# Binding of 11-cis retinaldehyde to the partially purified cellular retinaldehyde binding protein from bovine retinal pigment epithelium

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*Summary.* 11-cis retinaldehyde binding analysis was performed on a bovine retinal pigment epithelium preparation of cellular retinaldehyde binding protein (CRALBP), whose purity degree was estimated as 75%. Equilibrium binding studies were carried out measuring the replacement of tritium-labeled with unlabeled 11-cis retinaldehyde at 25 °C. Analysis of the experimental data both by a direct curve-fitting procedure utilizing a non linear least square regression analysis and by a conventional Scatchard plot revealed a single non-interacting binding site with an apparent equilibrium constant of $0.9 \times 10^{-7}$ M.
A binding stoichiometry of approximately 1 mol of 11-cis retinaldehyde/mol of binding protein can be calculated from the experimental data. Competition studies carried out in the presence of unlabeled 'trans' and 'cis' isomers of vitamin A derivatives confirm the high degree of specificity of the 11-cis retinaldehyde binding.
*Key words.* CRALBP; 11-cis retinaldehyde; retinal pigment epithelium.

Among the retinoid binding proteins the CRALBP of the retina and retinal pigment epithelium[1-4] is the only one known to bind and carry 11-cis retinaldehyde. This fact, its particular localization[5] and its ability also to carry 11-cis retinol in the retina[3] suggest that it has a physiological function related to the visual process, but this has not been proven. Although the CRALBP has been purified and many of its properties described, the binding of the 11-cis retinaldehyde has not been examined in detail. The aim of this work was to study the binding of the 11-cis retinaldehyde to the CRALBP partially purified from bovine retinal pigment epithelium and to examine the relative ability of other 'cis' and 'trans' retinoids to compete for the physiological ligand.

*Materials.* All-trans-1 [$^3$H] retinol (2.5 Ci/mmol) was purchased from New England Nuclear Corporation. Unlabeled retinol, retinaldehyde, retinyl palmitate (all in the all-trans configuration) were from SIGMA. 13-cis retinoic acid was kindly supplied by Hoffman-La Roche. 11-cis retinaldehyde, prepared by photoisomerization of all-trans retinaldehyde, was purified and its amount established by a Waters HPLC apparatus equipped with a Model 740 Data Module, using a μPorasil column (0.4 × 30 cm) and 2% diethyl ether in petroleum ether as the eluent[6] at a flow rate of 5 ml/min and a monitoring wavelength of 365 nm. Tritiated 11-cis retinaldehyde used in the binding assay was produced by photoisomerization (see above) from all-trans [$^3$H] retinaldehyde enzymatically obtained from all-trans [$^3$H] retinol[1]. 11-cis retinyl palmitate was synthesized as described by Groenendijk et al.[7] with minor modifications.

11-cis retinol was prepared by enzymatic reduction of purified 11-cis retinaldehyde. Reaction mixtures (0.4 ml) contained 62.5 μM 11-cis retinaldehyde in 0.5% (w/w) Tween 80 emulsion in water to give a final concentration of 0.125% Tween 80; 1 mM NADH; 100 mM $Na_2HPO_4$-$NaH_2PO_4$ buffer pH 5.9 and 64 U (40 mg) of equine liver alcohol dehydrogenase (SIGMA). The mixture, incubated in the dark at 37 °C for 60 min, was extracted by mixing successively with 4 ml of absolute ethanol, 15 ml of petroleum ether and 6 ml of water. From the upper phase, concentrated under nitrogen to a volume of several microliters, 11-cis retinol was separated from residual 11-cis retinaldehyde by descending thin layer chromatography and then purified and quantified by HPLC as described above using a monitoring wavelength of 320 nm and hexane:dioxane (95:5) as the eluent[8]. Sephadex G-100 and G-25 were from Pharmacia, DE 52 cellulose (DEAE cellulose) from Whatman. Protein standards for gel chromatography and SDS-PAGE were from Boehringer and from SIGMA respectively. All other reagents were of analytical grade (Merck).

Absorption spectra were recorded on a Beckman DU8 spectrophotometer; an Amicon UM10 membrane was used to concentrate samples before electrophoresis and spectroscopic analysis.

*Methods.* All operations were carried out in dim red light, wherever possible under nitrogen.

*Partial purification of cellular retinaldehyde binding protein.* Retinal pigment epithelium from freshly excised bovine eyes was homogenized in ice cold 32 mM sucrose/50 mM TRIS-Cl pH 7.4, with a glass/glass homogenizer at a constant speed of 2000 rev/min. The cytosol fraction was isolated by the experimental procedure described by Feeney-Burns and Berman[9]. The protein concentration of the cytosol averaged 4–6 mg/ml.

The identification of the CRALBP in the pigment epithelium was initially achieved by charging the cytosol with 11-cis [$^3$H] retinaldehyde ($1 \times 10^{-6}$ M) and applying it to a Sephadex G-100 column (1.8 × 50 cm) as described by Saari[10]. The binding with the exogenous [$^3$H]-retinoid was necessary, due to the absence of fluorescence which could be monitored from the pigment epithelium CRALBP and to the partial masking of its absorbance at 425 nm[3] by the strong absorbance of the hemoglobin eluted in the immediately preceding fractions. The protein fractions associated with the maximum radioactivity peak, and corresponding to a molecular weight of about 35,000[10] were identified as CRALBP.

In the preparative procedure to isolate the CRALBP, gel filtration on Sephadex G-100 was employed, followed by an ion exchange chromatography on DEAE cellulose column (0.9 × 60 cm) to detach the residual hemoglobin from the CRALBP.

Some experiments were designed to establish the quantitative contribution of the CRALBP to the partially purified protein preparation. Analytical SDS-gel electrophoresis was carried out on 8% polyacrylamide gels. The method used was essentially that described by Zahler[11]. Densitometric traces of the protein bands stained with Coomassie blue were obtained by scanning the gels for absorbance at 550 nm. Molecular weight of the

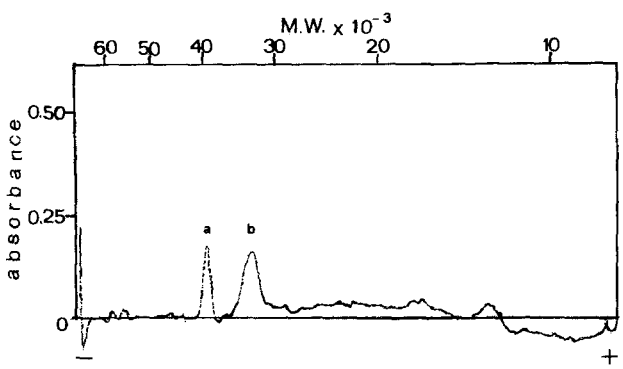


Figure 1. SDS gel electrophoresis of the partially purified CRALBP from retinal pigment epithelium. 50 μg of partially purified binding protein were used for the electrophoretic run. The gel was stained with Coomassie blue and scanned as described in Methods. The protein band labeled *b* showing a relative mobility corresponding to a molecular weight of 34,000 is identified as CRALBP.

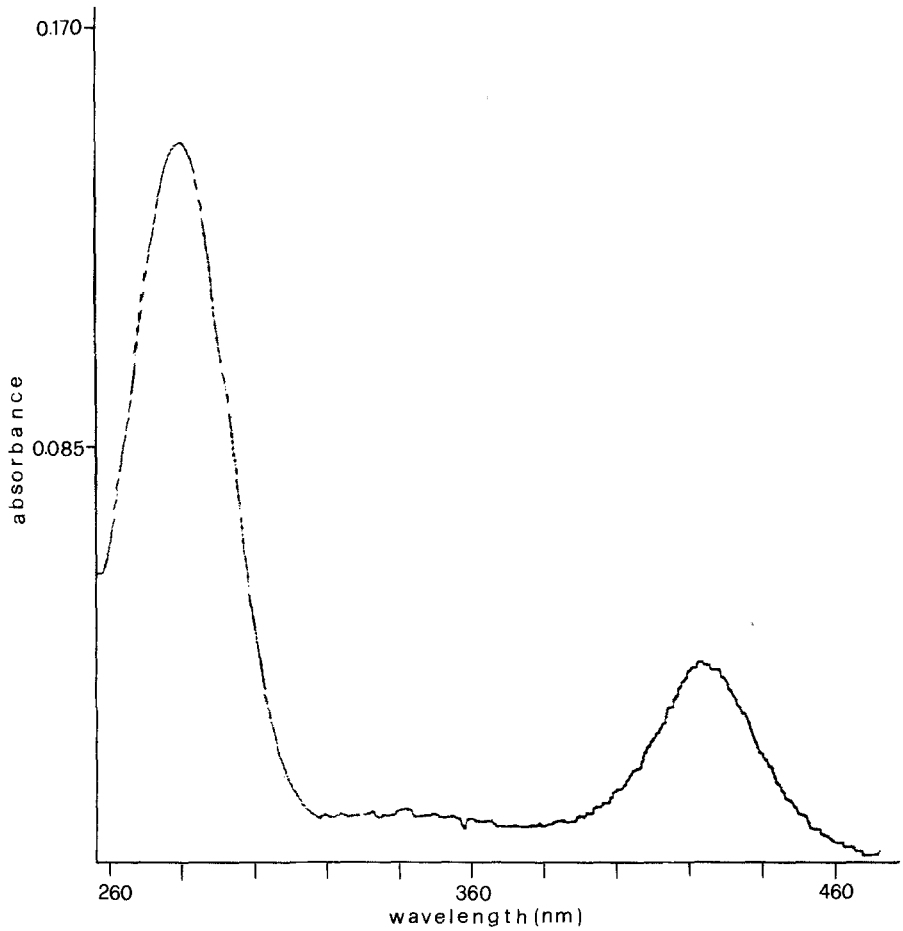


Figure 2. Absorption spectrum of partially purified CRALBP. The absorption spectrum is relative to partially purified CRALBP (140 µg/ml) fully saturated with 11-cis retinaldehyde. The presence of an absorption maximum at 425 nm is due to bound 11-cis retinaldehyde.

bands was estimated by the relative mobility, using bovine serum albumin (MW 69,000), ovalbumin (MW 45,000), glyceraldehyde-3-phosphate dehydrogenase (MW 36,000), carbonic anhydrase (MW 29,000) and lysozyme (MW 14,000) as protein markers.

To calculate the spectral ratio $A_{280}/A_{425}$ the absorbance spectrum of the partially purified CRALBP was recorded. For this purpose an aliquot of the partially purified protein from DEAE cellulose was employed, concentrated by ultrafiltration to a small volume, incubated with excess 11-cis retinaldehyde and passed over a column (1.0 × 30 cm) of Sephadex G-25 (see below) to remove the unbound 11-cis retinaldehyde.

*11-cis retinaldehyde binding.* To remove the endogenous ligand, the partially purified CRALBP (10 µg/ml) was exposed to the light from a 100 W incandescent lamp in an ice-bath for 2 min. Under these conditions the main photoproduct, all-trans retinaldehyde, is easily detached from the binding protein[12]. The addition of BSA to the CRALBP preparation, to avoid development of turbidity and precipitation of the protein during the bleaching[12], was not necessary, probably due to the low concentration of the CRALBP, and then of 11-cis retinaldehyde, in our binding fraction.

Preliminary experiments were carried out to optimize the amount of protein and the incubation time in the binding assay. In a standard binding assay 6 µg of the partially purified apoprotein in a total volume of 1.2 ml of TRIS-Cl pH 7.5 were added to 5 µl of ethanol solution of 11-cis [$^3$H] retinaldehyde to reach a final concentration of 1.4–88 × $10^{-8}$ M (800 to 50,000 cpm) and incubated in the dark at 25 °C for 6 h. From the pilot experiments evidence was achieved that this was the time of peak binding, in order to assure equilibrium even at the lowest of the retinoid concentrations.

At the same time, samples were made containing the same concentration of labeled 11-cis retinaldehyde in the presence of an excess of unlabeled ligand.

To further check the binding at the very low 11-cis [$^3$H] retinaldehyde concentrations (0.5–1.4 × $10^{-8}$ M) a preparation of labeled 11-cis retinaldehyde, whose specific radioactivity was increased 5-fold, was used under all other assay conditions described. Separation of bound from free retinaldehyde was achieved by the method of Bashor[13, 14]. Aliquots of the incubation mixture (5 µg protein) were applied to a column (0.8 × 10 cm) of Sephadex G-25 (Fine), equilibrated and then eluted with 50 mM TRIS-Cl pH 7.4 at a flow rate of 25 ml/h. Fractions of 0.5 ml were collected in counting vials and the radioactivity determined, after addition of 5 ml of liquid scintillation mixture, in a Nuclear Chicago scintillation counter. The radioactivity measured in a void volume of 8 ml was considered to be due to the bound 11-cis retinaldehyde. Non-specific binding, defined as that binding resistant to a 200-fold excess of non-radioactive 11-cis retinaldehyde, was subtracted from total binding to obtain the specific binding in all experiments.

In control experiments, labeled retinaldehyde was first applied to the gel column with no protein sample present. Not more than 2% of the total radioactivity applied to the column in each assay was ever recovered in the void volume. This value was then used to correct the values in the binding experiments.

*Competitive binding studies.* In the competitive binding studies, a variable excess of 11-cis retinaldehyde, all-trans and 11-cis retinol, 13-cis retinoic acid, and 11-cis retinyl palmitate, in 5 µl of

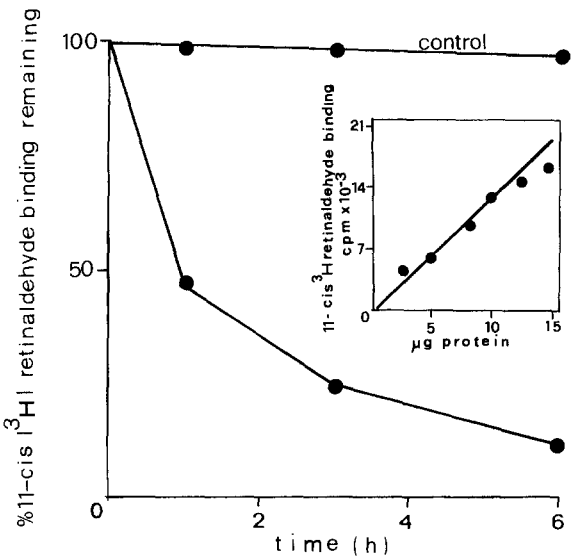


Figure 3. Reversibility of the 11-cis [$^3$H] retinaldehyde binding. Partially purified CRALBP (6 µg/ml) was incubated for 6 h at 25 °C with $88 \times 10^{-8}$ M 11-cis [$^3$H] retinaldehyde. Then a 200-fold excess of unlabeled 11-cis retinaldehyde was added. Aliquots of the mixture were removed at various times thereafter and assayed for 11-cis [$^3$H] retinaldehyde binding. The initial sample, at time zero, was taken as the reference value and subsequent values were expressed as per cent of [$^3$H] retinaldehyde binding remaining. The inset shows the dependence of the retinaldehyde binding on the amount of binding fraction expressed as µg protein per incubation mixture. A single binding protein preparation was assayed systematically at several dilutions in the presence of a constant amount of 11-cis [$^3$H] retinaldehyde of $88 \times 10^{-7}$ M.

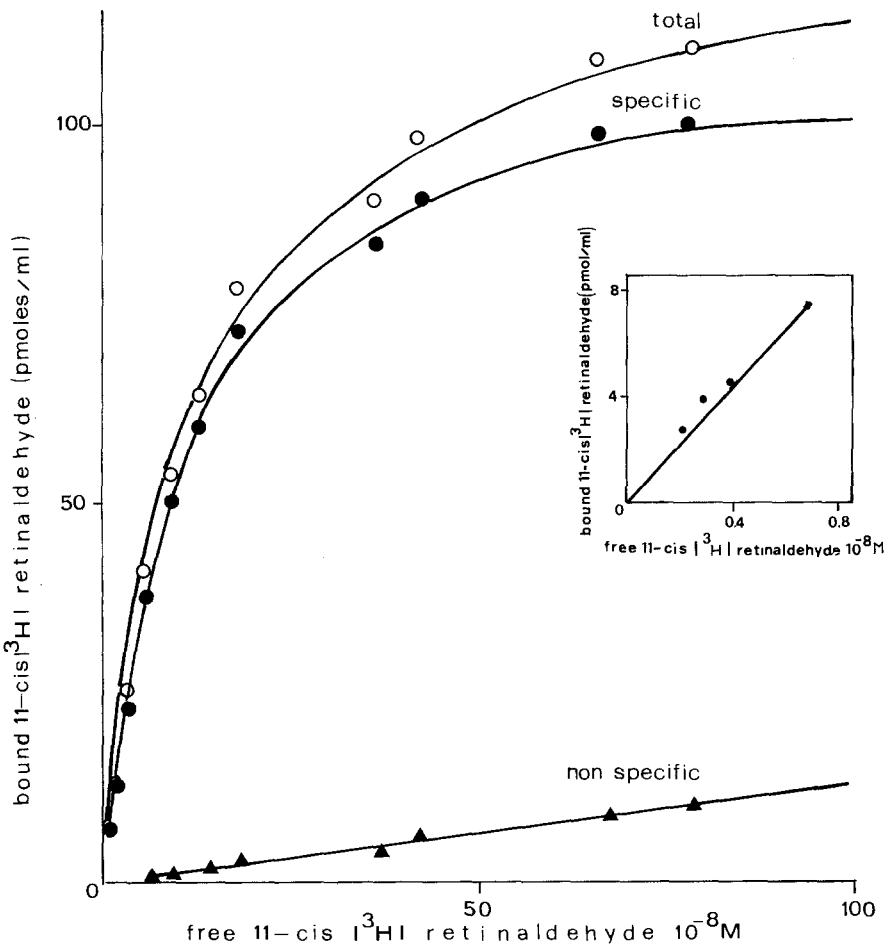


Figure 4. Saturation curve for the binding of 11-cis [$^3$H] retinaldehyde to partially purified CRALBP from pigment epithelium. Specific binding, derived by subtraction of non specific from total binding, was used to generate the Scatchard plot. Each point represents the mean of 4–5 experiments. The inset shows the specific binding curve relative to 11-cis retinaldehyde concentrations ranging between 0.5 and $1.4 \times 10^{-8}$ M (see also Methods).

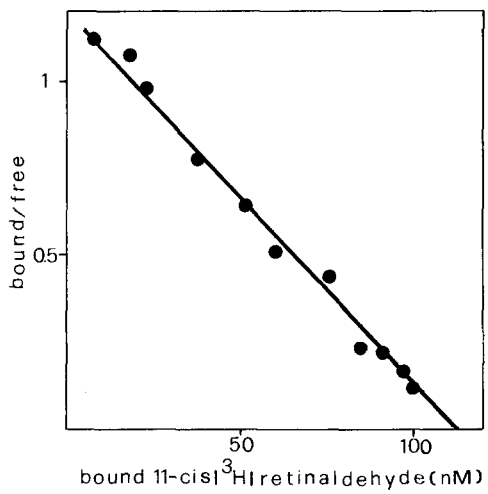


Figure 5. Scatchard analysis of specific 11-cis [$^3$H] retinaldehyde binding to partially purified cellular retinaldehyde binding protein. Data from the specific binding curve were plotted according to the method of Scatchard using a linear regression to find the lines which best fit the experimental values. Correlation coefficient ($r^2$) was 0.994.

ethanol, was alternatively added to the reaction mixture containing the partially purified CRALBP charged with 11-cis [$^3$H] retinaldehyde. Control samples received ethanol alone.

Protein concentration was determined throughout by the Coomassie blue method of Bradford[15] with 5 µg of $\gamma$-globulin as a standard (micromethod).

*Results and discussion. Degree of purity of CRALBP in the partially purified protein preparation.* Figure 1 shows the SDS-polyacrylamide gel electrophoresis pattern obtained with the partially purified 11-cis retinaldehyde binding protein after elution from DEAE cellulose. The binding protein (MW 34,000)[10] and a contaminant of a molecular weight of about 40,000 were the major polypeptide species present. Scanning of the Coomassie blue stained gel revealed that the area under the 11-cis retinaldehyde binding protein was approximately 72% of the total area under all peaks. Spectral analysis of the partially purified CRALBP is shown in figure 2. As expected for a partially purified protein preparation the spectral ratio $A_{280}/A_{425}$ was higher than the experimental value of 3.0 observed with a purified fully saturated binding protein[3]. Typically, the observed ratio was around 3.9, corresponding to a degree of purity of CRALBP of 77%. Taken together these results allow us to consider that in our preparation the degree of purity of CRALBP averaged 75%.

*Binding studies with partially purified CRALBP.* The binding of 11-cis [$^3$H] retinaldehyde with the partially purified CRALBP was linear with respect to the analyzed range of protein concentration (fig. 3, inset). Figure 3 shows the displacement of bound 11-cis [$^3$H] retinaldehyde from the CRALBP. The partially purified binding protein was incubated with 11-cis [$^3$H] retinaldehyde to achieve maximum binding, followed by the addition of 200-fold excess of cold ligand. Bound 11-cis [$^3$H] retinaldehyde could be nearly fully displaced by the addition of unlabeled retinoid, demonstrating the reversibility of the binding and indicating that the binding is non-covalent.

The concentration dependence of the 11-cis [$^3$H] retinaldehyde binding was examined. Incubation of the partially purified CRALBP with increasing concentrations of tritiated 11-cis retinaldehyde showed a limited number of binding sites which, under our experimental conditions, approached saturation at $88 \times 10^{-8}$ M 11-cis [$^3$H] retinaldehyde (fig. 4). The non-specific binding increased linearly with increased retinaldehyde concentration. In the inset is shown the portion of the binding curve relative to 11-cis [$^3$H] retinaldehyde concentrations ranging between 5 and $14 \times 10^{-9}$ M. The binding was linear, indicating that there are no cooperative site interactions at very low concentrations of ligand.

Scatchard analysis of the binding data, using a least squares regression analysis, yielded a straight line ($r^2$ 0.994) indicating a single class of binding sites with a Bmax of $112 \times 10^{-9}$ M. From the slope of the line an apparent equilibrium constant of $0.9 \times 10^{-7}$ M was calculated. A representative Scatchard plot from five separate experiments is shown in figure 5.

The saturation data were also analyzed by the Hill plot, carried out by fitting the Hill equation directly to the experimental data using a non-linear regression method[16]. From the latter analysis the Kd and Bmax values were found to be in close agreement with the values from the Scatchard analysis.

On the basis that the CRALBP has a molecular weight of 34,000, the amount of 11-cis retinaldehyde binding sites per molecule of binding protein was also calculated from the Scatchard plot and found 0.75.

The apparent binding stoichiometry was further investigated by incubating portions of partially purified protein with excess 11-cis retinaldehyde, followed by the removing of the unbound chromophore. The bound 11-cis retinaldehyde extracted and quantified by HPLC allowed us to calculate an apparent binding stoichiometry of 0.76 moles of 11-cis retinaldehyde/mol of CRALBP. The less than stoichiometric amount of bound 11-cis retinaldehyde was not unexpected since the sample had not been purified to homogeneity.

However, as the purity of the CRALBP with which we were dealing has been verified as being not less than 72%, a binding stoichiometry of approximately 1 mol of 11-cis retinaldehyde/mol of binding protein can be calculated.

*Competitive binding assays.* The degree of specificity of the retinaldehyde binding was also tested. Both 'all-trans' and 'cis' isomers of some vitamin A derivatives were incubated with the partially purified CRALBP and allowed to inhibit binding of $8.8 \times 10^{-7}$ M of tritiated 11-cis retinaldehyde to its binding sites. The competition profiles are shown in figure 6. One hundred percent binding capacity (control) represents the specific binding of 11-cis retinaldehyde in the absence of any competitor. The relative affinity for the receptor was derived by comparing the

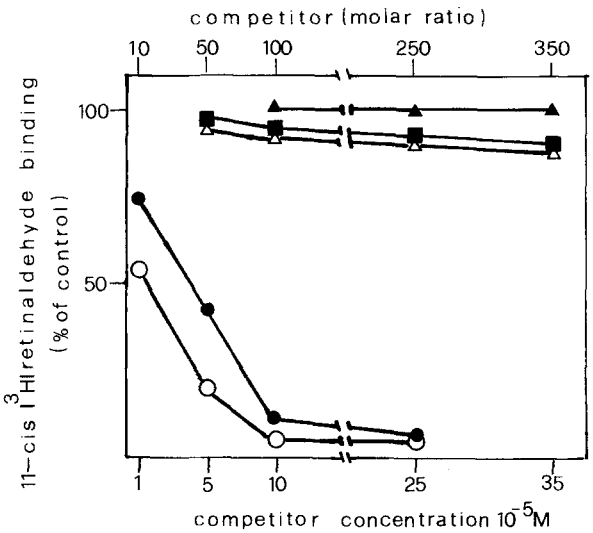


Figure 6. Competition for 11-cis [$^3$H] retinaldehyde binding site by unlabeled retinaldehyde analogues. Protein binding fraction was incubated as reported in Methods with $88 \times 10^{-8}$ M 11-cis [$^3$H] retinaldehyde alone and in the presence of unlabeled competitors (○—○, 11-cis retinaldehyde; ●—●, 11-cis retinol; △—△, trans retinol; ▲—▲, 11-cis retinyl palmitate; ■—■, 13-cis retinoic acid). The control value of specific binding in the absence of competing analogues was taken as 100% (18 ± 1.9 nmol/mg of protein). Each point represents the mean of duplicate determinations.

concentration of unlabeled compound required to reduce the 11-cis retinaldehyde binding to 50% of the control value.
None of the retinoids were found to compete significantly at any of the tested concentrations, except 11-cis retinol. This was not unexpected when it is considered that, in the retina, the CRALBP is found to carry 11-cis retinaldehyde and 11-cis retinol as the endogenous ligands, and in the pigment epithelium is also able to bind 11-cis retinol, in addition to the endogenous 11-cis retinaldehyde, following exposure to the light[3].
Although the different compartmentalization of the 11-cis retinoids in the retina and in the pigment epithelium[17] could be sufficient to explain the different complement of its endogenous ligand, the competition profile would also indicate for the CRALBP some difference in the affinity towards 11-cis retinaldehyde and 11-cis retinol.

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## Induction of chromosomal aberrations by the anthracycline antitumor antibiotics N,N-dimethyldaunomycin and aclacinomycin A

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*Summary.* The clastogenic effect of the anthracycline antitumor antibiotics, N,N-dimethyldaunomycin and aclacinomycin A, was studied in a murine hemopoietic cell line (Friend leukemia cells). A dose-dependent increase in chromatid lesions, i.e., achromatic lesions, chromatid breaks, chromatid deletions and triradial or quadriradial chromosomal exchange figures, was found. It appears that the clastogenicity of N,N-dimethyldaunomycin and aclacinomycin A is lower than that of the classic anthracycline, daunomycin, which is also a potent mutagen and carcinogen. The data demonstrate that the capacity of chemicals to induce point mutations and chromosomal aberrations may not necessarily be correlated: aclacinomycin A is devoid of mutagenic activity in bacterial (Salmonella typh.) and mammalian cell (HGPRT) mutagenesis assays, and is non-carcinogenic in rats. Nevertheless, it was now found to possess clastogenic activity.
*Key words.* Aclacinomycin A; anthracyclines; chromosome aberrations; daunomycin; N,N-dimethyldaunomycin.

The anthracycline antitumor antibiotics, adriamycin and daunomycin, are potent mutagenic, clastogenic and carcinogenic compounds[1,2]. On the other hand, N-substituted anthracyclines are only weakly mutagenic or nonmutagenic[3,4]. However, some of these N-substituted anthracyclines, such as cyanomorpholino-adriamycin[3], are potent crosslinking agents and induce unscheduled DNA-synthesis and chromosomal aberrations[3,5]. It appears, therefore, that a discrepancy may exist between the mutagenic properties of N-substituted anthracyclines (as determined with bacterial or mammalian cells) and their clastogenic potential. To further clarify this, we studied the clastogenicity of the N-alkylated anthracycline N,N-dimethyldaunomycin, and the N-alkylated oligosaccharide anthracycline, aclacinomycin A. The latter is of particular biological and clinical interest because of its differentiation-inducing capacity in vitro[6-8] and possibly in vivo[9]. It should be noted that both N,N-dimethyldaunomycin and aclacinomycin A are nonmutagenic in bacterial and mammalian cells[3,4] and that, furthermore, aclacinomycin A is also noncarcinogenic in rats[10].
*Materials and methods. Chemicals.* Aclacinomycin A and N,N-dimethyldaunomycin were kindly provided by Dr T. Oki, Sanraku-Ocean Co. Ltd., Tokyo, Japan, and by Dr E. Acton, Stanford Research Institute, Menlo Park, California, USA, respectively. Daunomycin was obtained by courtesy of Dr F. Arcamone, Farmitalia-Carlo Erba, Milano, Italy. Alpha medium without nucleosides and fetal calf serum were purchased from Gibco Co., Darmstadt, FRG.
Before use the anthracyclines were dissolved in 0.9% NaCl solution. A stock solution (1 mg/ml) was prepared and further dilutions were carried out using alpha medium without fetal calf serum.
*Cell line.* The Friend erythroleukemia clone F4-6 employed by us has been described[11,12].
*Induction of chromosomal aberrations.* Cell cultures were set up in duplicate by plating 2.5–3 × $10^5$ viable cells in plastic petri dishes (diameter, 5 cm), containing 5 ml of cell culture medium supplemented with 10% fetal calf serum. Three days later, the cultures were treated for 1 h with different concentrations of the test compounds. Thereafter, the cultures were rinsed twice with the medium to remove these compounds, and fresh culture medium was added. After 24 h, the cells were incubated for 2 h in the presence of colcemid (1 μg/ml) and harvested by decanting suspended cells or by scraping off the adherent cells with a rubber policeman. The cells were centrifuged, resuspended in the residual volume of 0.25–0.5 ml medium and treated for 12 min at room temperature with 4 ml of a hypotonic solution of KCl (0.075 M). After hypotonic treatment the cells were spun down again, resuspended in the remaining fluid and fixed by the addi-