Isolation and Characterization of Binding Proteins for Retinol from the Cytosol, Nucleosol and Chromatin of the Oviduct Magnum of Laying Hens

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Protein fractions that bind retinol were isolated from the cytosol, nucleosol and chromatin of the oviduct magnum of laying hens. The proteins isolated from the three sources showed similar elution profiles on chromatography through Sephadex G-75 and G-50 columns, and comparable mobility during electrophoresis on sodium dodecyl sulphate/polyacrylamide gels. Their molecular weights were calculated to be around 14 500. When oviducts from vitamin A-depleted and vitamin A-repleted immature chicks given oestrogen injections for 6 consecutive days were incubated with [3 H]retinyl acetate, uptake of the radioactivity in the nuclei of the vitamin A-depleted tissue was severalfold higher than that in the nuclei from the vitamin A-repleted tissue.

Ganguly (1974) suggested that most of the classical effects of vitamin A deficiency described in the literature can be explained if it is assumed that vitamin A is required for controlled division and differentiation of cells. Later by using oestrogen-induced development of the oviduct of immature chicks as a typical model system for rapid division and differentiation of cells, Joshi *et al.* (1976) showed that the growth of the oviduct in terms of increase in net weight and also in DNA, RNA and protein contents is markedly less in the chicks deprived of vitamin A. Subsequently, it was reported by Ganguly *et al.* (1978) and by Das *et al.* (1979) that the number of tubular gland cells is markedly less in such deficient oviducts. It was also reported by Ganguly *et al.* (1978) that when the oviduct explants of immature chicks treated with oestrogen are incubated with [3 H]thymidine *in vitro*, incorporation of the radioactive nucleoside is significantly lower in the tissue of the vitamin A-deprived chicks, compared with the corresponding vitamin A-repleted controls. The same workers have further shown that addition of retinol to the incubation medium restores the thymidine incorporation to near normal values in the deficient tissue, with retinol showing no such effects in the vitamin A-repleted tissue. It is thus clear from these observations that DNA metabolism as well as normal proliferation and differentiation of the primitive epithelial cells in the oestrogen-treated oviduct require the presence of retinol.

On the other hand, Bashor *et al.* (1973) pointed out that, like the steroid hormones, retinol may

function through the mediation of cellular binding proteins and in fact were the first to isolate such cellular binding proteins for retinol from various animal tissues. Since then several such cellular binding proteins for retinol and retinoic acid have been isolated in a few laboratories from many tissues of different species of animals (Chytil & Ong, 1978; Ong & Chytil, 1978) and we have also isolated such a protein from hen oviduct cytosol (Das *et al.*, 1978). If retinol were to function like a steroid hormone, and at the same time if steroid hormones require retinol for their normal function during development of the oviduct, it follows that, like the steroid hormones, there should be binding proteins for retinol in the chromatin of the oviduct. We report in the present paper isolation of such binding proteins for retinol from the nucleosol and chromatin of the nuclei isolated from the oviduct of laying hens.

Materials and Methods

White Leghorn laying hens (30–35 weeks old) were obtained from the Central Poultry Farm, Hesarghatta, near Bangalore, India, and were used immediately without any further treatment.

[11,12- 3 H]Retinyl acetate (sp. radioactivity 61 mCi/mmol) and other unlabelled retinol derivatives were gifts from Hoffmann–La Roche, Basel, Switzerland. [1(n)- 3 H]Retinol (sp. radioactivity 2.27 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A.

Triton X-100 was supplied by Centron Research Laboratory, Bombay, India, and Sephadex G-75 and G-50, cytochrome *c*, bovine serum albumin and Hepes were from Sigma Chemical Co., St. Louis,

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

MO, U.S.A. Sources of other materials used were as described previously (Das *et al.*, 1978).

Incubation of the oviduct minces with [^3H]retinyl acetate and isolation of nuclei

The laying hens were killed by decapitation, after which the magnum portion of the oviduct was cut into very small pieces with a pair of scissors and about 200mg of such pieces was incubated at 37°C for 2h in 2ml of Krebs–Ringer bicarbonate buffer (0.01M, pH7.2), which also contained 1mg of glucose/ml, 5mg of Hepes/ml and 1mg of bovine serum albumin/ml, together with 30nmol of [^3H]retinyl acetate. After the incubation, the pieces were washed with cold Krebs–Ringer bicarbonate buffer (0.01M, pH7.2) and homogenized in a Potter–Elvehjem homogenizer in 10vol. of 0.34M-sucrose in TM buffer [0.05M-Tris/HCl (pH7.4)/0.01M-MgCl₂].

The nuclei were isolated from the homogenate essentially by the method of Blobel & Potter (1966) with minor modifications. After filtration through four layers of cheesecloth the homogenate was centrifuged at 800g for 10min in a Sorvall RC-2B centrifuge, after which the crude nuclear pellet was suspended in 2.2M-sucrose in the TM buffer by gentle homogenization in a Potter–Elvehjem homogenizer with a loose Teflon pestle and the suspension was centrifuged for 1h at 105000g in a Spinco model L-50 ultracentrifuge with rotor 40. The pellet thus obtained was suspended in 1.0M-sucrose in the TM buffer and the suspension was centrifuged for 10min at 10000g in a Sorvall RC-2B centrifuge; the pellet obtained at this step contained the nuclei. The outer membranes of the nuclei thus isolated were removed by two successive washings with 0.34M-sucrose in the TM buffer containing 1% Triton X-100. When examined under an optical microscope these nuclear preparations appeared free from any cytoplasmic contamination and on the basis of DNA content the recovery of the nuclei at this stage was consistently found to be about 30–40% of the total homogenate.

Fractionation of the nuclei into subnuclear fractions

The detergent-washed nuclei (containing the ^3H radioactivity) were fractionated into the nucleosol and chromatin fractions by the procedure of Huang & Huang (1969) and briefly it was as follows. The nuclei were suspended in 10vol. of NaCl/EDTA buffer [0.075M-NaCl/0.025M-EDTA/0.01M-Tris/HCl (pH8.0)/0.2mM-phenylmethanesulphonyl fluoride] and the suspension was incubated at 0°C for 15min. The pellet obtained after centrifugation of the suspension at 10000g for 10min in a Sorvall RC-2B centrifuge was twice extracted with 10vol. of the same NaCl/EDTA buffer each time.

The supernatant fractions that contained the nucleosol were pooled and saved. The pellet represented the chromatin fraction.

The chromatin fraction thus obtained was suspended in 10vol. of 0.4M-NaCl in Tris/HCl buffer (0.01M, pH8.0), containing 0.2mM-phenylmethanesulphonyl fluoride and incubated at 0°C for 30min. After centrifugation of the incubated material at 10000g for 10min the supernatant was saved, whereas the pellet was extracted twice more by following the same procedure. The supernatant fractions were pooled and the radioactivity was measured in the pooled supernatant and pellet.

Purification of the cellular retinol-binding protein of the cytosol, nucleosol and the 0.4M-NaCl extract of the chromatin fraction

Large-scale purification of the binding proteins was now carried out by using the oviduct magnum of freshly killed laying hens. The procedure described by Das *et al.* (1978) for the purification of the cytosol-cellular retinol-binding protein of the magnum was used for the purification of the proteins from all the three sources. The cytosol protein was purified from the precipitate obtained by 0–60% saturation with (NH₄)₂SO₄ of the 100000g supernatant fraction of the whole homogenate of the magnum. For the nucleosol protein and for the chromatin-bound protein, the pooled NaCl/EDTA extracts of the nuclei and the 0.4M-NaCl extracts of the chromatin were dialysed for 16h against cold Tris/HCl buffer (0.01M, pH8.0) containing 0.2mM-phenylmethanesulphonyl fluoride, and were then freeze-dried.

The (NH₄)₂SO₄-precipitated fraction of the oviduct cytosol and the freeze-dried preparation of the nucleosol were separately dissolved in 2ml of 0.2M-NaCl in Tris/HCl buffer (0.05M, pH7.4) containing 0.2mM-phenylmethanesulphonyl fluoride, and after incubation with 10nmol of [^3H]retinyl acetate were chromatographed on Sephadex columns as described by Das *et al.* (1978). For purification of the binding protein in the chromatin extract, a similar procedure was adopted except that the solubilization and elution were carried out with 0.4M-NaCl in Tris/HCl buffer (0.05M, pH7.4) containing 0.2mM-phenylmethanesulphonyl fluoride.

Proteins were determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard and DNA was determined by the method of Burton (1956) with calf thymus DNA as standard.

All radioactivity measurements were done in an LS-100 liquid-scintillation counter by using 0.5% (w/v) 2,5-diphenyloxazole and 0.05% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in a 2:1 (v/v) mixture of toluene and Triton X-100. The counting efficiency for ^3H was about 8%.

Fig. 1. Purification of the binding proteins by gel filtration on Sephadex G-75 columns

The freeze-dried residues of the cytosol (500mg) and nucleosol (200mg) were dissolved in 2ml of buffer A (0.2M-NaCl/0.05M-Tris/HCl (pH 7.4)/0.2mM-phenylmethanesulphonyl fluoride) and incubated with 30nmol of [3 H]retinyl acetate at 22°C for 4h (Das *et al.*, 1978). The freeze-dried residue of the 0.4M-NaCl extract of the chromatin (45mg) was similarly incubated with 30nmol of [3 H]retinyl acetate except that here the buffer contained 0.4M-NaCl instead of 0.2M-NaCl. After the incubation, 0.4ml of dextran-coated charcoal (0.5% charcoal and 0.05% dextran T-70 prepared in 0.05M-Tris/HCl buffer, pH 7.2) was added to each mixture, after which the samples were allowed to stand at 0°C for 10min. The charcoal was removed by centrifugation at 600g for 5min and the clear supernatant was loaded on a column (50cm $\times$ 4cm) of Sephadex G-75 that had previously been equilibrated with buffer A. The column was eluted with buffer A at a flow rate of 30ml/h and 2ml fractions were collected in an LKB fraction collector. The fractions were monitored for A_{280} , and 100 μ l portions of each fraction were used for radioactivity measurements. The cellular retinol-binding protein in the chromatin extract was eluted with a buffer containing 0.4M-NaCl/0.05M-Tris/HCl (pH 7.4)/0.2mM-phenylmethanesulphonyl fluoride. For the dilution experiments 50mg of the freeze-dried residue of the nucleosol and 20mg of the residue of the chromatin extract were similarly incubated with 1nmol of [3 H]retinol together with a 100-fold molar excess of unlabelled retinol and further processed as described above. The cytosol preparation was not used here, because Das *et al.* (1978) have already reported such dilution effects with the cytosol cellular retinol-binding protein. [3 H]Retinol was used, because the specific radioactivity of the [3 H]retinyl acetate preparation was not satisfactory in these experiments. Symbols: ●, A_{280} ; △, radioactivity from the [3 H]-

Results

Subnuclear distribution of the radioactivity of [3 H]-retinyl acetate in hen oviduct magnum

Preliminary experiments, where the oviduct minces were incubated with [3 H]retinyl acetate, showed that approx. 3.5% of the total radioactivity of the whole homogenate was localized in the nuclear preparation. Fractionation of the isolated nuclei revealed that about one-third of the total nuclear radioactivity was associated with the NaCl/EDTA extract, whereas about 50% of the radioactivity left in the chromatin fraction could be extracted with Tris/HCl (0.01M, pH 8.0) containing 0.4M-NaCl (Table 1).

Chromatography of the protein fractions on Sephadex G-75 and G-50 columns

Large-scale isolation and purification of the cellular retinol-binding protein from the cytosol, nucleosol and chromatin were attempted by using the oviduct magnum of eight laying hens. Fig. 1 shows that, when chromatographed through Sephadex G-75 columns, these preparations gave two peaks of absorbance at 280nm in the chromatographic profile (peaks A and B). The radioactivity profile, on the other hand, revealed that in addition to the radioactivity associated with the major

Table 1. Uptake of radioactivity of [3 H]retinyl acetate by oviduct minces and subnuclear distribution of the radioactivity

Fraction	3 H radioactivity (c.p.m.)	
	Expt. 1	Expt. 2
Homogenate	533 000	520 000
Nuclei	14 784	14 355
NaCl/EDTA extract of nuclei	4955	4494
0.4M-NaCl extract of the chromatin	5368	4830
Chromatin residue	5742	5166

retinyl acetate; ○, radioactivity from the [3 H]retinol (in the dilution experiments only). (a) Cytosol; (b) nucleosol; (c) chromatin.

protein peak eluted in the void volume (peak A), a large peak of radioactivity (peak C, fractions 110–130) appeared after the protein peak B. These fractions showed highest specific radioactivity when expressed on the basis of mg of protein.

The fractions in peak C from the three sources were separately dialysed against Tris/HCl buffer (0.01 M, pH 7.4) containing 0.2 mM-phenylmethanesulphonyl fluoride and were then freeze-dried. After reincubation with 10 nmol of [3 H]retinyl acetate these fractions were rechromatographed through Sephadex G-50 columns (Fig. 2), after which the major protein peak (fractions 36–42), which eluted

after cytochrome *c* dimer and contained the bulk of the radioactivity, was freeze-dried.

Electrophoresis on SDS/polyacrylamide gel

Analysis on SDS/polyacrylamide gels of the protein fractions thus isolated revealed that all the three samples moved as major bands with comparable mobility and all the samples stained to some extent in the higher-molecular-weight regions (Fig. 3).

On the basis of the mobility on the SDS/polyacrylamide gel against the standard proteins, the molecular weights of the binding proteins from all three sources were calculated to be approx. 14 500.

Identification of the chemical form of the radioactivity bound to the various fractions

For this purpose the oviduct homogenate incubated with [3 H]retinyl acetate and the pooled fractions of the radioactive peak C of Fig. 1 derived

Fig. 2. Gel filtration on Sephadex G-50 columns of the pooled fractions from Fig. 1

The fractions from Sephadex G-75 filtration in Fig. 1 representing the highest specific radioactivity (peak C) were pooled and dialysed against 0.01 M-Tris/HCl buffer, pH 7.4, containing 0.2 mM-phenylmethanesulphonyl fluoride and were then freeze-dried. The freeze-dried residues were dissolved in 0.5 ml of buffer B [0.05 M-Tris/HCl (pH 7.4)/0.2 mM-phenylmethanesulphonyl fluoride] and re-incubated with 10 nmol of [3 H]retinyl acetate and the unbound retinyl acetate was removed by dextran-coated charcoal treatment as described in the legend to Fig. 1. The supernatant obtained after centrifugation was subjected to chromatography on a Sephadex G-50 column (45 cm $\times$ 1.5 cm) with buffer B as the eluent. The flow rate was adjusted to 30 ml/h and 1 ml fractions were collected. Both A_{280} and radioactivity in each fraction were determined as described in Fig. 1. Symbols: $\bullet$, A_{280} ; Δ , radioactivity from the [3 H]retinyl acetate. (a) Cytosol; (b) nucleosol; (c) chromatin.

Fig. 3. Polyacrylamide-gel electrophoresis of the binding proteins isolated from the cytosol, nucleosol and chromatin. SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970). Of the purified cellular retinol-binding proteins of the three sources obtained after the Sephadex G-50 chromatography (Fig. 2) 20 μ g was subjected to electrophoresis on 7.5% gel along with the marker proteins bovine serum albumin, ovalbumin and cytochrome *c*. The amounts of proteins usually used for such experiments (2–3 μ g) consistently do not take stain satisfactorily with Coomassie Blue in the case of the cellular retinol-binding protein. Therefore much larger amounts had to be used. (a) Molecular-weight markers; (b) cytosol cellular retinol-binding protein, (c) nucleosol cellular retinol-binding protein, (d) chromatin cellular retinol-binding protein.

from the nucleosol and from the 0.4M-NaCl extract of the chromatin respectively were extracted with chloroform/methanol (2:1, v/v), after which portions of the extracts were subjected to t.l.c. on silica gel C plates together with known samples of the reference radioactive retinol in a solvent system of 6% acetone in light petroleum (b.p. 40–60°C), when the radioactivity of the test samples moved parallel to that of the reference sample. It is noteworthy, however, that, when subjected to similar t.l.c. the [^3H]retinyl acetate used in these experiments, was found to contain about 10% of the radioactivity in the retinol fraction. Therefore it is possible that the protein-bound retinol could have arisen from hydrolysis of the [^3H]retinyl acetate or from the small amounts of the radioactive retinol found in the [^3H]retinyl acetate sample used. Moreover, it has been shown in Fig. 1 that, when dilution experiments were carried out with [^3H]retinol in the presence of a 100-fold excess of unlabelled retinol, there was no radioactivity in the protein fractions in peaks A and C derived from nucleosol and chromatin. Preliminary experiments in our laboratory with similar protein fractions isolated from the nucleosol and chromatin of sheep testes and with [^3H]retinol have yielded similar results. Therefore it would appear that the actual form that binds these protein fractions is retinol.

Uptake of the radioactivity of the incubated [^3H]retinyl acetate in the oviduct nuclei of vitamin A-deprived chicks treated with oestrogen

The preliminary experiments described above on the subcellular distribution of the radioactivity of [^3H]retinyl acetate in the magnum were carried out with the tissue isolated from normal laying hens, that presumably had received sufficient supply of vitamin A, and consequently their binding proteins should have contained normal amounts of vitamin A. Experiments were therefore designed to examine the relative uptake of the radioactivity of [^3H]retinyl acetate in the nuclei of the oviduct magnum of vitamin A-deprived and vitamin A-repleted chicks given oestrogen, and the results presented in Table 2 clearly demonstrate that the radioactivity in the nuclear fraction isolated from the oviduct of the vitamin A-deprived birds was severalfold higher than in the vitamin A-supplemented birds.

Discussion

We have previously shown that the cytosol of the magnum portion of the oviduct of laying hens contains a protein that binds retinyl acetate and has mol.wt. 14500. The present results further show that the nucleosol and chromatin isolated from the nuclei of the same tissue also contain similar binding

Table 2. Nuclear uptake of radioactivity of [^3H]retinyl acetate in the oviduct magnum of normal and vitamin A-deficient chicks treated with oestrogen

The procedures for making 1-day-old chicks deficient in vitamin A and for subsequent oestrogen treatment were as described by Joshi *et al.* (1976). After oestrogen administration for 6 consecutive days the birds were killed, after which 200mg pieces of the oviduct were incubated with the radioactive retinyl acetate and the nuclei were isolated as described in the Materials and Methods section. Four pieces of oviduct were pooled after incubation and analysed. The values are expressed as c.p.m./mg of DNA.

Expt.	Radioactivity in the nuclear fraction (c.p.m./mg of DNA)
(I) Normal	1880
Vitamin A-deficient	31250
(II) Normal	1488
Vitamin A-deficient	8720
(III) Normal	4750
Vitamin A-deficient	14450

proteins. But, whereas in our previous work we did not investigate the actual chemical form of vitamin A that binds the cytosol protein, in the present paper we have shown that the vitamin is bound to the proteins in the form of retinol.

During the process of purification through Sephadex G-75 and G-50 columns the proteins isolated from the three sources revealed similar elution profiles, and their mobilities during electrophoresis on SDS/polyacrylamide gel were also similar. Although these observations would suggest that their molecular weights are comparable, they do not indicate whether they are identical, or for that matter of common origin. Work in our laboratory has revealed that similar binding protein is present in the nucleosol and chromatin of sheep testes (R. Shinde, R. C. Das & J. Ganguly, unpublished work). It is, however, significant that the molecular weights of the binding proteins for retinol isolated by other workers from several animal tissues appear to be of the same order (Chytil & Ong, 1978).

It has also been shown in Fig. 1 that the radioactivity of the [^3H]retinol in the protein fractions isolated from the nucleosol and chromatin of the oviduct could be displaced by a 100-fold molar excess of unlabelled retinol. But, whereas in our previous work an additional peak of radioactivity, which appeared during the Sephadex G-75 chromatography, could not be displaced by excess retinyl acetate (Das *et al.*, 1978), in the present experiments a minor peak, which appeared during similar Sephadex filtration, lost its radioactivity when treated with excess unlabelled retinol. Since Das *et al.* (1978) used the cytosol fraction and since

ovalbumin is the major cytosol protein of hen oviduct, it is conceivable that the additional peak was due to non-specific binding of [^3H]retinol to ovalbumin. On the other hand, since in the present work such binding of the radioactivity could be displaced by excess unlabelled retinol it is clear that in this particular case the binding was specific. But such fractions correspond to higher-molecular-weight proteins and therefore it is possible that they might represent aggregates of the cellular retinol-binding protein of both sources. Such arguments assume greater significance when one considers staining with Coomassie Blue in the higher-molecular-weight regions in the polyacrylamide gel. Here also, it is possible that such minor bands could be given by aggregates of the cellular retinol-binding protein that did not completely dissociate under the present conditions of electrophoresis. Such conclusions receive further support from the claims of Ong & Chytil (1978) that the purified cellular retinol-binding protein of rat liver aggregates at higher protein concentrations.

Although there has been considerable work on the retinol-binding proteins isolated from the cytosol of various animal tissues, there are very few reports on the binding proteins of the cell nucleus. Thus Sani (1977) claimed the presence of a cellular retinoic acid-binding protein in the chromatin fraction of chick embryo skin, whereas Wiggert *et al.* (1977) detected such proteins in the nucleosol fraction of cultured human retinoblastoma cells. But in both cases only sucrose-density-gradient centrifugation was employed and no further characterization of the proteins was attempted. In the present work binding proteins for retinol have been purified from the cytosol, nucleosol and chromatin of hen oviduct magnum.

When compared with the predominant localization of steroid hormones in the nucleus of target tissues (Jensen *et al.*, 1974), only about 3% of the radioactivity of the [^3H]retinyl acetate found in the whole homogenate of the oviduct magnum of laying hens could be recovered in the nuclear fraction. It should, however, be pointed out that, since 10% of the radioactivity of the [^3H]retinyl acetate preparation used was found in the retinol fraction, and if retinol is readily transported to the nucleus, it is obvious that this value would represent minimum nuclear

uptake. But the nuclear uptake of the radioactivity increased severalfold in the tissue of the vitamin A-depleted chicks given oestrogen, compared with the corresponding vitamin A-repleted chicks. It is thus clear that the very low nuclear uptake in the laying hens was due to the retinol already present there. Furthermore, it has been our experience that nuclear uptake of the radioactivity shows a great deal of individual variation in the depleted birds. Here also, it is possible that this is due to variations in the amounts of retinol still present in the tissues after the period of depletion.

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