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Determination of Binding Affinities of Retinoids to Retinoic Acid-Binding Protein and Serum Albumin

By BRAHMA P. SANI, BELINDA C. TITUS and CHANDRA K. BANERJEE
Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205, U.S.A.

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Binding affinities of retinoic acid and its synthetic analogues to intracellular retinoic acid-binding protein, which is a possible candidate for mediating their biological function, and to serum albumin, the plasma transport protein, were evaluated. A quantitative method involving elimination of interfering serum albumin by immunoprecipitation was developed to measure the binding efficiency of these retinoids, some of which are active in modifying epithelial differentiation and preventing tumorigenesis. Two cyclopentenyl analogues of retinoic acid and 13-*cis*-retinoic acid showed, like retinoic acid, a binding efficiency of 100% for the cellular binding protein. With the phenyl, dichlorophenyl and trimethylmethoxyphenyl analogues of retinoic acid, the binding efficiency increased as the substituents on the aromatic ring increased; thus the trimethylmethoxyphenyl analogue binds almost as efficiently as retinoic acid itself. However, the trimethylmethoxyphenyl analogue with a sulphur atom on the side chain has a much decreased binding affinity. The correlation noticed between the binding efficiency of these retinoids and their biological activity in differentiation and/or in the control of tumorigenesis particularly enhances the confidence in the present method of determining the relative binding efficiencies. None of the vitamins, hormones and cofactors tested, showed appreciable affinity for the retinoic acid-binding site. Studies on binding of retinoic acid and its analogues to serum albumin indicate that no correlation exists between binding affinity for albumin and their biological potency.

Retinol (vitamin A), retinoic acid and some synthetic retinoids are of intrinsic importance for control of epithelial differentiation and prevention of neoplastic growth (Dawling & Wald, 1960; Thompson *et al.*, 1964; Bollag, 1972; Sporn *et al.*, 1976). The biological activity of retinoic acid in general growth and differentiation parallels that of retinol, except that it cannot replace retinol in the formation of visual pigments or in restoring reproductive processes (Dawling & Wald, 1960; Thompson *et al.*, 1964). In fact, retinoic acid has been shown to be more active than retinol in the reversal of keratinized metaplasia into mucous metaplasia in a chick skin organ-culture system (Wilkoff *et al.*, 1976). Further, retinoic acid and some of its analogues inhibit and reverse the hyperplasia induced in mouse prostate in organ culture by 3-methylcholanthrene (Lasnitzki & Goodman, 1974; Chopra & Wilkoff, 1976).

Work on the molecular mechanism of action of retinoids, other than in vision, has revealed that their function may be mediated by specific cellular binding proteins (Bashor *et al.*, 1973; Sani & Hill, 1974; Ong & Chytil, 1975; Sani & Hill, 1976). A cellular protein that binds retinol and a specific binding protein for retinoic acid have been partially characterized (Sani & Hill, 1974; Ong & Chytil, 1975). The two proteins have similar molecular weight (14000) and sedimentation coefficient (2S), but different tissue distribution (Ong & Chytil, 1975; Sani, 1976; Sani & Corbett, 1977). Retinoic acid-binding protein has been localized in the nuclei of certain normal tissues and experimental tumours (Sani, 1977). A retinoic acid-like structure with a ring, side chain and terminal free carboxyl group is essential for maximal binding to retinoic acid-binding protein (Sani & Hill, 1974, 1976).

In the present project, a method involving immunoprecipitation of interfering serum albumin and quantitative determination of binding affinity of retinoids to retinoic acid-binding protein has been developed. Since these binding affinities correlated with the biological potencies of these retinoids, it was also desirable to obtain data for comparative purposes on their binding efficiency with serum albumin, which functions as the plasma transport protein (Smith *et al.*, 1973; Sani & Hill, 1976).

Experimental

Retinoic acid, [11,12-^{3}H]retinoic acid (1.28Ci/mmol), retinol, retinal, methyl retinoate, diethyl-

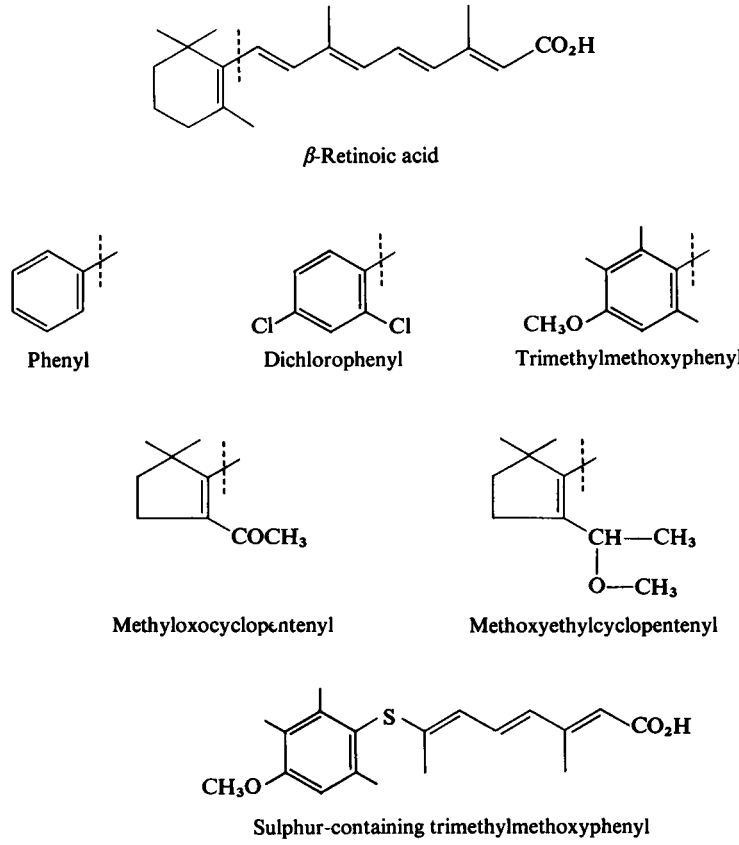

Fig. 1. *Structures of retinoic acid and analogues*

retinamide, 13-*cis*-retinoic acid, and 1-methyloxocyclopentenyl, 1-methoxyethylcyclopentenyl, 2-ethylcyclopentenyl, phenyl, dichlorophenyl, trimethylmethoxyphenyl analogues of retinoic acid and the trimethylmethoxyphenyl analogue with a sulphur atom at position 7 on the side chain, as shown in Fig. 1, were supplied by Hoffman–La Roche, Nutley, NJ, U.S.A., through the Lung Cancer Branch, National Cancer Institute, Bethesda, MD, U.S.A. These retinoids, except 13-*cis*-retinoic acid, possessed all-*trans*- conformations. Various hormones, vitamins and cofactors, and once-crystallized defatted bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. γ-Globulins from specific antiserum to chicken serum albumin (produced in rabbit) was purchased from Cappel Laboratories, Downington, PA, U.S.A.

Chick-embryo skin extracts, and incubation with [^{3}H]retinoic acid, its analogues and other test compounds

Skins from 13-day chick embryos were homogenized, freeze-dried and extracted with phosphate-buffered saline [0.03M-sodium phosphate (pH 7.2/0.1M-NaCl] (Sani & Hill, 1976), followed by centrifugation at 100000$g_{av.}$ for 60min (Sani & Hill, 1974, 1976). The supernatant was diluted with phosphate-buffered saline to a protein concentration of 12mg/ml. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Various concentrations of chromatographically homogeneous [11,12-^{3}H]retinoic acid (Sani & Hill, 1976) in 3μl of dimethyl sulphoxide were added to the skin extract (3mg of protein). In competition experiments, 300pmol of [^{3}H]retinoic acid and a 100-fold molar excess of the unlabelled

test compound in 5μl of dimethyl sulphoxide were added simultaneously to the skin extracts, and the preparations were incubated and dialysed as described by Sani & Hill (1976). Binding of [^{3}H]retinoic acid to macromolecules was determined on linear 5–20% (w/v) sucrose density gradients, after centrifugation in a Spinco SW 50.1 rotor at 180000$g_{av.}$ for 18h (Sani & Hill, 1974, 1976). The 2S peak corresponding to retinoic acid-binding protein and the 5S peak corresponding to albumin were located by using bovine serum albumin and ovalbumin as external markers (Sani & Hill, 1974, 1976). To eliminate the serum albumin peak, the skin extract (25mg of protein) was made to interact with immunoglobulins (10mg of protein) from antiserum to chick serum albumin for 1h at 25°C and then at 4°C for 48h. The precipitates were removed by centrifugation at 20000g for 30min. Samples of the supernatants were incubated with [^{3}H]retinoic acid and subjected to sucrose-gradient sedimentation as described above.

Albumin-binding assays

Defatted bovine serum albumin (50μg) in 0.3ml of phosphate-buffered saline was mixed with 3nmol of [^{3}H]retinoic acid with or without a 100-fold molar excess of unlabelled compound. After incubation at 4°C for 5h and dialysis for 20h against phosphate-buffered saline, 500μg of albumin was added to the incubation mixture, so that denaturation due to high dilution would be lessened. These preparations were centrifuged on 5–20% sucrose gradients, fractionated and examined as described above for the 5S albumin peak.

Results

Chick-embryo skin extracts after incubation with various concentrations of [^{3}H]retinoic acid and sedimentation on sucrose gradients give rise to the 5S peak corresponding to the albumin–[^{3}H]retinoic acid complex and the 2S peak corresponding to retinoic acid-binding protein (Fig. 2). The sizes of both peaks are proportionately increased as the amount of [^{3}H]retinoic acid in the incubation mixture is increased. The sedimentation profiles indicate that 300pmol of [^{3}H]retinoic acid (0.38μCi) saturate almost all the binding sites on retinoic acid-binding protein present in 3mg of protein of chick-embryo skin extract, whereas the binding sites on albumin remain at a sub-saturation point. Hence this amount of retinoic acid was chosen in our competition experiments for measuring the efficiency of binding of the retinoic acid analogues to retinoic acid-binding protein. With 300pmol of [^{3}H]retinoic acid, competition with 1-, 5- or 20-fold molar excesses of unlabelled retinoic acid revealed a corresponding decrease in the 2S retinoic acid-binding protein peak (Fig. 3). A 100-fold molar

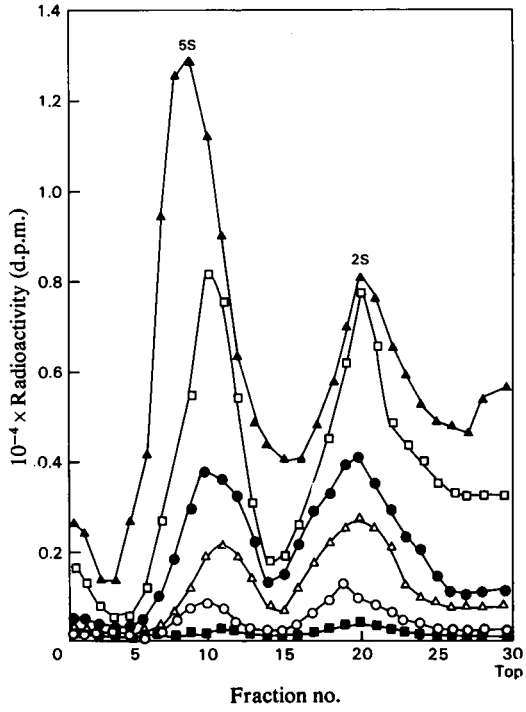

Fig. 2. *Sucrose-density-gradient-centrifugation patterns of chick-embryo skin extract (3mg of protein) plus different concentrations of [^{3}H]retinoic acid*

Concentrations were: ■, 10pmol; ○, 30pmol; △, 90pmol; ●, 150pmol; □, 300pmol; ▲, 600pmol.

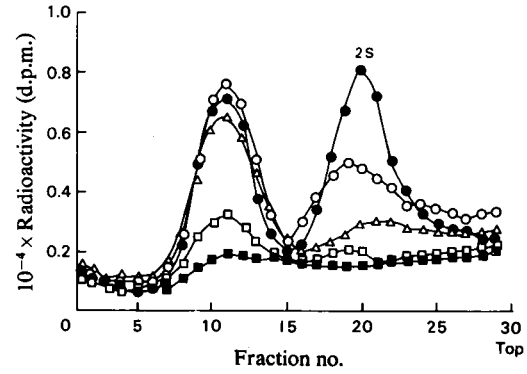

Fig. 3. *Sucrose-density-gradient sedimentation patterns of chick-embryo skin extract (3mg of protein)+[^{3}H]retinoic acid (300pmol) with and without various molar excesses of unlabelled retinoic acid*

●, Extract+[^{3}H]retinoic acid; ○, extract+[^{3}H]-retinoic acid+1-fold molar excess of unlabelled retinoic acid; △, extract+[^{3}H]retinoic acid+5-fold molar excess of unlabelled retinoic acid; □, extract+[^{3}H]-retinoic acid+20-fold molar excess of unlabelled retinoic acid; ■, extract+[^{3}H]retinoic acid+100-fold molar excess of unlabelled retinoic acid.

excess of retinoic acid gives a pattern similar to that of 20-fold molar excess of retinoic acid.

To establish the optimum conditions for binding of retinoic acid to the binding protein, incubation of [^{3}H]retinoic acid with skin extract was carried out at different ionic concentrations. Although no apparent difference in ligand binding was noticed up to 0.8 M-NaCl concentrations (result not shown), we chose 0.03 M-sodium phosphate buffer (pH 7.2)/0.1 M-NaCl, which was close to the physiological conditions, for our binding assays.

The albumin–[^{3}H]retinoic acid complex that constitutes the 5S peak on the sucrose-density-gradient profiles is virtually eliminated on immunoprecipitation of the chick skin extracts with immunoglobulins from antiserum to chick serum albumin (Fig. 4). Under these conditions, 100-fold molar excesses of various analogues of retinoic acid were allowed to compete. As shown by the size of the 2S peaks, the phenyl analogue had the least competitive effect, whereas the dichlorophenyl and trimethylmethoxyphenyl analogues were stronger competitors (Fig. 4). However, the sulphur-containing trimethylmethoxyphenyl analogue showed much less competitive effect than the analogue without sulphur. Using the chick skin extracts after immunoprecipitation, we have also observed that 13-*cis*-retinoic acid and the methyloxocyclopentenyl and methoxyethylcyclopentenyl analogues of retinoic acid compete as efficiently as retinoic acid for the retinoic acid-binding site (results not shown).

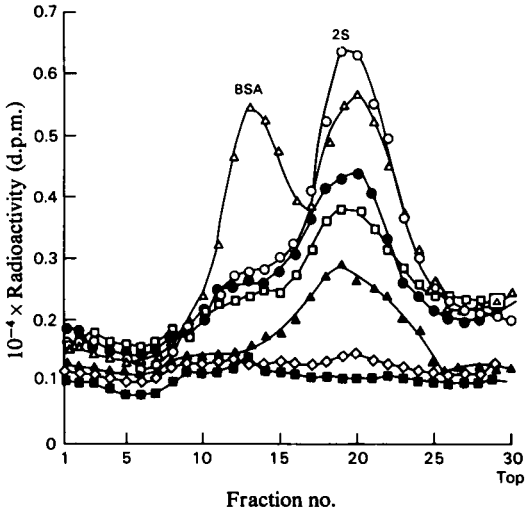

Fig. 4. *Sucrose-density-gradient-centrifugation patterns showing the effect of competition of 100-fold molar excess of unlabelled retinoids on the binding of [^{3}H]retinoic acid with chick skin extract*

○ and △, Controls containing 300 pmol of [^{3}H]retinoic acid plus skin extract (3 mg of protein) respectively with and without immunoprecipitation with antiserum to chick serum albumin. All the other radioactivity profiles are the control with immunoprecipitation plus 100-fold molar excess of the following analogues of retinoic acid: ●, phenyl; □, sulphur-containing trimethylmethoxyphenyl; ▲, dichlorophenyl; ◇, trimethylmethoxyphenyl; ■, retinoic acid. BSA, Bovine serum albumin.

To measure the competitive binding affinity of retinoic acid analogues for retinoic acid-binding protein, the inhibition of [^{3}H]retinoic acid binding caused by 100-fold molar excess of unlabelled retinoic acid has been regarded as 100% inhibition. The inhibition caused by similar excesses of various analogues is expressed as relative inhibition to the above standard as shown in Table 1. Although the trimethylmethoxyphenyl analogue showed 95% inhibition, which is almost as high as retinoic acid itself, the analogue in which the C=C in the chain is replaced by a sulphur atom showed only 38% inhibition. The dichlorophenyl analogue exhibited 55% inhibition and the phenyl analogue had the least competitive effect (30%). As well as retinoic acid, 100% inhibition is expressed by 13-*cis*-retinoic acid, methyloxocyclopentenyl and methoxyethylcyclopentenyl analogues of retinoic acid. In accordance with our earlier observations (Sani & Hill, 1974, 1976), those retinoic acid derivatives in which the free carboxyl group is blocked (esters, amides, oxime, lactone etc.) did not compete for the retinoic acid-binding site.

The presence of retinoic acid-binding protein in tissues, such as testis, ovary, brain and breast, that specialize in producing hormones prompted an investigation of possible binding of hormones, vitamins and cofactors to the binding protein. This was accomplished by competition experiments using a 100-fold excess of these unlabelled test compounds on retinoic acid-binding protein of chick

Table 1. *Percentage inhibition of binding of [^{3}H]retinoic acid to retinoic acid-binding protein by 100-fold molar excess of unlabelled retinoids*

Retinoid	Inhibition (%)
β-Retinoic acid	100
Phenyl analogue	30
Dichlorophenyl analogue	55
Trimethylmethoxyphenyl analogue	95
Sulphur-containing trimethylmethoxyphenyl analogue	38
Methyloxocyclopentenyl analogue	100
Methoxyethylcyclopentenyl analogue	100
13-*cis*-Retinoic acid	100
Retinal	5
Retinol	0
Methyl retinoate	0
Diethylretinamide	0

Table 2. *Effects of competition for [^3H]retinoic acid-binding sites on retinoic acid-binding protein by 100-fold molar excesses of hormones, vitamins and cofactors*

	Competing compounds	Effect of competition
1.	Cortisone, deoxycorticosterone, testosterone, dexamethasone, progesterone, oestradiol-17α, oestradiol-17β, prednisolone	No effect
	Cortisol	Slightly stimulated the binding
2.	Nicotinic acid, D-pantothenic acid, folid acid, *p*-aminobenzoic acid	No effect
	Thioctic acid	Competes slightly
3.	Cyclic AMP, cyclic GMP, prostaglandins E_1, E_2, B_1 and A_1	No effect

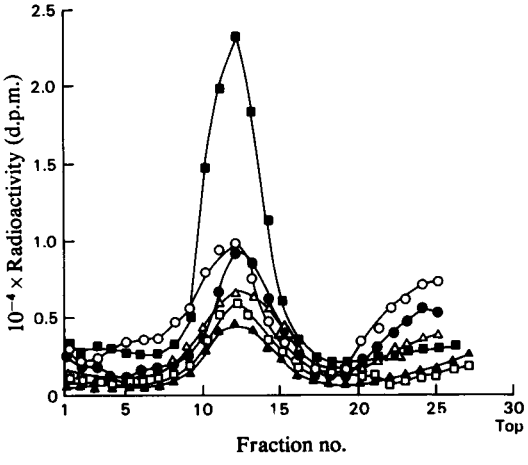

Fig. 5. *Sucrose-density-gradient sedimentation profiles of [^3H]retinoic acid (3 nmol)+50 μg of bovine serum albumin with and without 100-fold molar excess of the unlabelled retinoid*

■, Albumin+[^{3}H]retinoic acid; ●, albumin+[^{3}H]-retinoic acid+unlabelled retinoic acid; ○, albumin +[^{3}H]retinoic acid+phenyl analogue of retinoic acid; △, albumin+[^{3}H]retinoic acid+trimethyl-methoxyphenyl analogue of retinoic acid; □, albumin +[^{3}H]retinoic acid+13-*cis*-retinoic acid; ▲, albumin +[^{3}H]retinoic acid+dichlorophenyl analogue of retinoic acid.

skin extract. Most of the hormones tested showed no significant inhibitory effect (Table 2), although the presence of cortisol slightly stimulated binding of [^{3}H]retinoic acid to the binding protein. Compounds such as nicotinic acid, pantothenic acid, folic acid, *p*-aminobenzoic acid, prostaglandins, cyclic AMP and cyclic GMP did not compete for the retinoic acid-binding site, although some of these compounds have free carboxyl groups. Thioctic acid showed some inhibition of the binding.

Retinoic acid, like many fatty acids, is transported by serum albumin in the blood (Smith *et al.*, 1973). It was decided to study the binding efficiency of various analogues to albumin in order to make a comparison of their binding affinity to retinoic acid-binding protein. The presence of 100-fold molar excesses of retinoic acid analogues diminishes the binding of [^{3}H]retinoic acid to the binding protein (Fig. 5). A 100-fold molar excess of either retinoic acid or its phenyl analogue showed similar degrees of competition for albumin binding. The trimethylmethoxyphenyl and dichlorophenyl analogues of retinoic acid and 13-*cis*-retinoic acid competed more efficiently than did retinoic acid for the binding sites.

Discussion

The correlation between the binding affinity of retinoic acid and its analogues for retinoic acid-binding protein and their activity in epithelial differentiation (Sani, 1975; Sani & Hill, 1976; Wilkoff *et al.*, 1976) suggests that the general action of these retinoids could be mediated by retinoic acid-binding protein. If retinoic acid is functioning as a differentiation hormone and if retinoic acid-binding protein is essential for mediating its biological activity within the cell, then it should be possible to design, synthesize and test new analogues of retinoic acid that may have greater potency by studying their binding affinity to the binding protein. We have reported earlier a qualitative expression of relative amounts of retinoic acid-binding protein as well as the relative binding affinities of some retinoic acid analogues to the binding protein (Sani & Hill, 1976; Sani & Corbett, 1977). Others have measured the tissue contents of retinoic acid-binding protein and the binding efficiency of retinoic acid analogues to it using tissue preparations from which albumin was not removed (Ong & Chytil, 1975; Chytil & Ong, 1976). Such measurements may involve some approximations necessarily introduced by variation in the size of the 2S peak, owing to the presence of variable amounts of serum albumin, which binds retinoic acid with high affinity and constitutes the 5S radioactive peak (Sani & Hill, 1976; Sani & Corbett, 1977). The quantitative method involving immunoprecipitation of serum

albumin described here for measuring the binding efficiency of retinoids to retinoic acid-binding protein may provide a better assessment of their biological potencies.

Ligand-binding studies with increasing amounts of retinoic acid revealed that 300 pmol of [^{3}H]retinoic acid caused a 100% binding efficiency under the experimental conditions described. With this concentration of [^{3}H]retinoic acid, increasing concentrations of unlabelled retinoic acid exhibited a corresponding inhibition of binding of [^{3}H]retinoic acid to the binding protein. A 50% inhibition with a 1-fold molar excess of unlabelled retinoic acid (Fig. 3) explains the stoicheiometry of binding of retinoic acid to this protein. A 20-fold or a 100-fold molar excess of unlabelled retinoic acid gives rise to 100% inhibition. We have reported that similar inhibition has also been brought about by a 200-fold molar excess of unlabelled retinoic acid (Sani & Hill, 1976). The present data on relative binding efficiency of various retinoids is based on competition with 100-fold molar excess of the test compounds.

Aromatic analogues of retinoic acid competed for the binding site with an increasing order of affinity as the substituents on the ring increased; thus the trimethylmethoxyphenyl analogue had the highest binding efficiency. This observation, as well as the fact that the cyclopentenyl analogues and 13-*cis*-retinoic acid bind efficiently to retinoic acid-binding protein, parallels the ability of these compounds to reverse the metaplasia of hamster trachea and to reverse the keratinization of chick skin induced by vitamin A deficiency (Sporn *et al.*, 1975; Wilkoff *et al.*, 1976). The high binding efficiency of 13-*cis*-retinoic acid and of the trimethylmethoxyphenyl analogue to retinoic acid-binding protein is in particular agreement with their pronounced effects on the inhibition of experimental carcinogenesis (Bollag, 1974, 1975; Sporn *et al.*, 1977). When C=C on the hydrocarbon side chain is replaced by a sulphur atom at position 7 (see Fig. 1), whereby the chain length is not greatly affected, the molecule has a significantly lower binding efficiency compared with retinoic acid or its trimethylmethoxyphenyl analogue. This indicates a specific and essential requirement for the hydrocarbon chain of retinoic acid, in addition to a free carboxyl group, for maximal binding. This point is further supported by the present findings that compounds such as nicotinic acid, pantothenic acid, folic acid, *p*-aminobenzoic acid and prostaglandins, which contain free carboxyl groups, did not compete for the retinoic acid-binding site. The general correlation between the binding affinity of various retinoids for retinoic acid-binding protein and their biological activity in epithelial differentiation and in the control of tumorigenesis particularly enhances the confidence in our present assay procedure for measuring the relative binding efficiency of retinoic acid analogues.

Absorption, distribution and elimination of any drug *in vivo* will be greatly influenced by the extent of its binding to plasma proteins, by which the transport of many drugs is facilitated. Retinoic acid and its phenyl analogue showed a similar degree of affinity for albumin binding, whereas the trimethylmethoxyphenyl and dichlorophenyl analogues and 13-*cis*-retinoic acid showed even higher affinity than retinoic acid for the binding sites. Since we now know that some of these compounds bind poorly to retinoic acid-binding protein, and that they have very little biological activity (Sporn *et al.*, 1975; Wilkoff *et al.*, 1976), it is likely that no correlation exists between binding of retinoids to serum albumin and their biological activity.

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