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A Low-Molecular-Weight Protein from Rat Liver that Resembles Ligandin in its Binding Properties

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A protein of $s_{20,w}$ 1.6S and mol.wt. 14000, which binds covalently a metabolite of the aminoazodye carcinogen *NN*-dimethyl-4-amino-3'-methylazobenzene, was isolated from rat liver cytosol from both carcinogen-treated and normal rats. The protein binds non-covalently palmitoyl-CoA, fatty acids, bilirubin, sex steroids and their sulphates, bile acids and salts, bromosulphophthalein, diethylstilboestrol and 20-methylcholanthrene with a wide range of affinities. The protein is isolated as three components with isoelectric points of 5.0, 5.9 and 7.6 by a method involving isoelectric focusing. All three components have closely similar amino acid analyses, tryptic-peptide 'maps' and u.v. spectra. Each single component redistributes into all three on further electrophoresis. However, the three forms differ in their binding characteristics, the form of pI 7.6 having much the highest affinity for compounds bound non-covalently. The protein was identified immunologically in rat liver, small intestine, adipose tissue, skeletal muscle, myocardium and testis. The protein was compared with other hepatic binding-protein preparations of similar molecular weight.

The covalent binding of metabolites of aminoazodye hepatocarcinogen to liver protein was first observed by Miller & Miller (1947). Sorof *et al.* (1951) showed that, in the soluble supernatant, aminoazodye carcinogens were bound specifically to basic protein in what was called the 'h' fraction. Since then it has been shown that aminoazodye binds to only three principal proteins. Two were isolated by Ketterer *et al.* (1967) and the third by Sorof *et al.* (1972).

The best characterized of the three, aminoazodye-binding protein B (Ketterer *et al.*, 1967; Ketterer, 1971) is now called ligandin because of its wide range of binding affinities (Litwack *et al.*, 1971). The present paper concerns the low-molecular-weight aminoazodye-binding protein A, which was first described by Ketterer *et al.* (1967) and further reported on in brief by Ketterer *et al.* (1971). It was shown to bind aminoazodye carcinogen covalently through a methionine residue (Ketterer & Christodoulides, 1969) and to bind non-covalently several compounds also bound by ligandin (Ketterer *et al.*, 1975*a*).

Other low-molecular-weight binding proteins have also been described. Levi *et al.* (1969) identified a protein fraction from rat liver designated Z fraction, which bound bromosulphophthalein, bilirubin and thyroxine. Ockner *et al.* (1972) showed the presence of a protein in both the small intestine and liver which bound bromosulphophthalein and fatty acids. Mishkin & Turcotte (1974*a*,*b*) described a purification procedure for Z protein, its affinity for fatty acyl-CoA derivatives and its possible involvement in phospholipid biosynthesis. Warner & Neims (1975) also described a purified Z-protein preparation with affinities for bilirubin, thyroxine and hexachlorophene.

The aim of the present work was to characterize further the chemical and physical properties of protein A, to examine its binding specificity and affinities, to consider its possible roles in physiology and carcinogenesis and its relationship to other low-molecular-weight binding-protein preparations which have been reported. To assist in this comparison the binding properties of purified protein A were compared with those of a crude protein A, which was similar in its method of preparation to that of the Z fraction of Levi *et al.* (1969) and the fatty acid-binding protein fraction of Ockner *et al.* (1972).

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Materials and Methods

Chemicals

Unless otherwise stated all chemicals were analytical reagent grade and were obtained from BDH Chemicals, Poole, Dorset, U.K.

NN-Dimethyl-4-amino-3′-methylazobenzene and sodium dodecyl sulphate were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. *NN*-Dimethyl-4-amino-3′-methylazobenzene was purified before use by passage through alumina in benzene and recrystallization from methanol. Ampholines were obtained from LKB Instruments, Croydon, Surrey, U.K., Sephadex G-100 and Ficoll from Pharmacia (G.B.) Ltd., London W5 5SS, U.K., and acrylamide and *NN′*-methylenebisacrylamide from Kodak, Liverpool, U.K. Riboflavin, bovine serum albumin, ovalbumin, chymotrypsin, myoglobin, cytochrome *c*, sodium deoxycholate (3α,12α-dihydroxy-5β-cholan-24-oic acid; sodium salt), sodium cholate (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; sodium salt), sodium taurodeoxycholate (3α,12α-dihydroxy-5β-cholan-24-yl taurine, sodium salt), potassium dehydroepiandrosterone sulphate (potassium 3-sulpho-oxy-androst-5-en-17-one), potassium oestrone sulphate [potassium sulpho-oxy-oestra-1,3,5(10)-trien-17-one] and Triton X-100 were purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Disodium bromosulphophthalein [disodium 3,4-(1,2,5,6-tetrabromobenz)-5,5-bis-(4-hydroxyphenyl-3-sulphonate)furan-2-one], Naphthalene Black, Coomassie Blue and Carbowax 20M were obtained from G. T. Gurr, High Wycombe, Bucks., U.K. Pronase B was obtained from Calbiochem, San Diego, CA, U.S.A. Toluene and 2,5-diphenyloxazole were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. Agarose was from Miles Seravac Pty., Maidenhead, Berks., U.K. The following radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.: potassium [6,7-^{3}H]oestrone sulphate (potassium 3-sulpho-oxy-[6,7-^{3}H]oestra-1,3,5(10)-trien-17-one), specific radioactivities 480 and 5800Ci/mol; potassium dehydro-[7-^{3}H]epiandrosterone sulphate (potassium 3β-sulpho-oxy-[7-^{3}H]androst-5-en-27-one), 4600Ci/mol; [9,10-^{3}H]palmitic acid ([9,10-^{3}H]hexadecanoic acid), 500Ci/mol; [9,10-^{3}H]oleic acid (*cis*-Δ^9-[9,10-^{3}H]-octadecenoic acid), 2500Ci/mol; [1,2-^{3}H]cortisol (11β,17α,21-trihydroxy[1,2-^{3}H]pregn-4-ene-3,20-dione), 42000Ci/mol: [6,7-^{3}H]oestrone (3-hydroxy-[6,7-^{3}H]oestra-1,3,5(10)-trien-17-one), 46000Ci/mol; [6,7-^{3}H]oestradiol ([6,7-^{3}H]oestra-1,3,5,(10)-trien-3,17β-diol), 45000Ci/mol; [1,2-^{3}H]testosterone (17β-hydroxy-[1,2-^{3}H]androst-4-en-3-one), 42000Ci/mol; [G-^{3}H]20-methylcholanthrene, 10500Ci/mol; [G-^{3}H]diethylstilboestrol ([G-^{3}H]3,4-bis-(4-hydroxyphenyl)hex-3-ene), 11000Ci/mol; disodium bromo-[^{35}S]sulphophthalein (disodium 3,4-(1,2,5,6-tetrabromobenz)-5,5-bis-(4-hydroxyphenyl-3-[^{35}S]sulphonate)furan-2-one), 25.3Ci/mol. [1-^{14}C]Palmitoyl-CoA (60Ci/mol) was from NEN Chemicals G.m.b.H., Frankfurt, West Germany.

Animals

Liver was taken from male Wistar rats inbred in the Courtauld Institute of Biochemistry and weaned on to a vitamin-supplemented Rowett 86 diet (Thompson, 1936) supplied by the North Eastern Agricultural Co-operative Society, Aberdeen, Scotland, U.K. *NN*-Dimethyl-4-amino-3′-methylazobenzene in solution in corn oil was administered at a dose of 250mg/kg body wt. by intraperitoneal injection. Animals were killed 16–48h later by cervical dislocation. Livers were perfused with cold 0.25M-sucrose, and, after passage through a tissue press, a 1:3 (w/v) homogenate was prepared for isolation of aminoazodye-binding protein. For studies on whole soluble supernatant fraction, a 1:1 (w/v) homogenate was prepared. The soluble supernatant was obtained from these homogenates by centrifugation for 90min at 30000rev./min in a Spinco 30 rotor.

Preparative isoelectric focusing

Protein fractions were dialysed against water and subjected to isoelectric focusing in the LKB 8100 Uniphor apparatus. A linear 0–40% (w/w) sucrose density gradient composed of twenty-one 5ml steps was set up. This gradient was 1% (w/v) with respect to Ampholine pH3.5–10.0 and contained the sample of protein distributed evenly throughout. The separation was carried out at 4°C over 5 days. On the first day the potential difference was 500V and for the remaining 4 days was increased to 1000V. Fractions (2ml) were collected and their pH values and absorbances at both 280 and 460nm determined. Optical absorption at 460nm was taken as an index of bound aminoazodye.

Ampholines were removed by passage through a column (90cm×2.6cm) of Sephadex G-100 equilibrated with the buffer 0.1M-KCl/0.025M-K_2HPO_4 adjusted to pH7.0 with H_3PO_4, or by exhaustive dialysis against the same buffer.

Polyacrylamide-gel electrophoresis in 0.1% *sodium dodecyl sulphate*/8M-*urea*

Protein separation according to polypeptide molecular weight was performed by the method of Shapiro *et al.* (1967) as modified by Tasserson *et al.* (1970). This was used as a test of purity and a means of checking the value for the molecular weight determined previously (Ketterer *et al.*, 1967). The rate of electrophoresis in this system bears a linear relation to the log of the molecular weight. Bovine serum albumin,

ovalbumin, α-chymotrypsin, horse heart myoglobin and bovine heart cytochrome *c* were used as standard proteins. Their mol.wts. were taken to be 67000, 43500, 21600, 16890 and 12000 respectively (Sober, 1968).

Isoelectric focusing in polyacrylamide gel

Isoelectric focusing in polyacrylamide gel was performed by a method based on those described by Wrigley (1969). The Shandon disc electrophoresis apparatus (Shandon Southern Instruments, Camberley, Surrey, U.K.) was modified to take tubes 11cm long. Into each tube was poured a solution containing 0.75ml of acrylamide solution, 30% (w/v) with respect to acrylamide and 1% (w/v) with respect to *NN'*-methylenebisacrylamide, 0.075ml of Ampholine pH5–8, 0.2ml of 1.4% (w/v) riboflavin, 0.25ml of approx. 0.5% (w/v) protein solution and 1.75ml of water. Gels were set by photopolymerization. Electrophoresis was carried out over 4h with 0.2% (v/v) H_2SO_4 as anode solution and 0.4% (v/v) ethanolamine as cathode solution. During this time a potential of 125V rising to 300V was applied. Gels were washed overnight with 5% (w/v) trichloroacetic acid to remove Ampholine and then stained with 0.1% (w/v) Coomassie Blue in acetic acid/ethanol/water (2:9:9, by vol.) and washed in acetic acid/ethanol/water (2:5:13, by vol.).

Immunology

Antiserum to the pI7.6 protein component emulsified with Freund's adjuvant was raised in the rabbit. Tissue samples from various sources were tested for the presence of protein by immunodiffusion as described by Ouchterlony (1958).

Amino acid analysis

The amino acid analysis of separated protein components was determined in hydrolysates prepared by heating freeze-dried material *in vacuo* in constant-boiling HCl for 24 and 48h at 105°C. Hydrolysates were analysed chromatographically on a micro scale by using the modification by Beale & Kent (1968) of the method of Spackman *et al.* (1958). The tryptophan content was determined by the method of Beaven & Holiday (1952).

Tryptic peptides

Protein fractions containing approx. 4mg of protein in 0.1M-NH_4HCO_3 were heated briefly in a boiling-water bath, digested with approx. 0.04mg of trypsin for 4h, freeze-dried and taken up in 0.1ml of pyridine/acetic acid/water (25:1:225, by vol.). A certain amount of the digest remained insoluble and was removed by centrifugation. The soluble peptides were subjected to high-voltage electrophoresis in the above solvent in one dimension and chromatography in butanol/acetic acid/ pyridine/water (15:3:10:12, by vol.) in the second dimension. Peptides were made visible by spraying with 0.2% (w/v) ninhydrin in acetone and allowing colour to develop overnight.

Analytical ultracentrifugation

Ultracentrifugal analysis was performed in the Spinco model E ultracentrifuge at 63650rev./min and a mean temperature of 18°C.

Spectrophotometry

All measurements were made in 1cm cells at room temperature with a Pye Unicam SP.1800 u.v. spectrophotometer.

Protein concentration

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Bovine serum albumin concentrations were determined from the extinction coefficient $E^{1\%}_{279} = 6.7$ (Reynolds *et al.*, 1967).

Measurement of radioactivity

Radioactivity was counted in a Packard Tri-Carb 3375 liquid-scintillation spectrometer. Batches of scintillation fluid were prepared by dissolving 10g of 2,5-diphenyloxazole in 2.5 litres of Triton X-100/toluene (1:4, by vol.). Quench correction was made by the external-standard method. A minimum of 1000 counts was collected for each sample.

Equilibrium dialysis

Equilibrium-dialysis measurements were made by using polytetrafluoroethylene dialysis cells made in our workshop and consisting of two hollowed-out discs separated by the dialysis membrane and clamped together. The two chambers so formed can each hold up to 0.3ml of solution. The ratio of membrane surface area to total solution volume is $5cm^{-1}$. Membranes cut from 18/32 Visking tubing were boiled in 1% (w/v) Na_2CO_3 solution and rinsed thoroughly in water before use. Experiments were carried out at 4 (±1)°C.

Stock solutions of proteins and all ligands except bilirubin and the bile acids were prepared in 0.1M-KCl/0.025M-K_2HPO_4 adjusted to pH7.0 with H_3PO_4. Bilirubin and the bile acids were first dissolved in a small quantity of 0.1M-KOH, and the resulting solutions were adjusted to pH7.0 with H_3PO_4 and then diluted with 0.1M-KCl/0.025M-K_2HPO_4 adjusted to pH7.0 with H_3PO_4.

Equilibrium was attained within 20h for all labelled ligands except for palmitoyl-CoA, which required 72h. In trial experiments with no protein present, bilirubin (a potential competitor for labelled ligands) was found not to diffuse through the membrane and substantial amounts were adsorbed by both membrane and dialysis cell.

After equilibration, a 0.1ml sample of solution from each side of the membrane was taken for determination of radioactivity and thence the concentrations of radioactive ligand.

Analysis of binding results

Results for the binding of a single ligand were analysed by the method of Scatchard (1949). Association constants for non-radioactive compounds competing for labelled ligands were derived by using eqns. (1) and (2), which hold for two ligands A and B competing for the same class of n binding sites on a protein (Steinhardt & Reynolds, 1969).

$$\frac{\bar{\nu}_A}{L_A} = (n - \bar{\nu}_A - \bar{\nu}_B)K_A \quad (1)$$

$$\frac{\bar{\nu}_B}{L_B} = (n - \bar{\nu}_A - \bar{\nu}_B)K_B \quad (2$$

where $\bar{\nu}_i$ is the number of molecules of substance i bound per molecule of protein, L_i is the free concentration of substance i, n is the number of binding sites per molecule of protein and K_i is the intrinsic association constant of substance i. Rearrangement of eqn. (1) gives:

$$\bar{\nu}_B = n - \bar{\nu}_A - \frac{\bar{\nu}_A}{K_A L_A} \quad (3)$$

If n and K_A are already known from a direct binding experiment and $\bar{\nu}_A$ and L_A are the measured quantities then $\bar{\nu}_B$ can be calculated. If the total amount of B is known then L_B can be calculated. Combination of eqns. (1) and (2) gives:

$$K_B = \frac{\bar{\nu}_B K_A L_A}{\bar{\nu}_A L_B} \quad (4)$$

Results

Preparation of low-molecular-weight aminoazodye-binding protein

The method used was a simplification of that described by Ketterer *et al.* (1967), where the low-molecular-weight protein was obtained as a by-product of the preparation of aminoazodye-bound ligandin (Litwack *et al.*, 1971). The soluble supernatant was dialysed against 2mM-triethanolamine/HCl, pH8.0; a precipitate was removed by centrifugation and the supernatant was passed through a DEAE-cellulose column (2.6cm×30cm) equilibrated with the above buffer. Part of the low-molecular-weight protein was retained by this column and was eluted with buffer made 0.05M with respect to NaCl. The rest passed through this column and also through a CM-Sephadex C-50 column equilibrated with 0.01M-sodium acetate buffer, pH5.8, 0.04M with respect to NaCl. The two fractions were combined and reduced in volume by dialysis against a concentrated solution of Carbowax. The concentrate was applied to a column (2.6cm×90cm) of Sephadex G-100 equilibrated with 0.1M-sodium phosphate buffer, pH7.4, made 0.2M with respect to NaCl. Three protein fractions were eluted; that of largest elution volume (approx. 350ml) contained the low-molecular-weight aminoazodye-binding protein (see Fig. 1).

This fraction was then dialysed and subjected to preparative isoelectric focusing. Three aminoazodye-bound fractions were obtained with isoelectric points of pH5.0 (form I), 5.9 (form II) and 7.6 (form III) (see Fig. 2). Similar patterns were obtained when the soluble supernatant fraction from normal livers and carcinogen-treated livers were subjected to the same isolation procedure.

Amino acid analysis of the three aminoazodye-binding fractions

Forms I, II and III were analysed for amino acids (Table 1). Tryptophan was not detected in the soluble tryptic peptides, nor in a chromatogram of a Pronase digest of insoluble tryptic peptides, nor by the spectrophotometric method of Beaven & Holiday (1952).

'Map' of soluble tryptic peptides

Forms I, II and III gave identical tryptic-peptide 'maps'; an example is shown in Plate 1. Approximately 18 peptides were obtained, which is in accord

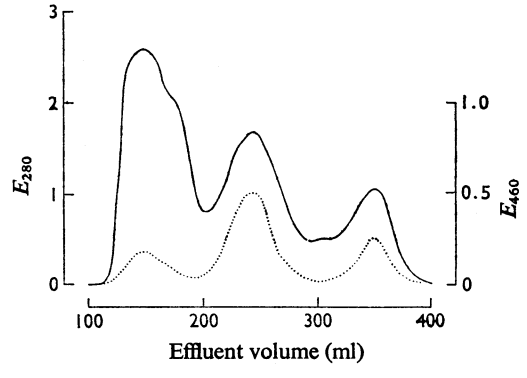
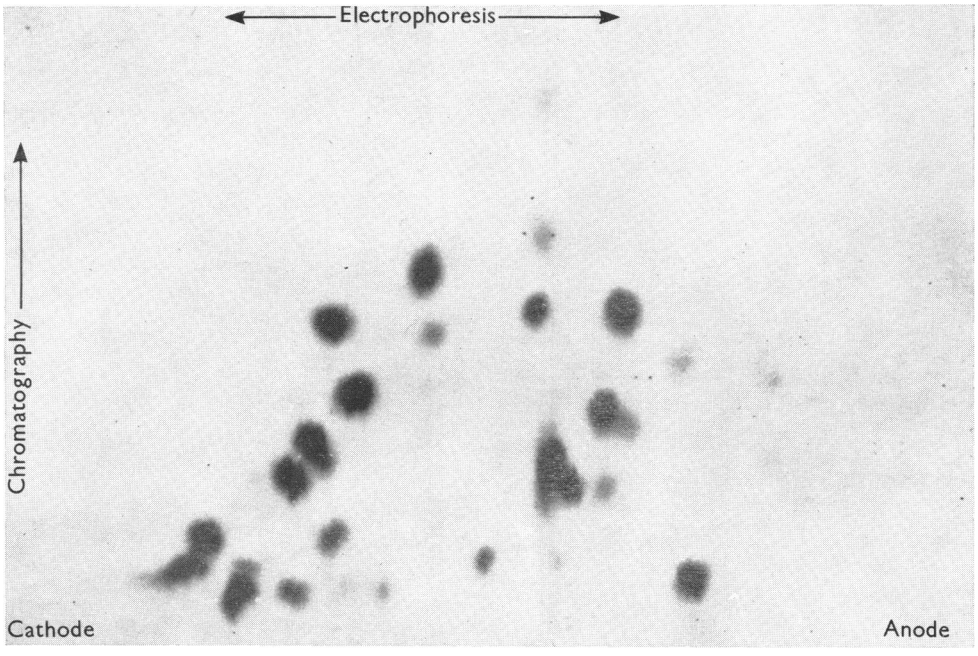


Fig. 1. *Sephadex G-100 gel filtration of the concentrated aminoazodye-binding protein A fractions from ion-exchange chromatography*

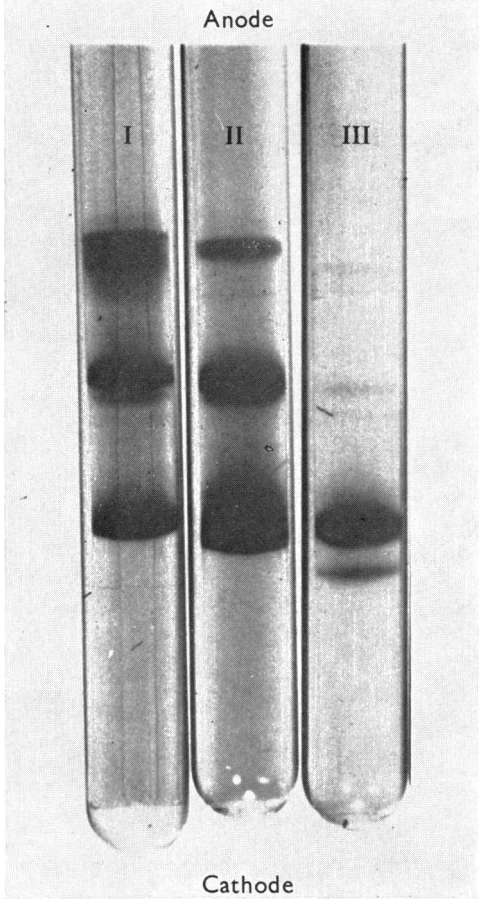
——, E_{280} (protein); ····, E_{460} (dye). For details, see the text.



EXPLANATION OF PLATE 1

'Map' of soluble tryptic peptides from aminoazodye-binding protein A

See the text for details.



EXPLANATION OF PLATE 2

Gel isoelectric focusing of purified aminoazodye-binding protein A forms I, II and III

See the text for details.

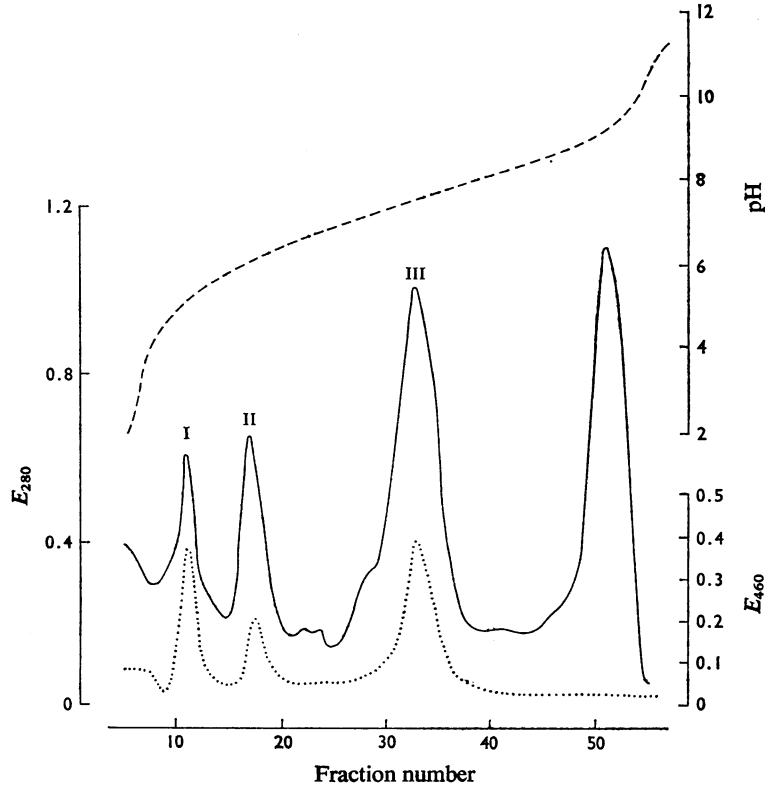


Fig. 2. *Isoelectric focusing of aminoazodye-binding protein A forms I, II and III*

——, E_{280} (protein); · · · ·, E_{460} (dye); – – – –, pH. For details, see the text.

with the number of lysine and arginine residues that can be calculated to be present per molecule of mol.wt. 14000, the value found by Ketterer *et al.* (1967).

U.v.-absorption spectrum

Each form of protein A, prepared from normal animals, gave an identical u.v. spectrum characteristic of a protein containing approx. 5 and 2 residues per 100 residues of phenylalanine and tyrosine respectively and no tryptophan. When protein A was prepared from *NN*-dimethyl-4-amino-3′-methylazobenzene-treated animals there was also an absorption maximum at 400nm, due to bound carcinogen.

Refocusing of the three components

Each component was refocused by zone electrophoresis either in polyacrylamide gels or on a sucrose density gradient and was found to redistribute so that the three original components were regained. There was, however, the tendency to give more of the pI7.6 component (see Plate 2).

Sodium dodecyl sulphate / urea / polyacrylamide-gel electrophoresis

When subjected to sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis each form of protein A gave rise to a single band, indicating that it did not consist of subunits. By comparison with standard proteins a mol.wt. of 14000 was estimated.

Sedimentation in the ultracentrifuge

In 0.15M-NaCl / 0.01M-Tris/HCl buffer, pH7.0, a 0.6% (w/v) solution of form III sedimented as a single boundary with $s_{20,w}$ 1.6S.

Binding results for the purified protein (form III)

All measurements were made on protein prepared from normal rats.

Direct measurements. Data for the binding of several ligands by protein A, plotted by the method of Scatchard (1949), are shown in Fig. 3. For both

Table 1. *Amino acid compositions of aminoazodye-binding protein A forms I, II and III*

nd, Not determined.

Amino acid residue	Form ...	Content (residues/100 residues) I	II	III	Approx. residues per mol. (mol.wt. 14000)
Gly		9.9	10.3	9.4	12
Ala		1.9	1.7	1.9	2
Val		8.5	8.2	7.9	10
Leu		5.1	4.8	5.1	6
Ileu		6.2	6.0	6.0	7
Phe		4.9	4.8	5.1	6
Trp		0	0	0	0
Tyr		1.9	2.0	1.9	2
Pro		nd	1.6	nd	2
Met		4.4	4.2	5.0	6
Cys		nd	1.6	nd	2
Ser		5.5	4.4	5.2	6
Thr		10.2	9.5	10.1	13
Asp		8.9	9.2	9.7	12
Glu		14.5	13.8	15.2	19
His		1.5	1.5	1.6	2
Lys		15.1	14.6	14.1	18
Arg		1.8	1.6	1.8	2

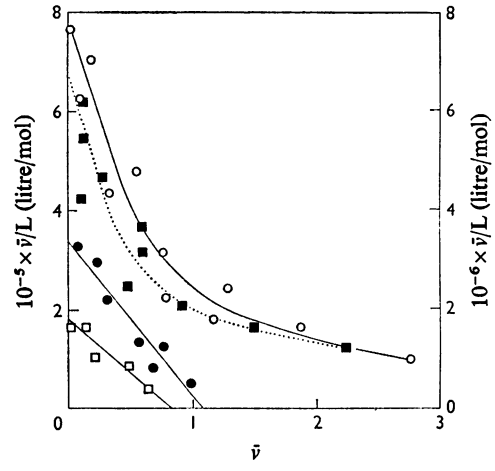


Fig. 3. *Scatchard plots for the binding of bromosulphophthalein* (○), *oestrone sulphate* (●), *dehydroepiandrosterone sulphate* (□) *and palmitoyl-CoA* (■) *by aminoazodye-binding protein A form III*

The left-hand axis (——) refers to bromosulphophthalein, oestrone sulphate and dehydroepiandrosterone sulphate; the right-hand axis (· · · ·) refers to palmitoyl-CoA.

oestrone sulphate and dehydroepiandrosterone sulphate there was a single binding site with association constants of 3×10^5 litre/mol and 2×10^5 litre/mol respectively. Bromosulphophthalein gave a more complex plot, indicative of a primary binding site with $K=7\times10^5$ litre/mol and several weaker sites. Palmitoyl-CoA also gave a complex Scatchard plot, indicating a primary site with $K=7\times10^6$ litre/mol and several weaker sites.

For ligands with low aqueous solubility and relatively low binding affinity, e.g. the fatty acids and unconjugated steroids, it was not possible to obtain the higher values of $\bar{\nu}$ necessary to construct complete Scatchard plots. In such cases measurements were made of $\bar{\nu}/L$ at low values of $\bar{\nu}$ (<0.01). Under these conditions, if a single class of binding sites is assumed, as found for oestrone sulphate and dehydroepiandrosterone sulphate, then the Scatchard equation

$$\frac{\bar{\nu}}{L} = (n-\bar{\nu})K$$

becomes

$$\frac{\bar{\nu}}{L} = nK$$

and $\bar{\nu}/L$ values are then directly proportional to the association constant. However, the assumption of a single class of binding sites may not be justified for all ligands (see, for example, the Scatchard plots for bromosulphophthalein and palmitoyl-CoA). Nevertheless, the parameter $\bar{\nu}/L$ at low $\bar{\nu}$ is a useful index of binding affinity, since it represents, in the absence of co-operative effects, the maximum ratio of bound to free ligand. Values for $\bar{\nu}/L$ at low $\bar{\nu}$ for a number of ligands are shown in Table 2.

Competition experiments. An excess (2000-fold) of non-radioactive oestrone sulphate was found to decrease markedly the binding of all ligands except palmitoyl-CoA (see Table 2). A number of unlabelled ligands were found to decrease the binding of [^{3}H]-oestrone sulphate. The association constants for these unlabelled ligands were calculated as described in the Materials and Methods section (see Table 3). The values obtained for oestrone sulphate, dehydroepiandrosterone sulphate and bromosulphophthalein are in good agreement with the directly measured quantities (see above). The value for palmitoyl-CoA is an order of magnitude lower than that obtained directly.

The estimated K for bilirubin was calculated assuming that there was no dialysis of this substance, as was observed in trial experiments in the absence of protein. Because of the adsorption of bilirubin to both membrane and dialysis cell, and its tendency to form aggregates at pH 7.0 (Brodersen & Theilgaard, 1969) the free monomeric concentration of bilirubin at equilibrium is certainly much lower than that calculated by subtracting bound from total bilirubin in the dialysis experiment. Therefore the true association constant is probably much greater than that shown in Table 3.

Table 2. *Binding of various ligands to purified aminoazodye-binding protein A form III (pI7.6) and to crude protein A, and competition effects with oestrone sulphate*

Results for the purified protein are expressed as $\bar{\nu}$/L (mol of ligand bound/mol of protein per free ligand concentration) (litre/mol), those for the crude protein as B/L (mol of ligand bound/g of soluble supernatant protein per free ligand concentration) (litre/g). The concentration of purified protein was 21 μM and that of the crude protein was 0.42 mg/ml. The concentration of radioactive ligand was 10 nM in all cases. In competition experiments with purified protein the concentration of oestrone sulphate was 21 μM, and in those with crude protein it was 100 μM.

	Purified protein		Crude protein	
Ligand	$10^{-5}\times\bar{\nu}$/L	$10^{-5}\times\bar{\nu}$/L (excess of oestrone sulphate)	B/L	B/L (excess of oestrone sulphate)
[^{14}C]Palmitoyl-CoA	67	70	9.3	9.1
Bromo[^{35}S]sulphophthalein	7.6	2.2	1.0	—
[^{3}H]Oestrone sulphate	3.4	1.3	0.36	0.10
[^{3}H]Diethylstilboestrol	3.2	1.3	0.36	—
[^{3}H]20-Methylcholanthrene	3.0	1.1	0.32	—
[^{3}H]Oestradiol	2.8	1.2	0.21	0.06
[^{3}H]Oleate	2.5	1.0	0.20	0.04
[^{3}H]Oestrone	2.4	0.8	0.15	—
[^{3}H]Dehydroepiandrosterone sulphate	1.9	0.7	0.20	—
[^{3}H]Palmitate	1.0	0.3	0.06	0.01
[^{3}H]Testosterone	1.0	0.25	0.06	—
[^{3}H]Cortisol	0	0	0.01	—

Table 3. *Competition by unlabelled ligands for [^{3}H]oestrone sulphate binding to purified aminoazodye-binding protein A form III (pI 7.6) and to crude protein A*

Results for the purified protein are expressed as $\bar{\nu}$/L (mol of oestrone sulphate bound/mol of protein per free oestrone sulphate concentration) (litre/mol), and those for the crude protein as B/L (mol of oestrone sulphate bound/g of soluble supernatant protein per free oestrone sulphate concentration) (litre/g). The concentration of purified protein was 21 μM, and that of crude protein 0.30 mg/ml. The concentration of [^{3}H]oestrone sulphate was 10 nM in all cases; the concentration of competitors in experiments with purified protein was 32.5 μM, and in experiments with crude protein it was 10 μM. K_c is the association constant in litre/mol estimated from competition results, assuming one binding site per mol of protein and an association constant for oestrone sulphate binding of 3.1×10^{5} litre/mol (see the text). K is the association constant determined directly. K_c for bilirubin was calculated assuming there was no diffusion of bilirubin through the dialysis membrane and no adsorption of bilirubin from solution (see the text).

	Purified protein			Crude protein
Competitor	$10^{-5}\times\bar{\nu}$/L	$10^{-5}\times K_c$	$10^{-5}\times K$	B/L
None	2.5	—	—	0.41
Palmitoyl-CoA	0.50	5.9	70	0.26
Bromosulphophthalein	0.50	5.9	7.0	0.05
Oestrone sulphate	0.79	2.9	3.1	—
Bilirubin	0.60	2.3	—	—
Dehydroepiandrosterone sulphate	1.07	1.6	2.2	—
Deoxycholate	2.00	0.25	—	—
Taurodeoxycholate	2.05	0.22	—	—
Cholate	2.32	0.10	—	—

Variation in binding affinity among the three isomers. Values of $\bar{\nu}$/L at $\bar{\nu}<0.01$ for the binding of several ligands by the three forms of aminoazodye-binding protein A are shown in Table 4. In each case the affinity rises in the order form I < II < III.

Binding results for crude protein A

[^{3}H]Oestrone sulphate (5 nmol) was added to 4 ml of supernatant from a 1:1 (w/v) homogenate of rat liver, and the resulting solution was applied to a column (2.6 cm × 90 cm) of Sephadex G-100 equi-

Table 4. *Variations in binding affinity among the three forms of aminoazodye-binding protein A separated by isoelectric focusing*

The protein concentration was 10–50 μM. Concentrations of ligand were 0.1 μM or less. See the text for details.

Ligand	$10^{-5} \times \bar{\nu}$/L (litre/mol) Form I (pI5.0)	Form II (pI5.9)	Form III (pI7.6)
Bromo[^{35}S]sulphophthalein	0.45	1.1	7.6
[^{3}H]Oestrone sulphate	0.09	0.45	3.4
[^{3}H]Dehydroepiandrosterone sulphate	0.07	0.35	1.9
[^{3}H]Palmitate	0.16	0.37	1.0

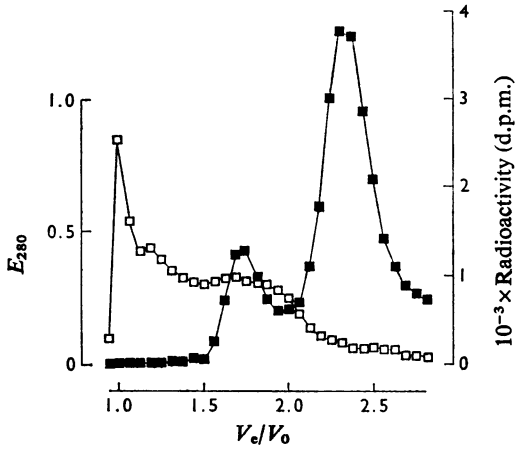


Fig. 4. *Sephadex G-100 gel filtration of rat liver soluble supernatant+[^{3}H]oestrone sulphate*

□, E_{280} (protein); ■, radioactivity (d.p.m./0.2ml of each fraction). See the text for details.

librated with 0.1M-KCl/0.025M-K_2HPO_4 adjusted to pH7.0 with H_3PO_4; 8ml fractions were collected. The elution pattern is shown in Fig. 4. The low-molecular-weight-binding fraction emerged from the column at a V_e/V_0 range of 2.0–2.8. This fraction, referred to as crude protein A, was subsequently prepared in the absence of added ligand and used as a source of binding protein for equilibrium-dialysis experiments.

Direct binding measurements. Scatchard plots for the binding of bromosulphophthalein, oestrone sulphate, dehydroepiandrosterone sulphate, palmitate and palmitoyl-CoA to crude protein A are shown in Fig. 5. For all ligands except palmitate the plots are curved, showing heterogeneity of binding sites. For palmitate a complete plot could not be obtained

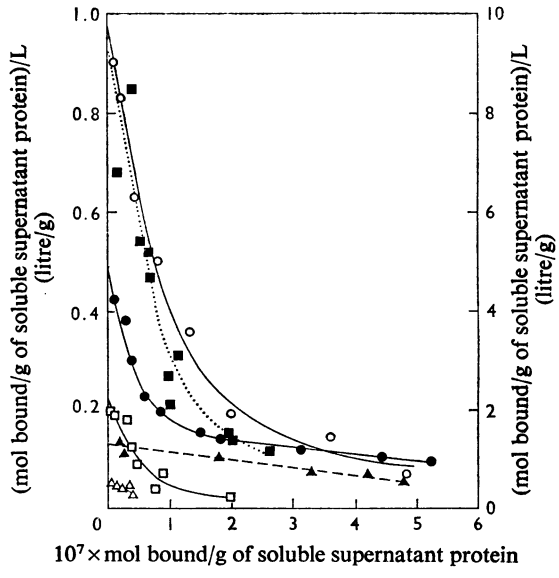


Fig. 5. *Scatchard plots for the binding of bromosulphophthalein, oestrone sulphate, dehydroepiandrosterone sulphate, palmitate and palmitoyl-CoA by crude aminoazodye-binding protein A*

The left-hand axis (——) refers to bromosulphophthalein (○), oestrone sulphate (●), dehydroepiandrosterone sulphate (□) and palmitate (△); the right-hand axis (····) refers to palmitoyl-CoA (■). ▲--▲, Scatchard plot for the binding of oestrone sulphate after exhaustive dialysis of crude protein A (left-hand axis).

because of its low aqueous solubility. The results suggest that for all the ligands there is a class of strong binding sites with a capacity of approx. 0.1 μmol/g of total soluble supernatant protein. Approximate association constants for these strong sites are bromosulphophthalein 8×10^6 litre/mol, oestrone sulphate 4×10^6 litre/mol, dehydroepiandrosterone sulphate 2×10^6 litre/mol, palmitate 10^6 litre/mol and palmitoyl-CoA 7×10^7 litre/mol.

Table 2 shows values of B/L at low B (B = mol of ligand bound/g of soluble supernatant protein) for the binding of a number of ligands to crude protein A. The parameter B/L is analogous to $\bar{\nu}$/L for the purified protein.

Competition studies. Also shown in Table 2 are results of competition experiments carried out in the presence of oestrone sulphate. Binding was decreased for oestradiol, oleate, palmitate and oestrone sulphate itself, but not for palmitoyl-CoA.

Table 3 shows results of experiments in which competition by bromosulphophthalein and palmitoyl-CoA for oestrone sulphate was examined. Both sub-

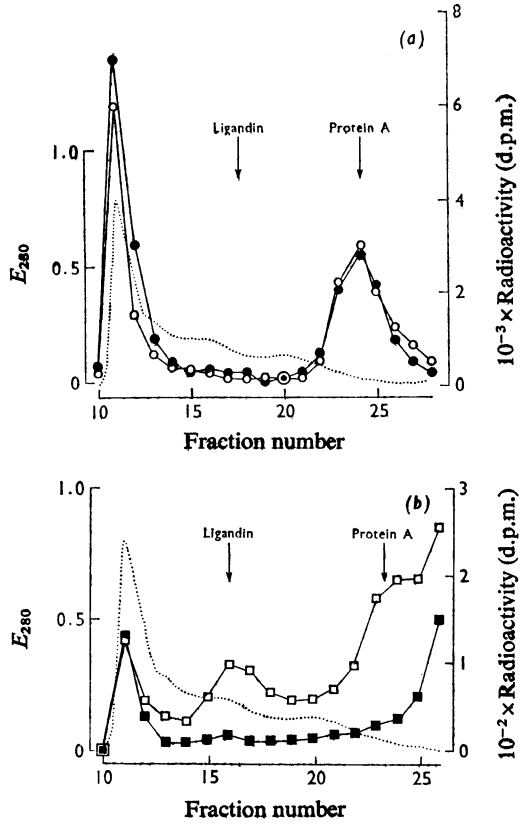


Fig. 6. *Effects of oestrone sulphate on the binding of* [^{14}C]*-palmitoyl-CoA and* [^{3}H]*oleate to rat liver soluble supernatant protein fractions measured by Sephadex G-100 gel filtration*

(*a*) Palmitoyl-CoA; ○, radioactivity (d.p.m./0.1 ml of each fraction) in the absence of oestrone sulphate; ●, radioactivity (d.p.m./0.1 ml of each fraction) in the presence of 1 mM-oestrone sulphate; ····, E_{280} (protein). (*b*) Oleate; □, radioactivity (d.p.m./0.1 ml of each fraction) in the absence of oestrone sulphate; ■, radioactivity (d.p.m./0.1 ml of each fraction) in the presence of 1 mM-oestrone sulphate; ····, E_{280} (protein). See the text for details.

stances decreased the binding of oestrone sulphate, but bromosulphophthalein was the more effective.

Competition effects with crude protein A were also studied by gel filtration on Sephadex G-100. Samples (0.5 ml) of soluble supernatant from a 1:1 (w/v) homogenate of rat liver were mixed with 0.5 ml samples of radioactive oleate or palmitoyl-CoA and subjected to gel filtration on columns (1.6 cm × 50 cm) of Sephadex G-100, equilibrated with either 0.1 M-KCl/0.025 M-K_2HPO_4 adjusted to pH 7.0 with H_3PO_4 or 1 mM-oestrone sulphate/0.1 M-KCl/0.025 M-K_2HPO_4 adjusted to pH 7.0 with H_3PO_4 (see Fig. 6). Oestrone sulphate markedly decreased the binding of oleate to the two binding fractions eluted after the void volume, but had little effect on palmitoyl-CoA binding.

Dialysis of crude protein A. After dialysis of a 5 ml sample of crude protein A (0.5 mg of protein/ml) against two changes of 5 litres of 0.1 M-KCl/0.025 M-K_2HPO_4 adjusted to pH 7.0 with H_3PO_4 the strong binding sites for oestrone sulphate were lost (see Fig. 5). A similar result was obtained for dehydroepiandrosterone sulphate.

Discussion

The protein described in this paper is unusual in that it exists in three forms with pI values as widely separated as 5.0, 5.9 and 7.6. That the three forms are identical polypeptides is shown by their closely similar amino acid analyses, identical tryptic-peptide 'maps' and u.v. spectra, and by the observation that, after isolation, each one of the isoelectric forms gave rise to a mixture of all three. It is difficult to envisage what could bring about such large differences in pI among these forms. That they result from the binding of Ampholines seems unlikely, since a similar electrochemical heterogeneity was observed when this protein was first isolated without their use (Ketterer *et al.*, 1967). As well as being of physicochemical interest this phenomenon may be of physiological importance, since the non-covalent binding affinity for all ligands so far studied increases in the order form I<II<III. Preliminary immunochemical experiments suggest that these forms are not artifacts of purification but may also exist in unfractionated soluble supernatant fraction.

The binding experiments show that aminoazodye-binding protein A (form III) has a number of ligands in common with ligandin (aminoazodye-binding protein B) (Ketterer *et al.*, 1975*a*,*b*) and similar affinities for many of these compounds. An exception is palmitoyl-CoA, which is the ligand with the highest affinity for protein A so far studied, but which does not bind strongly to ligandin, as indicated by the gel-filtration experiment shown in Fig. 6(*a*). It is noteworthy that the primary binding site for palmitoyl-CoA appears to be distinct from that for the other ligands studied. Competition studies show that oestrone sulphate will not displace bound palmitoyl-CoA, and palmitoyl-CoA displaces oestrone sulphate to a much lesser extent than would be expected if they shared the same site (see Fig. 6*a* and Table 3). This latter finding would be explained if the primary oestrone sulphate-binding site were a secondary site for palmitoyl-CoA.

Crude protein A binds the same compounds with the same specificities as does the purified protein, but with greater affinity (see Table 2 and Figs. 3 and 5).

The lower affinity of the purified compared with the crude protein may be an artifact of the isolation procedure; the simple act of dialysis of crude protein A against two changes of buffer results in the complete loss of the strong primary binding sites, and dialysis is one of the first steps of purification. Without dialysis crude protein A retains its strong primary binding sites on standing for several days.

The results of the binding experiments give some indication of the forces responsible for the binding. Because oestrone and oestrone sulphate gave similar association constants it is unlikely that ionic interactions are important; non-polar effects are probably the major driving force for binding. This view is supported by the results for 20-methylcholanthrene, diethylstilboestrol, testosterone and oestradiol, all neutral compounds with affinities similar to those of the charged steroid sulphates. The introduction of hydroxyl groups into a steroid nucleus can decrease affinity. Thus cholate binds less firmly than does deoxycholate, and cortisol binds very weakly if at all. For palmitoyl-CoA the CoA moiety is presumably an important factor for binding to the specific palmitoyl-CoA-binding site, whereas the palmitoyl part of the molecule may be more important for binding to a secondary site, which is apparently shared by the other ligands.

Several other groups of workers have studied low-molecular-weight binding proteins that might be identical with protein A; for example, crude and purified Z protein (Levi *et al.*, 1969; Mishkin *et al.*, 1972; Mishkin & Turcotte, 1974*a*,*b*; Kamisaka *et al.*, 1975; Warner & Neims, 1975) and crude and purified fatty acid-binding protein (Ockner *et al.*, 1972; Ockner & Manning, 1974). The published data enable comparisons to be made of five properties of the various purified preparations, namely molecular weight, isoelectric point, circular-dichroism spectrum, binding behaviour and tissue distribution. Molecular weights range from 10000 to 14000. Acidic pI values have been reported by Ockner & Manning (1974), Kamisaka *et al.* (1975) and ourselves, although in the present work the major component of protein A is slightly basic, with a pI of 7.6. Circular-dichroism spectra characteristic of a protein containing substantial β structure have been reported by Ketterer *et al.* (1975*a*) and Kamisaka *et al.* (1975). The various preparations have a number of ligands in common, for example, bilirubin, fatty acids and bromosulphophthalein. Fatty acyl-CoA derivatives bind with particular affinity to both Z protein (Mishkin & Turcotte, 1974*a*) and protein A. Z protein (Mishkin *et al.*, 1972), fatty acid-binding protein (Ockner & Manning, 1974) and protein A have been detected immunologically in rat liver, intestinal mucosa, myocardium, skeletal muscle and adipose tissue. These comparisons for the purified proteins suggest that they may all be the same. However, amino acid analyses and peptide 'maps' for preparations apart from our own, and quantitative immunochemical comparisons using antigens and antibodies from the various groups involved, are necessary before a definite conclusion can be reached.

The fact that crude protein A has the same binding specificities as purified protein A is further evidence that this protein is the principal if not the only binding protein in this molecular-weight range and therefore that the low-molecular-weight proteins which have been studied by the other authors are identical with it.

In view of the apparently specific binding site for palmitoyl-CoA it is possible that aminoazodye-binding protein A has more than one physiological function. Thus Mishkin & Turcotte (1974*b*) showed that the binding of palmitoyl-CoA by Z protein results in the stimulation of the acylation of α-glycerophosphate, a reaction catalysed by microsomal preparations, suggesting that the protein may play a role in lipid metabolism. On the other hand the relatively non-specific binding of substances with substantial hydrophobic moieties, e.g. bilirubin and steroids, may be part of a different physiological process. The binding of such compounds might result in an increase in their uptake from the blood and in subsequent intracellular transport, in a manner similar to that proposed for oxygen binding by myoglobin (Wyman, 1966). For example, Warner (1975) attributes perinatal toxicity of hexachlorophene to the low concentrations of Z protein in perinatal liver. The protein might also be important in regulating free concentrations of its ligands. These roles have been discussed in relation to ligandin (Ketterer *et al.*, 1975*b*).

The covalent binding of carcinogen by this protein may be related to its non-covalent binding properties. It is now known that the active form of the azodye carcinogen is the sulphate ester of *N*-hydroxy-*N*-methyl-4-aminoazobenzene (Miller & Miller, 1975). This is an organic anion similar to the steroid sulphates which bind non-covalently to both protein A and ligandin. It is possible that the carcinogen binds first at the non-covalent binding site and then subsequently reacts with a methionine residue in the same region to form a covalent bond. If, *in vivo*, there should be a time-lapse between non-covalent and covalent binding, this protein could behave as a transporting agent for activated carcinogen and thereby promote its carcinogenic action. If, on the other hand, covalent binding is instantaneous, the protein would inactivate the carcinogen and protect against carcinogenesis.

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