and oriented outwards, the so-called fibre (Valentine & Pereira, 1965).

The adenovirus capsid structures recognized by cell-surface receptors are most likely the vertex projections of the icosahedron, as suggested by the inhibition of virus attachment by highly purified free adenovirus fibres (Philipson *et al.*, 1968), by the neutralization of virus by anti-fibre immune serum (Pettersson *et al.*, 1968), and by selective chemical modification of protein components of the virion and of the cell membrane (Neurath *et al.*, 1970).

Since the adenovirus-fibre protein is synthesized in considerable excess by the infected cells and can be isolated from a cellular extract in good yield (Boulanger & Puvion, 1973), early attempts were made to utilize purified fibres coupled to an insoluble matrix to isolate the host-cell-surface receptors by affinity chromatography (Hughes & Mautner, 1973; Meager *et al.*, 1976). However, since the fibre is a rod-like structure terminated by a knob, and in the adenovirus capsid this knob is oriented outwards (Philipson & Pettersson, 1973), the orientation is an important factor when insolubilized fibres are bound to a gel matrix.

The aim of the present study was to characterize the protein subunits constituting the KB-cell plasma-membrane receptors for adenovirus, by using three different approaches: affinity chromatography, immunoadsorption and cross-linking with a cleavable bifunctional reagent.

Materials and Methods

Viruses

Wild-type human adenovirus type 2 was originally supplied by Dr. J. F. Williams (M.R.C. Virology

Unit, Glasgow). The adenovirus temperature-sensitive mutant ts-115 was isolated in our laboratory after nitrous acid treatment. This mutant is defective in fibre production (Martin *et al.*, 1975). Both wild-type and mutant ts-115 were propagated on KB cells. Wild-type adenovirus particles were labelled with 0.1 μCi of ^{14}C-labelled amino acid mixture/ml (specific radioactivity 42 mCi/mg-atom of C) from 8 to 30 h after infection. Labelled virions were purified by fluorocarbon extraction and two CsCl bandings, by conventional techniques (Green & Pina, 1963).

Cells

KB cells were grown in suspension culture at 2.5×10^5 cells/ml of minimum essential Joklik-modified medium (F-13; Gibco, Grand Island, NY, U.S.A.) supplemented with 5% (v/v) horse serum. The cells were labelled for 24 h either with L-[^{35}S]-methionine (0.5 μCi/ml; 460 Ci/mmol) or with ^{14}C-labelled amino acid mixture (0.5 μCi/ml; 42 mCi/mg-atom of C), in amino acid-deficient medium containing 10% of the concentration of amino acids in normal medium, and D-[6-^{3}H]glucosamine hydrochloride (2.5 μCi/ml; 12 Ci/mmol).

Adenovirus soluble antigens

Adenovirus capsid components hexons, pentons and fibres are synthesized in large excess in infected cells. Those which are not incorporated in the mature virion are defined as soluble antigens (Schlesinger, 1969). Free pentons and free fibres were isolated from the infected-cell pool of soluble antigens by a four-step procedure, comprising fluorocarbon extraction, $(NH_4)_2SO_4$ precipitation, DEAE-Sephadex A-50 chromatography and hydroxyapatite chromatography (Boulanger & Puvion, 1973).

Antisera

Anti-adenovirus rabbit polyspecific immune serum was obtained after repeated injections of adenovirus-particle suspension purified by three CsCl bandings. Rabbit monospecific anti-fibre serum was obtained by multiple injections of antigen–antibody complexes (fibre+anti-fibre antibodies) freshly formed within the agarose gel by using the two-dimensional immunoelectrophoresis technique of Laurell (1965): a wild-type infected-cell extract was the source of soluble antigens and was subjected to crossed immunoelectrophoresis against the polyspecific antiserum (Martin *et al.*, 1975). Rabbit monospecific anti-(penton base) serum was obtained in the same way, by injecting penton-base–anti-(penton base) immune precipitates: a mutant ts-115-infected cell extract of a culture maintained at non-permissive temperature, thus devoid of free fibre and of complete penton (penton base+fibre), was subjected to crossed immunoelectrophoresis against the polyspecific anti-adenovirus serum. The agarose gel containing the penton base–antibody immune precipitate was mixed with an equal volume of complete Freund's adjuvant and injected (Martin *et al.*, 1975).

Both sera were tested for specificity by the conventional techniques of double immunodiffusion, crossed immunoelectrophoresis and immunoprecipitation by using the *Staphylococcus aureus* protein A technique (Forsgren & Sjöquist, 1966). Both sera were devoid of detectable non-specific antibodies.

Isolation of KB-cell plasma membranes

All the operations were conducted in the cold (0°C) and 2 mM-phenylmethanesulphonyl fluoride was added to all buffers. KB-cell plasma membranes were prepared by a modification of the Tris method of Warren *et al.* (1966): 2 litres of labelled KB-cell suspension (approx. 5×10^8 cells) were sedimented and the cells washed once in phosphate-buffered saline (0.15 M-NaCl / 3 mM-KCl / 8 mM-Na_2HPO_4 / 15 mM-KH_2PO_4/0.5 mM-$MgCl_2$,$6H_2O$/1 mM-$CaCl_2$, pH 7.4). The cell pellet was resuspended in 7 ml of 0.01 M-Tris/HCl buffer, pH 7.4, containing 0.01 M-NaCl and 1.5 mM-$MgCl_2$, and left on ice to swell for 10 min. The cells were then lysed in the same buffer by 15 strokes in a tight-fitting Dounce glass homogenizer, and the resulting homogenate, adjusted to 10% (w/v) sucrose by addition of 1 ml of 80% (w/v) sucrose solution in 0.01 M-Tris/HCl buffer (pH 7.4)/5 mM-$MgCl_2$, was loaded (4 ml per gradient) on top of two preformed gradients made of 5 ml of 50% and 7.5 ml of 30% (w/v) sucrose in 0.01 M-Tris/HCl buffer (pH 7.4)/5 mM-$MgCl_2$. The gradients were centrifuged in the Sorvall RC 2B centrifuge with a HB-4 swinging-bucket rotor for 15 min at 4°C and 1500*g*. The membrane band at the 30%/50% interface was harvested with a Pasteur pipette, diluted with 3 vol. of cold 0.01 M-Tris/HCl buffer (pH 7.4)/5 mM-$MgCl_2$, and centrifuged at 5860*g* for 20 min. The membrane pellet was resuspended in 18 ml of 65% sucrose in 0.01 M-Tris/HCl buffer (pH 7.4)/5 mM-$MgCl_2$ and homogenized in a loose-fitting Dounce glass homogenizer (three strokes). A portion (9 ml) of this homogenate was placed at the bottom of a preformed discontinuous sucrose gradient made of 9 ml each of 55, 45 and 40% (w/v) sucrose in 0.01 M-Tris/HCl buffer (pH 7.4)/5 mM-$MgCl_2$. The gradients were centrifuged in the Beckman L2 65 K centrifuge with a SW 27 rotor for 2 h at 24000 rev./min and 4°C. The plasma-membrane band was visible at the 45%/55% interface. Fractions (1 ml) were collected by pumping from the bottom of the gradient and were assayed for 5'-nucleotidase activity (Michell & Hawthorne, 1965), and also examined by electron microscopy after

fixation in 2.5% (v/v) glutaraldehyde in 0.1M-sodium cacodylate buffer, pH7.2, and post-fixation in 1% (w/v) osmium tetroxide (Warren *et al.*, 1966).

Extraction of plasma-membrane glycoproteins

The gradient fractions containing the plasma membranes were diluted with 3vol. of 0.01M-Tris/HCl buffer (pH7.4)/5mM-$MgCl_2$ and the membranes pelleted at 5860*g* for 20min at 4°C. The membrane pellet was resuspended in 0.01M-Tris/HCl buffer, pH7.4, containing 0.5% sodium deoxycholate and homogenized in a tight-fitting Dounce glass homogenizer (three strokes), then maintained at 4°C for 30min with magnetic stirring. The excess of sodium deoxycholate was eliminated by dialysis in the cold for 48h against 0.01M-Tris/HCl buffer, pH8.0, containing 0.05M-NaCl.

Protein and radioactivity determinations

Protein content was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Radioactivity counting was carried out in 5ml of Bray's (1960) scintillation fluid.

Resistivity determination

The chromatographic elutions with NaCl gradients were monitored with a model E-382 Metrohm conductometer.

Affinity chromatography on Sepharose–fibre of KB-cell plasma-membrane extract

Preparation of the Sepharose matrix. Because some steric hindrance can impair the fixation of a macromolecular ligand such as the fibre on to the insoluble matrix, the use of an arm has been recommended (Cuatrecasas & Anfinsen, 1971). The arm used in the present experiment was bis(aminopropyl)amine. Dry CNBr-activated Sepharose 4B (6g; Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen overnight in 600ml of 1mM-HCl at room temperature (20°C) then washed thoroughly with 1mM-HCl. The gel (20ml) was rapidly resuspended in 5vol. of cold (4°C) 35mM-bis(aminopropyl)amine and left overnight on ice with slow stirring. The bis(aminopropyl)-amino-Sepharose thus obtained was washed with 200vol. of water. The fixation of bis(aminopropyl)-amine was controlled as follows: to 0.3ml of bis(aminopropyl)amino-Sepharose suspended in 2ml of saturated sodium borate was added 0.15ml of an aqueous 3% (w/v) solution of sodium 2,4,6-trinitrobenzenesulphonate. After 2h at room temperature the gel was washed with water, and if good fixation had occurred, it was a deep-orange colour.

Chemical modification of the ligand: citraconylation of adenovirus type-2 fibre. To prevent possible inter-fibre linkages occurring during the coupling reaction between the fibre ligand and the bis(aminopropyl)-amino-Sepharose matrix in the presence of carbodi-imide derivative, the amino groups of the fibre protein were reversibly blocked by reaction with citraconic anhydride (methylmaleic anhydride).

To a solution of purified free fibre in 0.1M-NH_4HCO_3 buffer, pH8.2 (50mg of protein in 5ml of buffer), were added six 5μl portions of citraconic anhydride at 20min intervals with stirring and pH monitoring with a pH-stat. After an additional 2h incubation at room temperature, the solution was dialysed at 4°C overnight against 100vol. of water adjusted to pH9 with aq. NH_3 (sp.gr. 0.885), then against 100vol. of 0.1M-sodium borate, pH8.5, containing 1M-NaCl.

Coupling of citraconylated fibre to bis(aminopropyl)-amino-Sepharose. Citraconylated fibre (30mg) in 0.1M-sodium borate buffer (pH8.5)/1M-NaCl was mixed with 20ml of hydrated bis(aminopropyl)amino-Sepharose, and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-*p*-toluenesulphonate was added to a final concentration of 0.1M. The reaction proceeded at room temperature for 3h with stirring. Fibre–bis(aminopropyl)amino-Sepharose was filtered on a sintered-glass filter, washed five times successively with 0.1M-sodium borate buffer (pH8.5)/1M-NaCl, and then with 0.1M-sodium acetate buffer (pH4.5)/1M-NaCl. The amount of fibre coupled to the bis(aminopropyl)amino-Sepharose was 4.5mg/g of hydrated gel, i.e. a fixation yield of about 90%.

Deblocking of the citraconylated fibre. The reaction with citraconic anhydride has been proved to be reversible on acidic treatment, with complete recovery of free amino groups, immunochemical properties and native conformation (Singhal & Atassi, 1971). Fibre–bis(aminopropyl)amino-Sepharose was washed on to a sintered-glass filter with 0.2M-glycine/HCl buffer (pH2.8)/1M-NaCl, then maintained overnight at 4°C in the same buffer with stirring. The gel was then washed and stored in 0.01M-Tris/HCl buffer, pH8, containing 0.05M-NaCl. Less than 0.5mg of fibre/g of Sepharose gel was eluted from the gel during this reaction of decitraconylation, implying that most of the fibre was attached through its amino acid carboxylic groups and not through its citraconyl groups (Scheme 1*d*).

Biological activity of the fibre–bis(aminopropyl)-amino-Sepharose. To determine if the fibre coupled to Sepharose was still immunologically active, 1ml of anti-fibre serum was chromatographed on a 10ml column of fibre–bis(aminopropyl)amino-Sepharose. The first fraction, eluted with the rinsing buffer [0.01M-Tris/HCl (pH8.0)/0.05M-NaCl], was dialysed

overnight against water, freeze-dried and dissolved in 1ml of phosphate-buffered saline, and exhibited no reaction with purified fibre in double immunodiffusion and in immunoprecipitation of [^{3}H]valine-labelled fibre. Most of the specific anti-fibre antibodies were eluted with 0.2M-glycine/HCl buffer (pH2.8)/1M-NaCl.

Affinity chromatography of KB-cell plasma-membrane extract. A sodium deoxycholate extract of plasma membranes in 0.01M-Tris/HCl buffer (pH8)/0.05M-NaCl (2–3ml total volume, 10–15mg of protein) was chromatographed on the fibre–bis(aminopropyl)amino-Sepharose column (20ml of gel) at a slow rate (0.5ml/h) and at room temperature to favour the adsorption of specific proteins. The column was first rinsed with the starting buffer (25ml), then successively with 25ml of 0.15M-NaCl and 25ml of 0.6M-NaCl, both in 0.01M-Tris/HCl, pH8. Before re-use, the column was thoroughly washed with 25ml of 0.2M-glycine/HCl buffer (pH2.8)/1M-NaCl. Fractions (3ml) were collected and the fractions corresponding to each buffer were dialysed against water, freeze-dried, and dissolved in 1ml of phosphate-buffered saline. Unlabelled KB-cell plasma-membrane extract was used for biological assays, to avoid interference with adenovirus label. Labelled cell material was used for sodium dodecyl sulphate/polyacrylamide-gel analysis of chromatographic fractions. The elution was monitored by protein determination, radioactivity counting and resistivity measurement.

Immunoadsorbent

Activation of Bio-Gel P-300. The method described by Ternynck & Avrameas (1972) was used. For this, 5g of dry polyacrylamide gel (Bio-Gel P-300, 100–200 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) was swollen in water at room temperature for 24h, then washed with 3vol. of water. To 100ml of hydrated gel was added 500ml of 6% (v/v) glutaraldehyde (Merck Laboratories, Darmstadt, West Germany) in 0.1M-sodium phosphate buffer, pH7.0. The suspension was incubated at 37°C overnight with gentle stirring, then washed with 20×500ml of water.

Fixation of anti-(penton base) antibodies. A crude preparation of anti-(penton base) immunoglobulins was obtained by precipitation of a rabbit anti-(penton base) immune serum with 33%-satd. $(NH_4)_2SO_4$, pH7.0. The precipitate was dissolved in phosphate-buffered saline and dialysed overnight against the same buffer. To 10ml of glutaraldehyde-activated Bio-Gel P-300 was added 30mg of immunoglobulins diluted in 10ml of phosphate-buffered saline and the adsorption was left to proceed at 4°C for 72h with gentle stirring. The gel was then centrifuged (3500*g*, 10min, 4°C) and washed repeatedly with phosphate-buffered saline until the A_{280} was negligible. The first supernatant and those of the two following washings were pooled and the amount of immunoglobulin conjugated to the gel particles was estimated from the difference between the initial amount of protein added and that found in the pooled supernatants. A 25mg sample of globulins was conjugated to 10ml of hydrated Bio-Gel. To block any residual free active aldehyde groups on the gel beads, the gel was resuspended in an equal volume of 0.1M-lysine, pH7.4, and left overnight at room temperature. The gel particles were then thoroughly washed with phosphate-buffered saline, followed by two washings with 5vol. of cold 0.2M-glycine/HCl buffer, pH2.8, one washing with 2vol. of 0.2M-K_2HPO_4 and two washings with phosphate-buffered saline.

Immunoadsorption of penton. To 10ml of anti-(penton base) antibody-conjugated Bio-Gel P-300 was added 20mg of purified free pentons in 10ml of phosphate-buffered saline and the suspension was gently stirred at 4°C for 24h. The gel was centrifuged (3500*g*, 10min, 4°C) and the supernatant collected to determine the amount of non-adsorbed penton: 5mg of penton was adsorbed on 10ml of antibody–Bio-Gel.

Fixation of the penton–anti-(penton base) antibody complex. To prevent the release of penton during the following steps of isolation of the adenovirus cell receptor, the antigen–antibody complexes attached to polyacrylamide-gel particles were covalently linked together with glutaraldehyde. Antigen–antibody Bio-Gel (10ml) was dispersed drop by drop in a large volume (250ml) of 2.5% (v/v) glutaraldehyde solution in 0.1M-sodium phosphate buffer, pH7.4, and stirred continuously for 1h at room temperature. Then 25ml of 1M-lysine, pH7.4, was added and the suspension was stirred overnight at room temperature. The gel was then exhaustively washed with phosphate-buffered saline and stored in the same buffer with 0.01% NaN_3. The colour of this gel immunoadsorbent was light-brown (Fig. 1).

Biological activity of the penton-immunoadsorbent. If the fibre projection of the penton was appropriately oriented outwards, the immunoadsorbent must retain anti-fibre antibodies. A portion (0.5ml) of anti-fibre immune serum was diluted to 2ml with phosphate-buffered saline and adsorbed on 2ml of immunoadsorbent at 4°C overnight with stirring. The gel was centrifuged (3500*g*, 10min, 4°C), washed with phosphate-buffered saline and the adsorbed proteins were eluted with 0.2M-glycine/HCl buffer (pH2.8)/1M-NaCl. The eluate was dialysed overnight against phosphate-buffered saline and assayed for immunoprecipitation of [^{3}H]valine-labelled fibre. The eluate was found to have the same specific activity in immunoprecipitation of labelled fibre as a crude preparation of anti-fibre antibodies obtained by 33%-satd.-$(NH_4)_2SO_4$ precipitation at pH7.0.

Adsorption of KB-cell plasma-membrane extract on immunoadsorbent. A portion (10ml) of a sodium deoxycholate extract of KB-cell plasma membranes in phosphate-buffered saline (15–20mg of protein) was adsorbed on to 10ml of the immunoadsorbent-Bio-Gel overnight at 4°C with stirring. The supernatant was removed after centrifugation and the gel was washed with 3×20ml of phosphate-buffered saline. The adsorbed material was eluted with 0.2M-glycine/HCl buffer, pH2.8, containing 1M-NaCl for 30min at 4°C with stirring, then dialysed against cold water, freeze-dried and dissolved in 1ml of 0.01M-Tris/HCl buffer, pH8. The protein concentration of this eluted fraction was usually 100–200μg/ml.

Effect of fibre- and penton-binding material from cell surface on adenovirus attachment. The unlabelled membrane material isolated by affinity chromatography on fibre–bis(aminopropyl)amino-Sepharose or by batch adsorption on the immunoadsorbent was assayed for inhibition of attachment of adenovirus particles labelled with ^{14}C-labelled amino acid to intact KB cells.

In several sets of test tubes, 2.5×10^7 KB cells suspended at 37°C in 2ml of phosphate-buffered saline with magnetic stirring were mixed with portions of ^{14}C-labelled adenovirus suspension containing 1.5×10^{11} plaque-forming units corresponding to 7500c.p.m. (6×10^3 plaque-forming units/cell). The virus suspensions had been preincubated for 2h at 4°C with or without KB-cell plasma-membrane (starting deoxycholate extract), non-retained material (i.e. material contained in the excluded peak of affinity chromatography or in the supernatant of batch adsorption on immunoadsorbent), or material eluted from the affinity column or immunoadsorbent. In one series of experiments, material binding to fibre–bis(aminopropyl)amino-Sepharose or penton–immunoadsorbent was preincubated with 100μg of trypsin/ml of 0.1M-Tris/HCl buffer, pH8.0, for 16h at 37°C, followed by inhibition of trypsin by 2mM-phenylmethanesulphonyl fluoride. Samples were removed at intervals, centrifuged (2000*g*, 10min, 4°C) and the supernatant fluid was assayed for radioactivity.

Analytical sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Samples were heated at 100°C for 2min in an equal volume of 0.0625M-Tris/HCl buffer, pH6.8, containing 10% (v/v) mercaptoethanol, 4% (w/v) sodium dodecyl sulphate and 6M-urea, and run on slab gels made up with 17.5% acrylamide, 0.1% sodium dodecyl sulphate (ratio acrylamide:bisacrylamide, 150:1, w/w) overlayered by a spacer gel containing 5% acrylamide (ratio acrylamide:bisacrylamide, 4:1) in the discontinuous buffer system described by Laemmli (1970). In some experiments, slab gels made of 12% acrylamide (ratio acrylamide:bisacrylamide, 37.5:1) were used. Gels were analysed by radioautography or fluorography as described by Bonner & Laskey (1974).

Cross-linking of adenovirus and KB-cell receptors with a cleavable bifunctional reagent

The cleavable cross-linking reagent methyl 4-mercaptobutyrimidate hydrochloride (Pierce Chemical Co., Rockford, IL, U.S.A.) was used to link reversibly adenovirus particles attached to the receptor sites of KB-cell plasma membrane.

Saturation of KB-cell receptor sites by adenovirus particles. For this 2×10^8 KB cells labelled with [^{35}S]methionine were suspended in HMS buffer [0.05M-Hepes* (sodium salt) buffer, pH7.5, containing 0.05M-$MgCl_2$ and 0.25M-sucrose]. Then 4ml of virus-particle suspension (infectious titre, 10^{13} plaque-forming units/ml) containing at least 10^{14} physical particles was added to the cell suspension, and the adsorption was left to proceed for 2h at 0°C with stirring.

Amidination of the proteins of the adenovirus–receptor complex. The cells were centrifuged at low speed (2000*g*, 10min, 4°C), suspended in 10ml of HMS buffer containing 1.5mM-mercaptoethanol and 5mg of methyl 4-mercaptobutyrimidate hydrochloride/ml (Traut *et al.*, 1973) and maintained in this medium for 30min at 0°C.

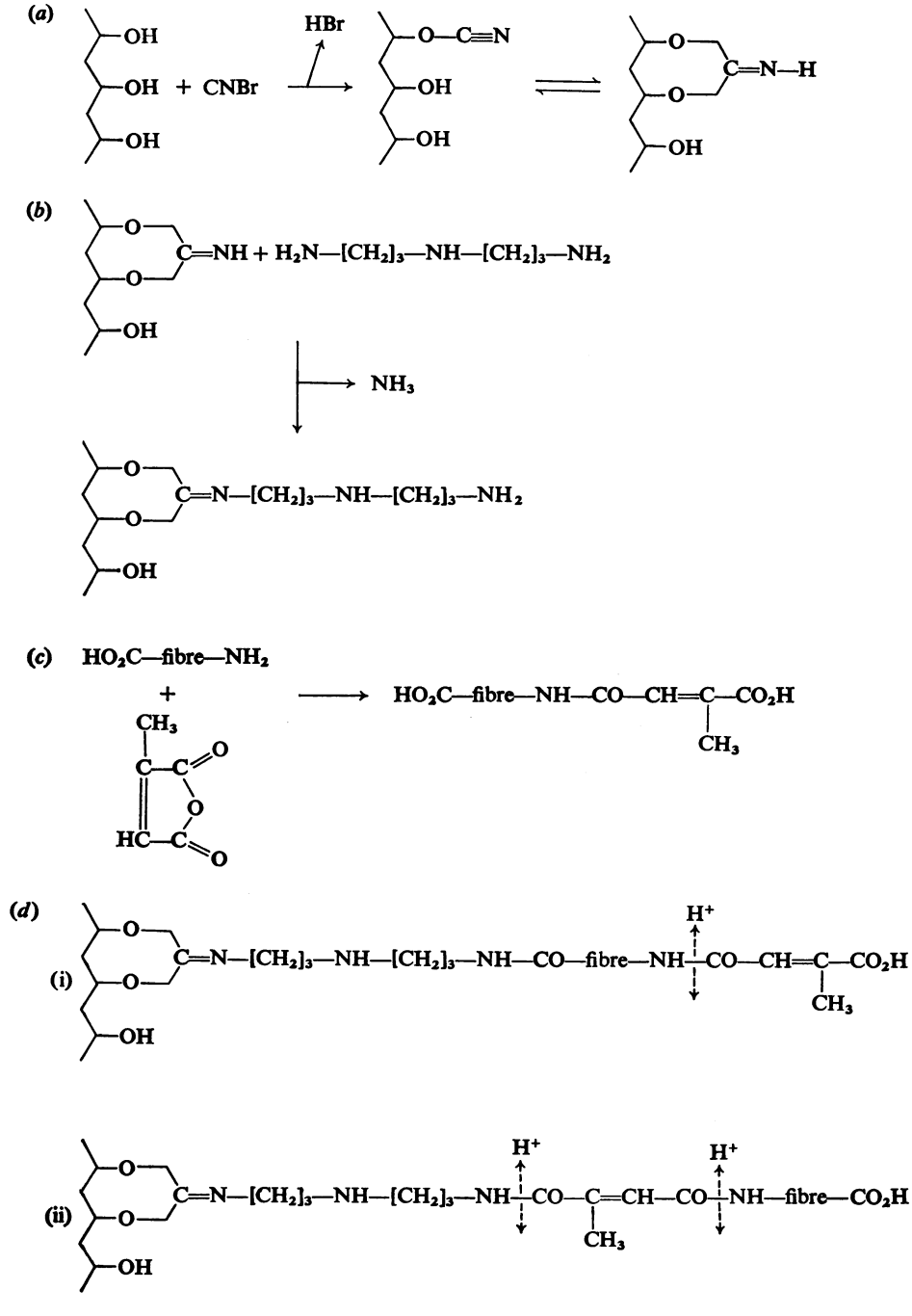
Formation of disulphide links. The cells were then centrifuged (2000*g*, 10min, 4°C) and resuspended in 10ml of HMS buffer containing 100mM-H_2O_2 for 90min at 0°C.

Extraction of the adenovirus–membrane-receptor complex. The cells were centrifuged (2000*g*, 10min, 4°C) and resuspended in 1ml of HMS buffer containing 0.25% Triton X-100 for 1h at 0°C, then centrifuged at 5600*g* for 10min at 4°C. A control cell suspension was subjected to the same treatment, except that virus was omitted in the first adsorption step.

Isolation of the adenovirus–membrane-receptor complex. The supernatant of the last centrifugation, containing cross-linked adenovirus–membrane-receptor complexes extracted with Triton, was loaded on 25–40% (w/v) sucrose gradients in 0.05M-Tris/HCl buffer, pH8, containing 0.2M-NaCl and 0.01M-EDTA (sodium salt) and centrifuged for 80min at 25000rev./min at 4°C in the SW-41 rotor of a Beckman centrifuge. Fractions (300μl) were collected and radioactivity was determined on 50μl portions. Fractions corresponding to the sedimentation of adenovirus marker were pooled, dialysed against water and freeze-dried.

Cleavage of disulphide linkages. The disulphide cross-links were cleaved by heating the adenovirus–receptor complexes for 2min at 100°C in 0.0312M-

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.



Scheme 1. *Preparation of the bis(aminopropyl)amino-Sepharose*
(*a*) Activation of Sepharose 4B with CNBr. (*b*) Coupling of the bis(aminopropyl)amine arm. (*c*) *N*-Citraconylation of the adenovirus type-2 fibre. (*d*) Coupling of the citraconylated fibre to the bis(aminopropyl)amino-Sepharose and deblocking of the amino groups at acidic pH (i). The fibres linked to the bis-(aminopropyl)amino-Sepharose through their citraconyl groups were liberated by acid treatment (ii).

Tris/HCl, pH6.8, containing 5% mercaptoethanol, 2% sodium dodecyl sulphate, 3M-urea and the receptor proteins analysed by electrophoresis on sodium dodecyl sulphate/polyacrylamide gel as described above.

Results

Affinity chromatography on adenovirus-fibre–bis-(aminopropyl)amino-Sepharose of KB-cell plasma-membrane extracts

In the affinity gel used here the adenovirus fibre was linked to a Sepharose matrix by an arm. The choice of arm was determined by two considerations. (i) To avoid possible steric hindrance between the fibre ligand and the Sepharose matrix, a bis(aminopropyl)-amine arm was fixed to activated Sepharose to form a bis(aminopropyl)amino-Sepharose (Scheme 1*b*). (ii) Direct binding of fibre on CNBr-activated Sepharose would occur by reaction of imidocarbonate groups of the activated Sepharose and amino groups of the fibre. It has been shown that in the process of adenovirus adsorption to erythrocytes, fibre reacted through amino groups with the carboxylic acid groups of the cell receptor glycoprotein(s) (Neurath *et al.*, 1970). It therefore seemed crucial to preserve as many amino groups as possible on the fibre protein. Fibre amino groups were first blocked by citraconylation, then citraconylated fibre was reacted with bis-(aminopropyl)amino-Sepharose in the presence of a carbodi-imide derivative, to form an amide linkage. The citraconyl groups were then eliminated by acidic treatment (Scheme 1*d*). The fibre thus bound to Sepharose could be considered as biologically active, since it retained its antigenic determinants, as proved by affinity chromatography of anti-fibre–antibody on a fibre–bis(aminopropyl)amino-Sepharose column.

It is known that KB-cell receptors for adenovirus can be removed by non-ionic detergents such as Triton X-100 and sodium deoxycholate (Hughes & Mautner, 1973; Butters & Hughes, 1974, 1975). A sodium deoxycholate extract of isolated KB-cell membranes was chromatographed on a fibre–bis-(aminopropyl)amino-Sepharose affinity column. The original extract, the unadsorbed material (at 0.05M-NaCl), and the material eluted at 0.15M- and 0.6M-NaCl were assayed for biological activity, i.e. for inhibition of attachment of labelled adenovirus particles to KB cells. Kinetics of attachment of virus particles, preincubated for 2h at 0°C with material eluted with 0.15M- or 0.6M-NaCl, showed that a maximum of 25–50% of virus label remained unadsorbed after 2h incubation with the cells at 37°C (Fig. 2*a*). No detectable inhibition of adsorption was found in the non-adsorbed material and in the material rinsed with 0.05M-NaCl, within the protein concentration range used for retained material (25–75 μg of protein).

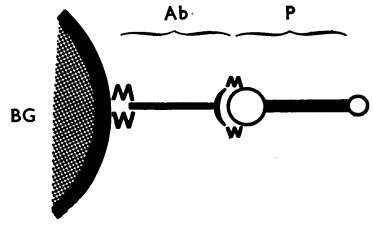


Fig. 1. *Structure of the penton–immunoadsorbent*
Anti-(adenovirus penton base) antibody (Ab) was coupled to polyacrylamide beads of Bio-Gel P-300 (BG) through glutaraldehyde cross-links shown by angular lines. Adenovirus penton (P) was adsorbed on the antibody–gel and the immune complex post-fixed with glutaraldehyde.

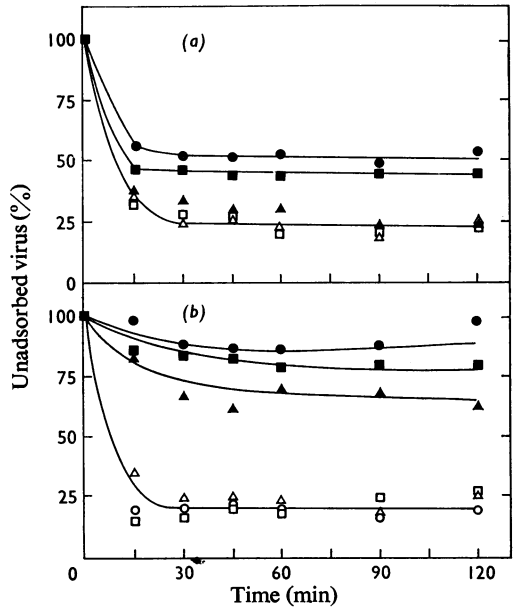


Fig. 2. *Effect of penton-fibre-binding material from the KB-cell surface on attachment of adenovirus*
The kinetics of attachment of ^{14}C-labelled adenovirus on KB cells was studied at 37°C in the absence (△) or in the presence of increasing amounts (μg of protein) of KB-cell plasma-membrane material retained on fibre–bis(aminopropyl)amino-Sepharose (affinity column) and on penton–immunoadsorbent. (*a*) Affinity column: ▲, 25μg; ■, 50μg; ●, 75μg of retained material; □, 75μg of retained material predigested with trypsin. (*b*) Immunoadsorbent gel: ▲, 5μg; ■, 10μg; ●, 15μg of retained material; □, 15μg of retained material predigested with trypsin; ○, adenovirus preincubated with 50μg of original deoxycholate extract; 100% corresponds to 7500 c.p.m. and 1.5×10^{11} infectious particles.

When [^{35}S]methionine- and [^{3}H]glucosamine-labelled extracts were passed through the affinity column, 16–17% of the ^{35}S and 9–10% of the ^{3}H label were eluted with 0.6M-NaCl. Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the retained material showed a complex pattern of polypeptide bands ranging from 100000 to 25000 in mol.wt., among which six major species of mol.wts. 78000, 74000, 55000, 42000, 40000 and 34000 could be distinguished (Plate 1, slot *d*).

Adsorption of KB-cell plasma-membrane extract on penton–immunoadsorbent

The second affinity model developed in this study consisted of an insolubilized immune complex bound to polyacrylamide beads. The immune complex was formed of penton + anti-(penton-base) antibody, linked to Bio-gel particles with glutaraldehyde. The structure of the complex was maintained by further glutaraldehyde cross-linking. In such a system, the fibre projections were oriented outwards as on the virus particle, the terminal knob on the fibre being accessible to proteins having an affinity for its electric charges and/or conformational structure (Fig. 1). Batch adsorption on this penton–immunoadsorbent of a plasma-membrane deoxycholate extract retained a fraction [eluted with 0.2M-glycine/HCl buffer (pH2.8)/1M-NaCl] inhibiting the adenovirus attachment with a high efficacy. Fig. 2(*b*) shows that at 37°C attachment of adenovirus was rapid and that 78% of the label was adsorbed in 30min, with an apparent adsorption rate constant of 12.8×10^{-3} ml$^{-1}\cdot$min^{-1}. When adenovirus particles (50μg of protein, 1.5×10^{12} physical particles) were preincubated with increasing amounts of retained material, 60–90% of the virus label remained in the supernatant, with apparent adsorption rate constants of 6.4×10^{-3}, 3.1×10^{-3} and 1.1×10^{-3} ml$^{-1}\cdot$min^{-1}, for respective protein amounts of 5, 10 and 15μg of retained fraction. This inhibition of attachment was destroyed by trypsin treatment, suggesting that this inhibiting factor was a protein.

The penton–immunoadsorbent retained 2–3% of the protein material extracted with deoxycholate from the cell plasma membrane. When [^{35}S]methionine- and [^{3}H]glucosamine-labelled plasma-membrane extract was adsorbed on the immunoadsorbent, 0.5–1.0% of the total ^{35}S and 1–1.25% of the total ^{3}H label was retained on the gel. Analysis of this retained material by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed four major

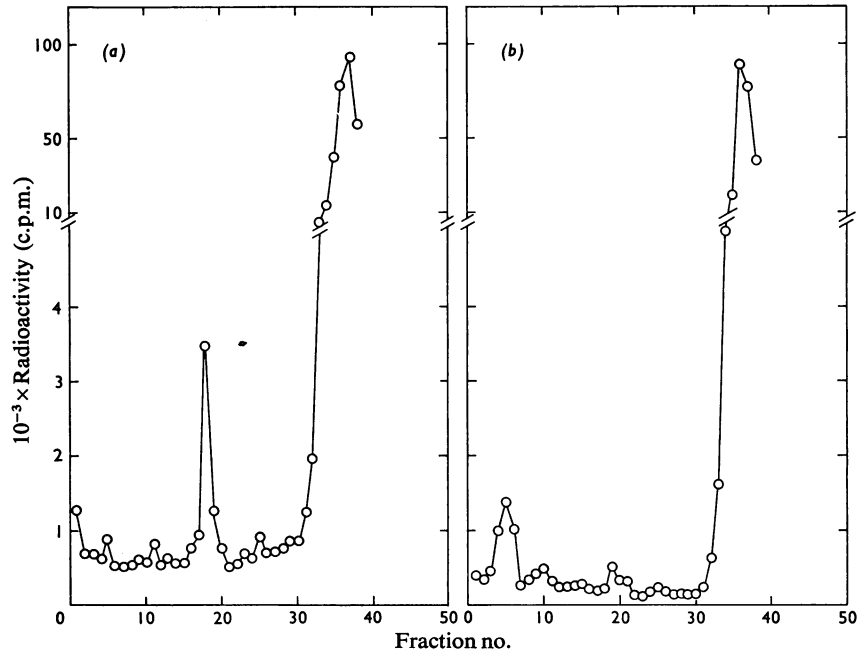
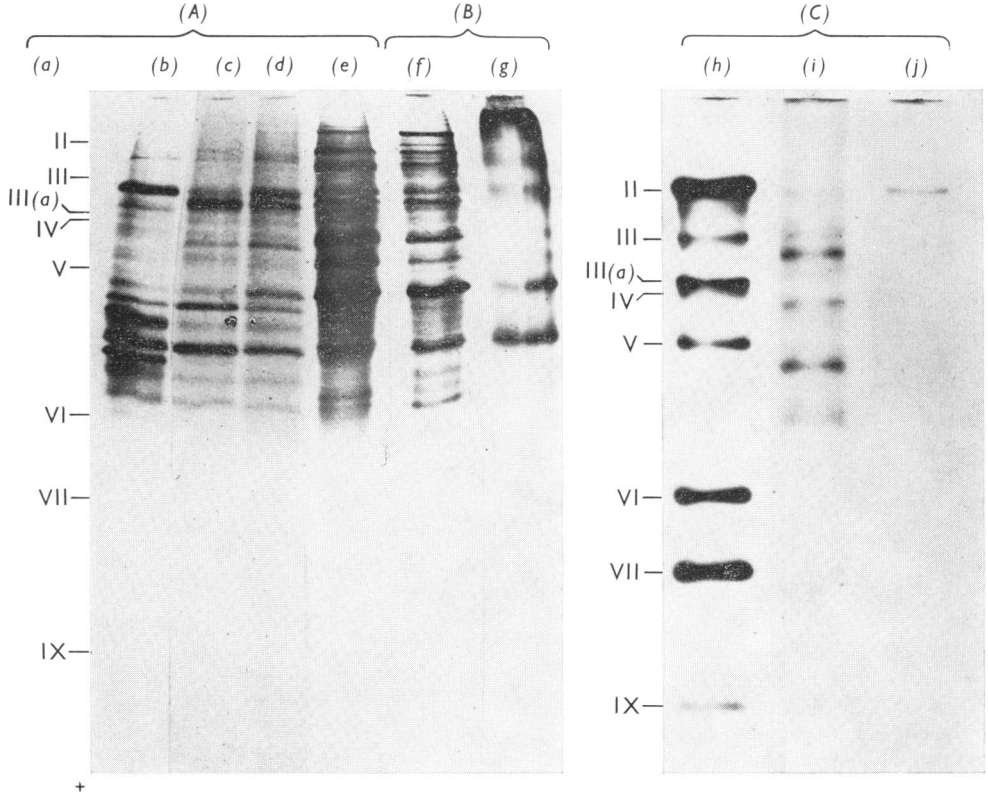


Fig. 3. *Isolation of cross-linked adenovirus-receptor complexes by velocity gradient centrifugation*
[^{35}S]Methionine-labelled KB cells saturated at 0°C with adenovirus particles (*a*) and control KB cells (*b*) were treated with cleavable di-imidoester and subjected to a Triton X-100 extraction. The resulting extracts were analysed on sucrose density gradients. Fractions (300μl) 17–20 of both gradients, corresponding to the position of adenovirus marker, were pooled, and analysed on sodium dodecyl sulphate/polyacrylamide gel, as shown in Plate 1.



EXPLANATION OF PLATE I

Polypeptide analysis on sodium dodecyl sulphate/polyacrylamide slab gel of penton-fibre-binding component(s) (*A*) Affinity chromatography on fibre–bis(aminopropyl)amino-Sepharose of deoxycholate extract from [^{35}S]methionine-labelled KB-cell plasma membranes. (*a*) Positions and mol.wts. of major adenovirus polypeptide markers visible on stained gel are: II, 120000; III, 85000; III(*a*), 68000; IV, 65000; V, 48500; VI, 24000; VII, 18500; IX, 12500. (*b*) Unadsorbed fraction eluted with 0.05M-NaCl. (*c*) Fraction eluted with 0.15M-NaCl. (*d*) Fraction eluted with 0.6M-NaCl. (*e*) Original deoxycholate extract. (*B*) Adsorption to penton–anti-(penton base)–immunoadsorbent: (*f*) unretained fraction; (*g*) retained fraction. (*C*) Cross-linking of adenovirus particles to KB-cell surface proteins: (*h*) ^{14}C-labelled adenovirion; (*i*) fractions 17–20 from gradient (*a*) of Fig. 3; (*j*) fractions 17–20 from gradient (*b*) of Fig. 3; slots (*a*)–(*g*) and (*h*)–(*j*) correspond to separate runs in two different gel systems: 17.5% acrylamide, ratio acrylamide:bisacrylamide 150:1, w/w, (*a*)–(*g*); 12% acrylamide, ratio acrylamide:bisacrylamide 37.5:1, w/w, (*h*)–(j).

protein subunits of apparent mol.wts. 100000, 78000, 42000 and 34000 (Plate 1, slot *g*).

Identification of the receptor components by cross-linking of adenovirus particles with KB-cell receptors

There are about 10^4 specific receptor sites for adenovirus type 2 on the KB-cell surface, and as many as 2×10^5 unlabelled adenovirus infectious particles per cell were incubated with [^{35}S]methionine-labelled KB cells, in order to saturate the sites on the host-cell membrane. After attachment of virus particles, carried out at 0°C to prevent virus engulfment (Lonberg-Holm & Philipson, 1974), the imidate function of the cleavable cross-linking reagent methyl 4-mercaptobutyrimidate hydrochloride was allowed to react with amino groups of proteins under reducing conditions. A mild oxidation formed disulphide linkages between neighbouring amidinated proteins on the receptor site and on the virus capsid.

By taking advantage of the sedimentation properties of adenovirus particles in a velocity gradient, the cross-linked virus–receptor complexes were extracted with Triton X-100 in iso-osmotic buffer and isolated by sucrose-gradient centrifugation. Most of the cellular protein label remained at the top of the gradient, but fractions containing radioactivity occurred in the position of free adenovirus marker (fractions 17–20, Fig. 3*a*). Since no radioactivity was detected in the corresponding gradient fractions of Triton extract from control KB cells, i.e. cells cross-linked without adenovirus particles adsorbed to their surface (Fig. 3*b*), it is probable that this label represented cell-surface proteins cross-linked to virus particles. The pooled fractions 17–20 represented 0.0015–0.002% of the total cellular [^{35}S]methionine label, and about 0.2% of the label in the original Triton extract of the membrane. The polypeptide composition of this labelled material cross-linked and co-sedimenting with adenovirus particles was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after heating for 2min at 100°C in 0.0625M-Tris/HCl buffer, pH6.8, containing 5% mercaptoethanol, 2% sodium dodecyl sulphate and 3M-urea. This latter treatment resulted in the cleavage of the disulphide cross-linkages. Plate 1 (slot *i*) shows that three major labelled cell protein subunits were found in the receptor–adenovirus complexes, of apparent mol.wts. 78000, 60000 and 42000, along with three discrete species of mol.wts. 115000, 88000 and 34000. The 115000-mol.wt. species was also found in the corresponding gradient fractions of the control cell extract (Plate 1, slot *j*): this protein subunit most probably represents cellular particulate material sedimenting like adenovirus particles. The five other species, of mol.wts. 88000, 78000, 60000, 42000 and 34000, seemed to be specific for adenovirus-attached cell-surface proteins.

Discussion

It has been shown by Butters & Hughes (1974, 1975) that the KB-cell plasma-membrane contains four major classes of glycoprotein subunits with apparent mol.wts. of about 90000, 70000, 60000 and 34000, easily extractable with sodium deoxycholate and Triton X-100. The specific receptors responsible for adenovirus adsorption were readily extracted under those conditions.

Two types of affinity-gel system have been compared in this study with regard to their ability to retain protein(s) of KB-cell plasma membrane involved in adenovirus attachment.

(i) Fibre–bis(aminopropyl)amino-Sepharose is the simplest affinity gel to obtain, considering the vast amount of fibre protein synthesized by the host cell and the relatively easy purification procedure. This material, however, appears to be poorly specific with regard to the protein species retained. The high percentage of [^{3}H]glucosamine label and [^{35}S]methionine label adsorbed on this gel was incompatible with the minor amount of protein receptor on the cell membrane: it has been calculated from binding data that the components responsible for the binding of adenovirus on the cell surface represent about 0.015% of the total KB-cell plasma-membrane protein (Hughes & Mautner, 1973). Sodium dodecyl sulphate/polyacrylamide-gel analysis of this retained material showed a polypeptide pattern too complex for us to assign the receptor function to a particular protein species. Nevertheless, affinity chromatography of a plasma-membrane extract on fibre–bis(aminopropyl)amino-Sepharose resulted in an enrichment of retained material in biologically active factor(s) inhibiting adenovirus attachment on KB cells, and in six polypeptide species of mol.wts. 78000, 74000, 55000, 42000, 40000 and 34000, compared with the original extract (Plate 1, slots *b–e*). The lack of specificity of the fibre–bis(aminopropyl)amino-Sepharose could be due in part to amino groups generated on the Sepharose by CNBr activation, and/or to unfavourable orientation of fibres.

(ii) The immunoadsorbent used in this study was a complex affinity gel consisting of anti-(penton base) antibodies insolubilized by cross-linking with glutaraldehyde on polyacrylamide particles, on which complete pentons were adsorbed and subsequently cross-linked. In such a structure, the fibre with its terminal knob was appropriately oriented outwards, as in the adenovirus particle. As expected, the yield of material adsorbed on the gel was much lower than on fibre–bis(aminopropyl)amino-Sepharose, but the specificity of binding seemed better: the immunoadsorbent selected four major protein subunits of apparent mol.wts. 100000, 78000, 42000 and 34000. This is in good agreement with the finding by Meager *et al.* (1976) of a major glycoprotein component of

approx. mol.wt. 75000, present in a partially purified KB-cell fluorocarbon extract, and retained on a fibre–Sepharose affinity column. The specific activity with respect to inhibition of adenovirus attachment was much higher with material eluted from penton–immunoadsorbent than with that eluted from fibre–bis(aminopropyl)amino-Sepharose.

(iii) To determine which polypeptide species were part of the specific receptor site for adenovirus, labelled KB cells were incubated with adenovirus particles at high multiplicity of infection to saturate the cell surface receptors (over 2×10^5 plaque-forming units/cell), and at low temperature to prevent adenovirus entry into the cell. Cross-linking of adenovirus particles attached to the cell receptors was carried out with a cleavable di-imidoester, and the adenovirus–receptor complexes, after extraction with the non-ionic detergent Triton X-100, were isolated by velocity-gradient centrifugation. The polypeptide composition of the receptor site, analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after cleavage of the disulphide cross-links, revealed three major polypeptide subunits of mol.wts. 78000, 60000 and 42000, along with two discrete species of 88000 and 34000. The 78000-, 42000- and 34000-mol.wt. subunits were therefore the three major polypeptide species common to the three selection systems. Although phenylmethanesulphonyl fluoride was added to all buffers, we cannot exclude the possibility that the 42000- and 34000-mol.wt. components might be proteolytic fragments of higher-molecular-weight glycoproteins, such as the 78000-mol.wt. fragment. The 42000-mol.wt. subunit could also correspond to actin subunits present in the cell membrane, which may be linked in some way with the glycoproteins or alternatively bind non-specifically to the fibre affinity column and the penton–immunoadsorbent.

In a model for virus attachment on cell membrane, proposed by Lonberg-Holm & Philipson (1974), virus might first interact reversibly with one or a few specific receptor subunits. In this case, the 78000-mol.wt. subunit retained on fibre-affinity gel and on penton–immunoadsorbent and found cross-linked with adenovirus particle would be a good candidate for this primary fibre-binding component. Additional receptor subunits able to diffuse through the cell membrane would further interact with the initial unstable virus–receptor complex to form multivalent binding. The results of the cross-linking reaction between adenovirus and its receptor do not allow us to assess whether the other subunits were present at the time of adsorption or appeared after primary interaction of the adenovirus fibre with the 78000-mol.wt. glycoprotein. However, the results of affinity chromatography and of immunoadsorption suggest that the 42000- and 34000-mol.wt. species are part of the primary fibre-binding structure. The 88000- and 60000-mol.wt. species could merely be neighbouring proteins cross-linked with the adenovirus-binding components. If the 42000-mol.wt. species corresponds to actin subunits, this would imply that actin tubules are closely related to adenovirus-receptor sites, and could be involved in adenovirus transport through the cytoplasm (Dales & Chardonnet, 1973).

The fact that di-imidoester reacting with amino groups on proteins was capable of cross-linking adenovirus to membrane receptor suggests, as previously observed (Neurath *et al.*, 1970; Boulanger *et al.*, 1972), that the carbohydrate moieties of the cell-surface glycoproteins are not directly involved in the adenovirus attachment, as also concluded by Meager *et al.* (1976).

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