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A rapid versatile microassay for cellular retinol-binding protein using Lipidex-1000 microcolumns

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Abstract A new, rapid and versatile microassay for cellular retinol-binding protein has been developed based on separation of bound and free ligand by means of Lipidex-1000, a hydrophobic Sephadex derivative. This requires quantitative manipulation of retinol in aqueous solution. The tendency of retinol to adhere to glass and plastic surfaces was overcome by addition of the detergent Ammonyx LO, which yields a micellar dispersion. Detergent concentrations up to 10 mM did not interfere with binding of retinol to Lipidex-1000 or binding protein. The binding capacity of Lipidex-1000 was found to exceed 400 nmol of retinol per ml of gel. Retinal pigment epithelium (RPE) cells were used as a source for cRBP (cellular retinol-binding protein). The binding protein is saturated with ligand by incubation for 60 min at room temperature at concentrations of free retinol over 180 nM. Separation of protein-bound retinol from free retinol is achieved via Lipidex-1000: protein-bound (specific and nonspecific) retinol is not retained and is eluted by buffer with the protein fraction. Free retinol is retained by Lipidex and is subsequently recovered by elution with methanol. Total recovery of ligand approaches 100%. Analysis time is about 4 hr for a maximum of ca. 50 samples. Nonspecific protein binding can be determined equally effectively either by incubation with 3 mM PCMBS or by addition of a 100-fold molar excess of nonlabeled retinol. The day-to-day reproducibility is within 5% and the detection limit is 7 μg of cRBP/liter using labeled retinol. With a 10-fold sacrifice in sensitivity, the assay can also be run in a nonradioactive mode using fluorescence detection. Estimation of the cRBP level in other tissues with this new assay gives values in the same range as reported elsewhere using RIA. The levels decrease in the order: retinal pigment epithelium > > kidney > testis > > brain. Even the low tissue levels in brain (23 pmol/g of tissue) can be measured with the Lipidex microassay. The very high levels in RPE (68,000 pmol/g of tissue) probably reflect the important role of this tissue in furnishing and recycling retinol for the visual process. — **Timmers, A. M. M., D. A. H. M. van Groningen-Luyben, F. J. M. Daemen, and W. J. De Grip.** A rapid versatile microassay for cellular retinol-binding protein using Lipidex-1000 microcolumns. *J. Lipid Res.* 1986. **27:** 979–987.

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The retinoids, vitamin A and derivatives, are a class of essential compounds with diverse biological activity. *a)* They have profound effects on cell differentiation and proliferation in many tissues and hence have become important targets of research in dermatology and oncology (1). *b)* The aldehyde derivative of retinol is directly involved in vision (2). *c)* Vitamin A or its esters are essential for reproduction and vision. The latter two processes cannot be supported by retinoic acid (2, 3).

Utilization of vitamin A in the body is regulated at several sites (4). Transport of vitamin A, a very lipophilic compound, from liver to target tissue occurs through the serum retinol-binding protein (sRBP) (5). This protein is synthesized and secreted by the liver. Entry of vitamin A from the blood circulation into the cells is mediated by an sRBP receptor (6). At the cellular level retinoids are transported by a cellular retinol-binding protein (cRBP) or a cellular retinoic acid-binding protein (cRABP) (7). The latter two retinoid-binding proteins are very common to many tissues (8). Two other retinoid-binding proteins appear to be exclusively located in the retina: the cellular 11-*cis*-retinoid-binding protein (cRA1BP) (9) and the interphotoreceptor matrix retinoid-binding protein (IRBP) (10, 11).

Currently the working mechanism of retinoids both at the cellular and at the molecular level is a matter of very active research. The selectivity of the retinol-binding proteins appears to be an important parameter in this con-

Abbreviations: cRBP, cellular retinol-binding protein; RPE, retinal pigment epithelium; sRBP, serum retinol-binding protein; cRABP, cellular retinoic acid-binding protein; cRA1BP, cellular 11-*cis*-retinoid-binding protein; IRBP, interphotoreceptor matrix retinoid-binding protein.

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text. The biological activity of a retinoid seems to be directly related to its affinity for the cellular retinoid-binding protein (12).

For the assay of retinoid-binding proteins, several procedures have been described: binding assays, employing separation of free and bound ligand by sucrose gradient centrifugation (13, 14), on charcoal-coated dextran (15), or by gel filtration (13, 16–18), and radioimmunoassays (19, 20), employing radiolabeled binding protein and specific antibodies.

In the context of our studies on retinal pigment epithelium (21), the need for a rapid, simple, and sensitive retinoid-binding protein assay was felt. Upon reviewing the existing methods, several disadvantages became obvious. Sucrose gradient centrifugation and gel filtration assays are lengthy and laborious. Even the recently introduced modification of the gel filtration approach—the much more rapid mini-gel column centrifugation procedure (22)—still requires laborious manipulation. Separation of free and bound ligand with charcoal-coated dextran may result in considerable loss of protein (23). Radioimmunoassays are very sensitive, but require purified and labeled retinoid-binding proteins as well as monospecific antibodies elicited specifically against every different retinoid-binding protein to be assayed.

Recently a reliable and rapid binding assay for the fatty acid-binding protein has been introduced (23), which is based on separation of the free from the protein-bound hydrophobic ligand (palmitate) by means of Lipidex-1000, a hydrophobic Sephadex-G25 derivative containing 10% (w/w) alkyl groups. Since this matrix has high affinity for hydrophobic compounds and has very low affinity for protein (23), its use could present a promising alternative for the retinoid-binding protein binding assays described so far. After improving conditions for manipulation of retinol in aqueous environment (inclusion of detergent), we found that under optimal conditions Lipidex-1000 indeed has a high capacity to bind free retinoids. This led to the development of a new binding assay for cellular retinol-binding protein, which is rapid, specific, and sensitive.

MATERIALS AND METHODS

Materials

Lipidex-1000 was obtained from Packard Instrument Co., Inc. (Downers Grove, IL). All-*trans*-[11,12-di-^{3}H]retinol (sp. act. 55 Ci/mmol) was from Amersham (Amersham, UK); unlabeled all-*trans* retinol was from Eastman Kodak Co. (Rochester, NY); Ammonyx LO was from Millmaster Onyx International (Fairfield, NJ); *p*-chloromercuribenzenesulfonic acid (PCMBS) monosodium salt was from Sigma Chemical Co. (St. Louis, MO); Con-A Sepharose was from Pharmacia AB (Uppsala, Sweden); and Aqua Luma Plus scintillation fluid was from Lumac:3M BV (Schaesberg, the Netherlands). All other reagents were of analytical grade. Incubation buffer contained 10 mM KH_2PO_4, pH 7.4, containing either 0.06% Ammonyx LO, (RPE) or 0.2% Ammonyx LO (rat tissue). Elution buffer contained 10 mM KH_2PO_4, pH 7.4.

As a source for cellular retinol binding protein (cRBP), retinal pigment epithelium cells were used (24, 25). They were isolated from bovine eyes obtained from the local slaughterhouse by the perfusion technique described previously (21). Their cytoplasmic proteins are released by sonication (30 sec, Branson B12 Sonifier with microtip) and, following centrifugation (30 min, 40,000 *g*, 4°C) the supernatant was used directly in the retinol-binding protein assay.

Bovine serum, isolated from blood collected at the local slaughterhouse, was used as a source of serum retinoid-binding protein.

Interphotoreceptor retinol binding protein (IRBP) was isolated from bovine retinas as described by Adler and Evans (26): the fraction released from Con-A Sepharose was directly used in the binding assay.

Preparation of rat tissue extracts

Male rats (3 months, 150–200 g, fed ad libitum on Hope Farm rat pellets RNH) were killed by cervical dislocation and immediately perfused through the left ventricle with an isotonic Ca^{2+},Mg^{2+}-free salt solution containing 2 mM EDTA (21). Several organs were dissected and, after wet weight determination, the organs were homogenized in 10 mM KH_2PO_4, pH 7.4, in a Potter-Elvehjem tube with tight-fitting plunger. The soluble proteins were extracted for 16 hr at 4°C. The supernatants obtained by centrifugation (60 min at 10^5 *g*) were stored at −20°C and used directly for the assay of cRBP.

A stock solution of all-*trans*-retinol in ethanol was stored under argon at −70°C. The aqueous working solution was prepared fresh for every experiment by diluting the ethanol stock in incubation buffer to 800 nM retinol and addition of ^{3}H-labeled retinol (about 6×10^5 cpm/ml).

PCMBS was dissolved in incubation buffer in a concentration of 8 mM.

Protein concentration was determined according to Lowry et al. (27) using BSA for calibration.

Preparation of Lipidex-1000 microcolumns

Glass Pasteur pipets were cut off at the beginning of the capillary part and at the other end leaving a total length of about 4 cm. Then 200 µl of a suspension of Lipidex-1000 (diluted 1:1 with MeOH) was pipetted into the column using a glass bead (2–3 mm diameter) for support. After the methanol had eluted, the microcolumn was washed three times with 0.7 ml of ice-cold elution buffer. Routinely, every microcolumn was used only once. However, repeated use is, in principle, possible. Ready-to-use columns can be stored for at least 1 week at 4°C.

Cellular retinol binding protein assay using Lipidex-1000

Assays were carried out in duplicate. In order to determine the "total (specific + nonspecific) retinol binding" capacity in the protein fraction, the following mixture was incubated for 30–60 min at room temperature: 160 μl of soluble protein extract in incubation buffer (0–200 μg of protein/ml), 80 μl of incubation buffer, and 80 μl of 800 nM [^{3}H]retinol. In order to freeze the binding equilibrium, the mixture was then cooled in ice and kept at 0°C for 10 min. The entire incubation was performed under dim red light (> 590 nm) in order to prevent photoisomerization of retinoids. Separation of protein-bound retinol from free retinol can be performed under fluorescent light. Two hundred fifty μl of the cooled incubation mixture was carefully pipetted on top of a Lipidex-1000 microcolumn (100 μl of gel) and the eluate was collected. The microcolumn was then washed with 250 μl and 500 μl of elution buffer, respectively. Elution and washing takes about 20 min. The three aqueous eluates were collected in a 20-ml scintillation vial. This represents "the protein-bound retinol" fraction. The "free retinol" fraction, which becomes bound to Lipidex-1000 during the procedure, was subsequently eluted with two volumes of 250 μl of methanol (5 min) and collected in a second scintillation vial. In order to equalize the radiation quenching factors, 500 μl of methanol was added to the protein-bound retinol fraction and 1 ml of elution buffer to the free retinol fraction. Then, 10 ml of scintillation fluid was added to each vial and, after thorough mixing, the amount of ^{3}H label present was determined in a Philips liquid scintillation counter.

The extent of "nonspecific binding" of retinol in the protein mixture was measured in parallel samples either by preincubation of the protein extract for 30 min at room temperature in the presence of 3 mM *p*-chloromercuribenzenesulfonic acid (PCMBS), a strong inhibitor of cellular retinol and retinoic acid binding proteins (cRBP and cRABP) (28), or by addition of a 100-fold molar excess of nonlabeled retinol to the incubation mixture simultaneously with the labeled all-*trans* retinol.

Sucrose gradient assay

The assay of retinol-binding protein via sucrose gradient centrifugation was performed as described by Wiggert and Chader (25). Briefly, 250 μl of cooled incubation mixture, prepared as for the Lipidex microassay, was layered on top of a 4.5-ml 5–20% (w/w) sucrose gradient prepared in 10 mM KH_2PO_4, pH 7.4. After centrifugation (16 hr, 220,000 *g*, 4°C) the bottom of the tube was punctured and fractions of five drops each were collected. Each fraction was mixed with 5 ml of scintillation fluid and counted as described above. Nonspecific binding was measured as described for the Lipidex microassay.

Calculation of specific binding

Lipidex microassay. Total protein binding (PT). Since the recovery in the eluates of the total label applied to Lipidex microcolumns is nearly quantitative (93–100%), total label (free + protein bound) was calculated by adding the radioactivity measured in the protein-bound fraction (PT) to the radioactivity measured in the free retinol fraction (FT). That percentage of total label, which represents total protein bound retinol (% PT), then becomes:

$$\%\mathrm{PT} = \frac{\mathrm{PT}}{\mathrm{PT} + \mathrm{FT}} \times 100\%.$$

Nonspecific protein binding (PN). The amount of nonspecific binding to the protein fraction was resolved either by performing the assay in the presence of excess nonlabeled all-*trans* retinol or by preincubation with a specific inhibitor (PCMBS). Again, total label was calculated by adding the radioactivity in the protein bound fraction (PN) to that in the free retinol fraction (FN). The percentage of nonspecific binding (% PN) was then:

$$\%\mathrm{PN} = \frac{\mathrm{PN}}{\mathrm{PN} + \mathrm{FN}} \times 100\%.$$

Specific protein binding (PS). PN + FN should equal PT + FT. The amount of retinol specifically bound to binding protein in the 250-μl reaction mixture (PS) was then calculated as follows:

$$\mathrm{PS} = (\%\ \mathrm{PT} - \%\ \mathrm{PN}) \times 50\ \mathrm{pmol}.$$

Sucrose gradient assay. The calculation of the specific binding of retinol as obtained by sucrose gradient centrifugation was also done by subtracting the nonspecific binding from the total binding.

cRBP (M_r ca. 15 kD) migrates with a 2 S-sedimentation constant. Hence, those fractions in the 2 S region, where the label density was higher in the assay for total binding than in the corresponding assay for nonspecific binding, were considered to represent cRBP. The radioactivity in these fractions was added together and represented total 2 S-bound label (PTS).

Likewise, the sum of the radioactivity in the corresponding 2 S fractions of the nonspecific binding assay was calculated and represented nonspecific 2 S-binding (PNS). These figures were normalized into percentages of total label layered on the gradients, yielding % PTS and % PNS. The specific binding in 250 μl of reaction mixture (PSS) was then calculated as follows:

$$\mathrm{PSS} = (\%\ \mathrm{PTS} - \%\ \mathrm{PNS}) \times 50\ \mathrm{pmol}.$$

RESULTS AND DISCUSSION

Retinol in aqueous media

Retinol is highly insoluble and rather unstable in aqueous solution. Consequently, it demonstrates a strong tendency in aqueous environment to aggregate and/or to adhere to whatever glass or plastic surfaces are available (29). This property makes it impossible to handle retinol quantitatively, and does not allow a calculation of the actual concentration of retinol in aqueous media from the amount added. These physical characteristics render assays of retinol-binding proteins based on binding of retinol troublesome and less reliable.

In order to overcome this disadvantage, we investigated whether the addition of detergents, which are routinely used for the solubilization of hydrophobic compounds in aqueous media, might also be suitable for aqueous dispersion of retinol. **Table 1** demonstrates that solubilization of retinol in aqueous media using low concentrations of detergent does indeed strongly suppress the tendency of retinol to adhere to glass and polyethylene surfaces. The detergent Ammonyx LO was selected for routine use in this respect since it is a commercially available, reasonably cheap, mild, and well-defined nonionic detergent. In the presence of at least 3 mM Ammonyx LO (0.06%), which is well above its critical micellar concentration of 1 mM (30), retinol appears to be completely in micellar form and behaves as a stable aqueous dispersion. Concentrations up to 0.5 mM retinol are easily attained. As deduced from spectral measurements, retinol solubilized in incubation buffer is stable for at least 4 hr at room temperature.

Retinol binding by Lipidex-1000

In order to explore the suitability of Lipidex-1000 in a binding assay for retinol-binding proteins, its capacity to bind retinol dissolved in Ammonyx LO must first be analyzed. This is performed by applying 250-μl aliquots of solutions with increasing retinol concentration in incubation buffer (0.06% Ammonyx LO) to 100 μl of Lipidex-1000 gel. Rapid and complete extraction of retinol out of its micellar solution could be achieved and no saturation of binding was observed up to the highest concentration tested (160 μM). Hence, the binding capacity of Lipidex-1000 for retinol amounts to at least 40 nmol of retinol per 100 μl of gel and easily satisfies the requirements for a binding assay for retinol-binding proteins. Higher detergent concentrations were also investigated; Ammonyx LO concentrations up to 0.5% (25 mM) did not affect the binding capacity of Lipidex-1000 for retinol.

A small percentage of leakage through the Lipidex microcolumn was observed, which appears to have been due to slow degradation of [^{3}H]retinol. This percentage was low when a fresh batch was used ($\leq$ 3%) but increased up to 10% in 6 months. The amount of protein-bound retinol (total and nonspecific binding) was corrected for this leakage.

Source of cBRP

Retinal pigment epithelium was selected as a source for the cellular retinol-binding protein since that tissue contains high amounts of this binding protein (24, 25) and we have developed a reliable procedure to isolate these cells in high yield and high purity (21).

Binding assay

Several conditions have to be fulfilled in order to allow reliable and quantitative determination of receptors or binding proteins by means of a binding assay. *1)* Separation of protein-bound ligand and free ligand must be achieved. *2)* Quantitation of binding protein requires saturation of the binding proteins with added ligand. Hence, incubation should be continued until a dynamic equilibrium between receptor and ligand has been attained and the concentration of added ligand should be sufficient to saturate the binding protein. *3)* Reliable discrimination

TABLE 1. Typical recovery of retinol in aqueous phase after 30 min incubation of 1 μm retinol at room temperature in some common vessel materials

Vessel Material	Recovery of Retinol (%)		
	Aqueous Phase[a]		
	Buffer Only	+ Nonylglucose (3 mM)[b]	+ Ammonyx LO (3 mM)[c]
Polystyrene	13	35	85
Glass	30	80	96
Polyethylene	12	90	98

[a] Aqueous phase: 10 mM KH_2PO_4, pH 7.4.
[b] Detergent concentration was below critical micellar concentration (6.5 mM, ref. 30).
[c] Incubation buffer; detergent concentration was above critical micellar concentration 1 mM, ref. 30).

between specific binding protein-dependent and nonspecific binding of the ligand should be accomplished.

The binding assay for retinol binding protein using Lipidex-1000 was developed along these lines.

The feasibility of separating protein-bound and free retinol with Lipidex-1000 was investigated by applying incubation mixtures containing cRBP and labeled retinol to the Lipidex microcolums. Subsequently the columns were eluted as described under Methods. The label not retained by the Lipidex represents protein-bound retinol. **Fig. 1** shows that a certain fraction of the retinol elutes in the "protein-bound retinol fraction." It further shows that a linear relation exists between the amount of total label eluting with the protein fraction and the amount of protein applied to the column. We conclude that, in agreement with the results described for the fatty acid-binding protein (23), separation of protein-bound and free retinol can be achieved by means of Lipidex-1000.

In order to determine the time to binding equilibrium during incubation of retinol with the binding protein at room temperature, the incubation time was varied from 5 min to 2 hr. The amount of protein-bound retinol was measured as described under Methods. Maximal binding was reached after 15–30 min of incubation at room temperature and remained constant for the next 2 hr (data not shown). In order to ascertain that equilibrium conditions are reached, incubation times of 60 min at room temperature were applied routinely.

Saturation of binding is achieved when the amount of specifically protein-bound retinol levels off to a constant level upon increasing the total amount of retinol in the incubation mixture at a fixed binding protein concentration. This permits selection of a retinol concentration where, rather independent of the amount of protein present in the assay, the amount of specifically bound retinol is a quantitative measure for the amount of binding protein present. This aspect was addressed by incubating cell supernatant with a fixed amount of protein in the presence of increasing retinol concentrations for 1 hr at room temperature. Subsequently, free and protein-bound retinol were separated over Lipidex-1000 microcolumns as described above. The amount of specifically protein-bound retinol first increased with increasing free retinol concentration, but then leveled off at concentrations over 180 nM (**Fig. 2**). Hence, a retinol concentration of 200 nM was used routinely in the binding assay. This concentration corresponds to 50 pmol of retinol per 250 μl sample, which is still far below the binding capacity of the amount of Lipidex-1000 gel used (40 nmol). Fig. 1 already confirmed that under these conditions a linear relation exists between amount of protein-bound retinol and protein concentration. Hence, saturation and thus quantitation of binding protein can be achieved at 200 nM retinol provided that total retinol binding does not exceed 10% of retinol added.

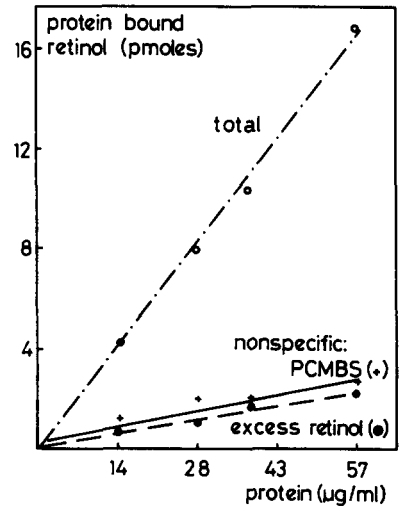

Fig. 1. Separation of free and protein-bound retinol by means of Lipidex-1000 and determination of nonspecific retinol-binding in protein fractions. A cRBP preparation (15–60 μg/ml) was incubated with 200 nM ^{3}H-labeled retinol for 60 min at room temperature. Total protein-bound retinol was determined by the Lipidex microassay as described in Methods. The total protein-bound retinol is plotted versus protein concentration. Nonspecific protein-bound retinol was determined by addition of 100-fold excess of nonlabeled retinol (●-●-●) and by preincubation with 3 mM PCMBS for 30 min at room temperature (+ - + - +).

The last condition to be investigated is whether one can easily discriminate between specific and nonspecific binding of retinol. The established method for determination of nonspecific binding in binding assays consists of incubating the binding protein under normal assay conditions, but thereby including a 100-fold excess of nonlabeled ligand. We have used the same approach by incubating the cRBP preparation in the presence of 200 nM [^{3}H]retinol and additional nonlabeled retinol (20 μM). It is evident from Fig. 1 that the amount of label eluting with the protein fraction is strongly reduced by incubation in the presence of a 100-fold excess of nonlabeled retinol. An independent approach confirmed that the latter curve represents nonspecific binding. It has been reported that retinol binding to the cellular retinol-binding protein is very sensitive to millimolar concentrations of PCMBS (28). Hence, treatment with PCMBS might be a suitable way to inhibit the specific binding of retinol to cRBP and thus to monitor nonspecific binding. Upon preincubation of the cRBP preparation with 3 mM PCMBS for 0.5 hr at room temperature, a binding curve was obtained which indeed nearly coincided with the one obtained in the presence of excess nonlabeled retinol (Fig. 1). Although different mechanisms are involved, PCMBS and excess nonlabeled retinol appear to be equally effective in monitoring nonspecific binding of retinol. Since the high concentrations of retinol required might be undesirable under certain conditions, nonspecific binding was further routinely determined by preincubation with 3 mM PCMBS. This concentration of

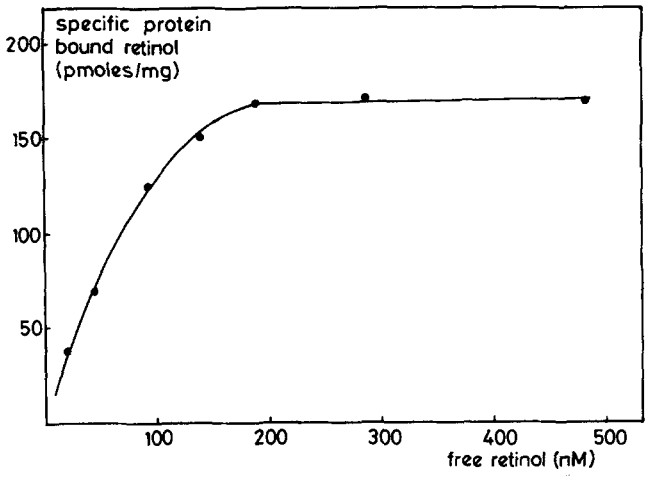

Fig. 2. Saturation of cellular retinol-binding protein with all-*trans* retinol. In this typical experiment, a cRBP preparation (57 μg/ml) was incubated with increasing retinol concentrations (25–500 nM) for 60 min at room temperature. Parallel samples were preincubated with 3 mM PCMBS to determine nonspecific binding. Specific binding was calculated and plotted versus free retinol concentrations. The amount of specific protein-bound retinol initially increased with increasing free retinol concentrations, but then leveled off at 180 nM retinol.

PCMBS did not affect the binding capacity of Lipidex-1000 for retinol (not shown).

In conclusion, the Lipidex microassay unequivocally satisfies all requirements set for a reliable binding assay.

Reproducibility and sensitivity

The Lipidex microassay had to be evaluated further with respect to reproducibility and sensitivity. The day-to-day reproducibility was evaluated by assaying the same cell supernatant with respect to binding activity at intervals of several days. The binding activity was thereby defined as the amount of specific bound retinol (total bound minus nonspecific bound as determined with PCMBS) per mg of protein. Plots of the amount of specific bound retinol versus protein concentration (**Fig. 3**) show that the curves obtained over a 7-day period coincided within the experimental error. The respective binding activities ± SD were calculated by linear regression (on five samples in duplicate per assay), and indeed did not change significantly over the various measurements. Similar results have since been obtained repeatedly, which demonstrates that the assay allows very reproducible measurements.

The detection limit of the assay, defined as the lowest binding protein concentration that shows a significant difference between total and nonspecific binding, was determined with the Student's *t*-test on data again obtained with RPE supernatants. For this typical cellular retinol binding protein preparation, a detection limit of 0.5 pmol of retinol/ml was calculated ($P < 0.05$). With a molecular weight of 15 kD for the cellular retinol-binding protein (8) and a binding stoichiometry of 1 to 1, a retinol concentration of 0.5 pmol/ml corresponds to 7 μg/l of cellular retinol-binding protein. Thanks to the fact that PCMBS can be used to measure nonspecific binding, the assay can be performed equally well with nonlabeled retinol, using fluorescence detection (λ_{ex} = 330 nm emission peaks at 480 nm). Under these conditions, the assay becomes about tenfold less sensitive (tenfold higher detection limit), but no special equipment for radioactive work and scintillation counting is required.

Comparison with other assays

In the evaluation of a new assay, there should be a comparison with the existing procedures for the determination of cellular retinol-binding protein. The approach based on sucrose gradient centrifugation has been employed most extensively, and has also been used by Miller et al. (22) for comparison with a radioimmunoassay (RIA) and their improved gel filtration method. Hence, we compared the performance of the sucrose gradient centrifugation and the Lipidex-1000 microcolumn procedure in the assay of RPE cell supernatant. Using the same typical RPE preparation, we measured a binding activity of 280 pmol of retinol/mg of protein with the Lipidex microassay and a binding activity of 200 pmol of retinol/mg of protein with the sucrose gradient procedure, i.e., about 71% of the Lipidex microassay. This lower value may originate

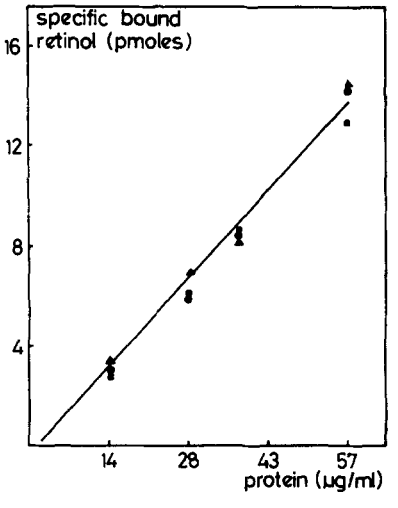

Fig. 3. Reproducibility of the Lipidex microassay. A dilution range of a cRBP preparation was assayed on 3 separate days (symbols representing days 1, 2, and 7 are ▲, ■, and ●, respectively). Nonspecific binding was determined by preincubation with PCMBS. Specific binding of retinol was calculated and plotted versus protein concentration. The curve obtained by linear regression analysis of all data points yielded an average binding activity of 231 ± 9 pmol of retinol/mg of protein. Similar analysis of the separate data points yielded values of 238 ± 18, 223 ± 12, and 234 ± 10 pmol of retinol/mg of protein at days 1, 2, and 7, respectively. These values are not significantly different and illustrate the reproducibility of the assay.

in the long separation times required by the sucrose gradient. Miller et al. (22) reported that under their conditions the sucrose gradient procedure yielded values of about 70% of the minicolumn assay and of about 60% of those values obtained by RIA. Since the conditions for the sucrose gradient procedure used as a reference may not be identical for the two laboratories and its reproducibility is not as good as that of the other assays, this comparison only allows general conclusions. Nevertheless, it is obvious that the Lipidex microassay may yield values comparable to gel filtration and RIA.

In order to test the performance of the Lipidex microassay on tissues with lower cRBP content, we assayed several rat tissues for which cRBP levels have been reported in literature (19, 31, 32). We selected three tissues with relatively high, intermediate, and low cRBP levels, i.e., kidney, testis, and brain, respectively. The results are given in **Table 2.** It is evident that the tissue levels correlate very well with the literature values. The relatively large variance is mainly due to biological variation. This probably also explains the relatively large variation between data obtained by the various radioimmunoassays. In one case we even measured a large difference between two kidneys from the same animal (1280 and 2420 pmol/g of tissue, respectively).

The lower cRBP content in these tissues implies that the protein concentration in the incubation mixture must be increased (500–1000 μg/ml) relative to RPE (≤ 200 μg/ml). Under these conditions, insufficient detergent was available at a total concentration of 3 mM (0.06%) to achieve complete solubilization of retinol and, therefore, the total detergent concentration in the incubation mixture was raised to 10 mM (0.2%) leaving the other conditions unchanged. The effect of the detergent concentration on the assay performance was tested on the RPE extract. Detergent concentrations in the incubation mixture up to 13 mM did not interfere with the assay. At higher concentrations, a concentration-dependent decrease in binding activity was observed.

Discrimination between the various retinoid binding proteins

Finally, the question was addressed as to whether the Lipidex microassay as described was selective for cRBP in the presence of other retinoid-binding proteins.

Considering cRAlBP (cellular 11-*cis*-retinoid binding protein) and cRABP, it can be safely stated that the high specificity of these binding proteins for their respective ligands (12) guarantees that they will bind only insignificant amounts of all-*trans* retinol and will not interfere with the cRBP assay. Indeed, while RPE contains a high level of cRAlBP (700 pmol/eye (33)), the value we measured with the Lipidex microassay for cRBP (550 ± 50 pmol/eye) was in excellent agreement with levels inferred from biochemical analysis (500 pmol/eye (33)). Similarly, the cRBP levels measured in the various rat tissues which contain significant amounts of cRABP (19, 32) agreed well with the literature values obtained by RIA.

Rather, one might expect interference of the other retinol-binding proteins described so far (cRBP-II in small intestine (34); the extracellular sRBP in blood and IRBP in the interphotoreceptor matrix). cRBP-II has only been detected in small intestine and its biochemical parameters (Kd, ligand specificity) have not yet been reported. If they turn out to be very similar to those of cRBP, a binding assay like the Lipidex microassay will not be able to distinguish between cRBP and cRBP-II and will measure the total. Interference with the extracellular RBPs, which might contaminate certain tissue extracts, is easily eliminated. An evaluation of the performance of the Lipidex microassay on serum (sRBP and serum albumin) and purified IRBP showed that binding of retinol to these extracellular RBPs is not inhibited by 3 mM PCMBS (data not shown). Hence, by preincubation with PCMBS, *only* the cellular retinol-binding protein is inactivated and will contribute to the specific binding activity determined by subtracting PCMBS-insensitive binding from total binding.

TABLE 2. Levels of cRBP in rat tissues. Comparison of the Lipidex-1000 microcolumn assay with literature values obtained by RIA

Species	Tissue	RIA Adachi et al. (Ref. 31)	RIA Kato et al. (Ref. 32)	RIA Ong et al. (Ref. 19)	Lipidex-1000 Assay	
			pmol/g wet weight ± SD			
Bovine	RPE				68,000 ± 6,200	(3)[a]
Rat	Kidney	3980 ± 1000	1631 ± 175	513 ± 16	1,702 ± 556	(4)
Rat	Testes	1270 ± 260	1113 ± 300	264 ± 23	394 ± 189	(2)
Rat	Brain	270 ± 150	275 ± 69	27 ± 2.2	23 ± 7	(1)

[a]Number in parentheses is the number of different tissue samples. Assays on the same sample were performed two to four times over a time interval of several months.

In pilot studies, we further investigated whether Lipidex-1000 can be applied to assay the other cellular retinoid binding proteins, viz. the 11-*cis*-retinoid-binding protein (cRAlBP) and cellular retinoic acid binding protein (cRABP), respectively. One of the major questions will be whether Lipidex-1000 has sufficient affinity for their ligands to allow separation of protein-bound and free ligand. Titration of Lipidex-1000 with ligands dissolved in incubation buffer (0.06% Ammonyx LO) have not yet established upper limits, but show complete extraction both of retinoic acid and of 11-*cis*-retinaldehyde up to at least 12.5 nmol per 100 μl per gel. We conclude that the Lipidex microassay can, in principle, be adapted to the other cellular binding proteins.

CONCLUSIONS

The Lipidex microassay that we have developed satisfies all criteria for a reliable binding assay for cellular retinol-binding proteins. It is simple, reproducible, yields a linear relation between protein concentration and retinol binding over a wide concentration range (2–1000 μg/ml), and it conforms to existing procedures. In addition, it has several definite advantages over the existing procedures (radioimmunoassay or free/bound ligand separation by sucrose-gradient centrifugation, gel filtration, or charcoal adsorption). *1)* It is rapid and requires relatively little equipment and manipulation. Manually, up to 50 samples can be easily handled simultaneously, with a processing time of ca. 4–5 hr (excluding scintillation counting, which can be performed overnight). The procedure lends itself well to automation. *2)* With some sacrifice in sensitivity (ca. tenfold decrease), the procedure can be run equally well in a nonradioactive mode, and then requires only a simple fluorometer for quantitative measurements. *3)* The Lipidex microassay can easily be applied to several tissues and, in contrast to immunoassays, no highly specific and/or rapidly decaying reagents are required. *4)* In principle, the Lipidex microassay is very versatile. Programming onto the other cellular retinoid-binding proteins (cRAlBP, cRABP) should be easy. Their ligands (11-*cis*-retinaldehyde and retinoic acid, respectively) can be extracted from the incubation mixture with the same efficiency as retinol, when not bound to protein.

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