(S10 lysate) was dialyzed for 2 hours against 100 volumes of 10 mM Hepes, 90 mM potassium acetate, 1.5 mM Mg acetate, and 2.5 mM DTT, pH 7.4. The dialyzed S10 lysate was again centrifuged for 10 min (10,000 rev/min, SS34 rotor), and the supernatant was stored at −80°C for at least 24 hours. The S10 lysate was thawed and centrifuged at 4°C for 10 min at 10,000 rev/min in an Eppendorf centrifuge. The supernatant (S10 extract) was treated with micrococcal nuclease (15 μg per milliliter of extract) in the presence of $CaCl_2$ (7.5 μl of 0.1 M $CaCl_2$ per milliliter of extract) at 20°C for 15 min. The reaction was terminated by the addition of 200 mM EGTA (15 μl per milliliter of extract). The S10 extract was adjusted to 10% glycerol and stored in small portions at −80°C. Stringent control of the pH of all buffers during the preparation of the extract was found necessary. The most important parameters for the efficiency of translation were the concentrations of viral mRNA and Mg^{2+}, as small deviations from maximal conditions led to drastically reduced protein synthesis.

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Intrasubunit Signal Transduction by the Aspartate Chemoreceptor

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Receptors that transmit signals across cell membranes are typically composed of multiple subunits. To test whether subunit interactions are required for transmembrane signaling by the bacterial aspartate receptor, dimers were constructed with (i) two full-length subunits, (ii) one full-length subunit and one subunit lacking the cytoplasmic domain, or (iii) one full-length subunit and one subunit lacking both the cytoplasmic and the transmembrane domains. Methylation of the cytoplasmic domain of all three receptor constructs was stimulated by the binding of aspartate. These findings demonstrate that transmembrane signaling does not require interactions between cytoplasmic or transmembrane domains of adjacent subunits and suggest that signaling occurs via conformational changes transduced through a single subunit.

SPECIFIC TRANSMEMBRANE RECEPTORS mediate the detection of a wide variety of extracellular ligands, such as growth factors, hormones, nutrients, and neurotransmitters (*1*). Binding of a ligand to the extracellular domain of such a receptor results in a change in the signaling properties of the cytoplasmic domain of the protein. The aspartate receptor from *Salmonella typhimurium* is a member of a family of transmembrane receptors in which globular extracellular and cytoplasmic domains are separated by one or two membrane-spanning segments (*2*). They form a class of receptors distinct from the receptors with seven transmembrane segments, such as rhodopsin and the β-adrenergic receptor (*3*). Transmembrane signal transduction by the aspartate receptor occurs within a stable dimeric receptor (*4*), but the question of how a conformational change is transduced across the membrane remains unanswered. Two possible mechanisms for signal transduction are: (i) an intersubunit mechanism in which a change in the relative position of the ligand-binding domains of the dimeric receptor leads to a change in the relative positions of the cytoplasmic domains of the receptor, or (ii) an intrasubunit mechanism in which a conformational change occurs independently within each subunit of the dimer. To differentiate between these possibilities, we constructed a series of hybrid receptors (Fig. 1) that contain subunits lacking one or two of the three domains of the receptor. The first hybrid, the 1 + 2/3 receptor, contained one full-length subunit and one truncated subunit consisting of the ligand-binding domain and the transmembrane domain (residues 1 through 259). A second hybrid, the 1 + 1/3 receptor, contained one full-length subunit and one subunit consisting of the ligand-binding domain alone. The wild-type receptor, 1 + 1 receptor, was included as a positive control in each experiment. To evaluate the requirement for interaction between subunits during signal transduction, we tested each of these receptors for its ability to transmit a transmembrane signal.

We constructed hybrid aspartate receptors by disulfide cross-linking full-length and

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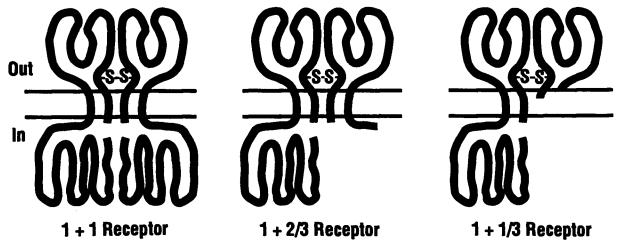

Fig. 1. Structures of full-length and hybrid aspartate receptor dimers. The full-length receptor (1 + 1 receptor) is identical to the wild-type receptor except that the asparagine at position 36 has been replaced by a cysteine through which the subunits are cross-linked; this modification does not affect the signaling properties of the receptor. The 1 + 2/3 receptor is made up of one full-length subunit and one subunit consisting of residues 1 to 259; essentially none of the cytoplasmic domain is present in this truncated subunit. The truncated subunit of the 1 + 1/3 receptor consists of residues 25 to 188 and contains essentially none of either the transmembrane or cytoplasmic domains.

truncated subunits in which a cysteine residue had been inserted at position 36 by means of site-directed mutagenesis (Cys^{36} receptors). The disulfide-linked Cys^{36} receptor functions normally in aspartate binding and in aspartate-mediated signaling events (*5*). The fragment containing the ligand-binding and transmembrane domains, corresponding to residues 1 to 259 in the full-length receptor, was generated by proteolysis of the full-length Cys^{36} receptor, as described (*6*). The ligand-binding domain of the aspartate receptor, consisting of residues 25 to 188, was generated by replacing the first transmembrane segment of the Cys^{36} aspartate receptor with the cleavable signal sequence from *Escherichia coli* alkaline phosphatase and inserting a stop codon after residue 188 (*7*).

The disulfide-linked full-length aspartate receptor was prepared by addition of the oxidizing agent copper phenanthroline (*5*). To produce the 1 + 2/3 receptor, a fragment containing residues 1 to 259 of the receptor was incubated with the full-length receptor, and the dimers were then trapped by addition of copper phenanthroline (*8*). When this procedure was applied to the 1 + 1/3 receptor, very little of the desired hybrid receptor was formed, because the full-length receptor subunits bind more readily to other full-length subunits than to the ligand-binding domain. To circumvent this problem the cysteine residue on the fragment was activated by reduction with dithiothreitol and treatment with dithionitrobenzoate. After the activation step the full-length Cys^{36} receptor (containing a free sulfhydryl) was mixed with the activated fragment, leading to formation of the hybrid receptor (*9*). This technique produced the desired hybrid receptor with high yield and could be used to direct the formation of specific hybrid dimers in other systems.

To evaluate the signaling properties of the various receptor types, mixtures of wild-type and hybrid receptors were reconstituted into lipid vesicles (*10*), and in vitro methylation reactions were conducted in the presence and absence of saturating concentrations of aspartate. Binding of aspartate to the extracellular domain of the receptor leads to an increase in the rate of methylation of specific glutamate residues located in the cytoplasmic domain. This process allows the detection of changes in aspartate concentration. Incubation of the aspartate receptor with the CheR methyltransferase and [methyl-^{3}H]-S-adenosylmethionine in vitro leads to methylation of the same glutamate residues. Aspartate binding approximately doubles the rate of methylation of the receptor (*11*). Hybrid dimers were prepared such that cross-linked wild-type and hybrid dimers were present in approximately equal amounts. Methylation of the hybrid 1 + 2/3 receptor was indistinguishable from that of the wild-type receptor (Fig. 2A). The rates of methylation of both the wild-type and the hybrid receptors were stimulated by the addition of aspartate, and the absolute amount of methylation of the two receptor types was similar. Normal signal transduction by a dimer containing only one cytoplasmic domain indicates that each subunit of the dimer can signal independently, without interaction between cytoplasmic domains. Similarly, the rate of methylation of the 1 + 1/3 receptor was stimulated by the presence of aspartate (Fig. 2B). Intersubunit interactions between the transmembrane domains of the receptor dimer also appear to influence receptor function because the basal methylation rate of the 1 + 1/3 hybrid receptor was approximately one-fifth of that of the wild-type receptor dimer.

Analysis of the signaling properties of these hybrid receptors depends on whether the truncated subunits can prevent association of full-length receptor subunits. Although the subunits of the hybrid dimers are

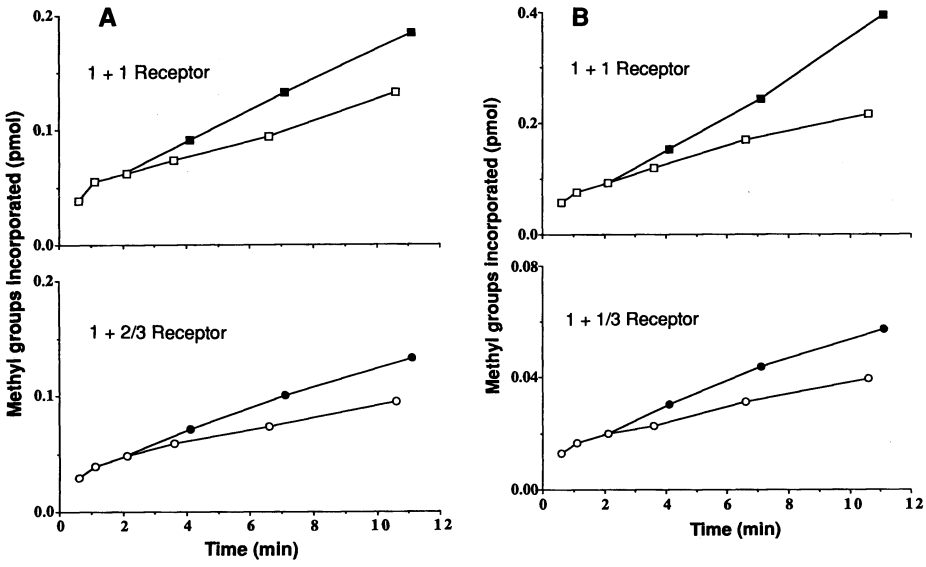

Fig. 2. Effect of aspartate on methylation of full-length and hybrid receptor dimers. Methylation reactions were performed by mixing equal volumes of reconstituted receptor vesicles (*10*) and a solution containing the CheR methyltransferase and 100 μM [*methyl*-^{3}H]-S-adenosylmethionine (Amersham, 15 Ci/mmole) in a buffer consisting of 50 mM sodium phosphate (pH 7.0), 10% glycerol, 1 mM PMSF, 1 mM EDTA. Portions were removed at various times, and aspartate was added to a final concentration of 1 mM to one-half of the reaction mixture after 2.5 min. Samples were boiled in SDS sample buffer containing 10 mM iodoacetamide, and the receptor dimers were separated on nonreducing SDS-polyacrylamide gels (7.5%) (*15*). After staining with Coomassie Blue and destaining, the appropriate regions of the gel were cut out, and methyl groups incorporated into the receptor were quantified by scintillation counting in a simple diffusion assay (*16*). Filled symbols indicate the presence of aspartate. (**A**) Methylation of the cross-linked 1 + 1 receptor (squares) and the cross-linked 1 + 2/3 receptor (circles). (**B**) Methylation of the noncross-linked 1 + 1 receptor (squares) and the cross-linked 1 + 1/3 receptor (circles). The 1 + 1 receptor was not cross-linked in this case because no oxidizing agent was used to construct the 1 + 1/3 receptor. These results are representative of four separate experiments showing only slight variations due to differences in the ratio of the various receptor types and variable reconstitution efficiency. In all cases aspartate approximately doubled the rate of methylation.

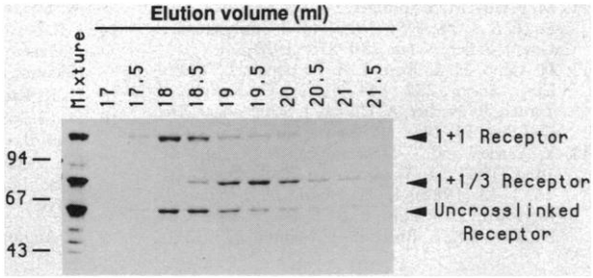

Fig. 3. Gel filtration analysis of covalently and noncovalently associated aspartate receptor dimers. A mixture of cross-linked full-length receptors (1 + 1 receptors), cross-linked hybrid receptors (1 + 1/3 receptors), and uncross-linked receptors (pretreated with 5 mM iodoacetamide to prevent disulfide formation) was applied to a 600- by 7.5-mm Bio-Rad Bio-Sil TSK-400 HPLC gel filtration column equilibrated in 100 mM sodium phosphate (pH 7), 1% β-octylglucoside at 1 ml/min. Fractions were collected at intervals of 0.5 min, and proteins were precipitated by addition of trichloroacetic acid (final concentration, 10%). Precipitates were resuspended in SDS sample buffer containing 5 mM iodoacetamide, and proteins were separated by nonreducing SDS–polyacrylamide gel electrophoresis (7.5% gels). The gel was stained with Coomassie Blue and destained. Positions of molecular size markers are shown on the left. The positions of migration of the various receptor types are shown to the right of the panel. Under the conditions of the gel filtration column, uncross-linked receptor subunits remained associated and co-eluted with the cross-linked full-length dimers; the cross-linked hybrid dimers appear not to associate with other subunits and thus eluted later.

linked by a disulfide bond, it is possible that the full-length subunits of two hybrid dimers could noncovalently associate to form a pseudo–wild-type receptor despite the presence of the cross-linked truncated subunits. However, the hybrid receptor eluted from a gel filtration column at later times (corresponding to a smaller molecular size) than either the cross-linked full-length dimer or the noncovalently associated full-length dimer, which migrated as a monomer on the SDS-polyacryamide gel used to analyze proteins eluted from the column (Fig. 3). Thus, the presence of the truncated subunit apparently prevents further association of the full-length subunits of the hybrid receptor under conditions that allow association between full-length subunits that are not cross-linked.

Intrasubunit signal transduction by the aspartate receptor indicates that transmembrane signaling can occur by propagation of a conformational change through one or both of the transmembrane segments of a single receptor subunit. Thus, association or dissociation of monomers and dimers is not necessary for signal transduction by this receptor. Also inconsistent with our results are mechanisms that involve changes in the relative positions of the two cytoplasmic domains in the receptor dimer, such as rotation of one subunit relative to another or lateral motion of one subunit toward or away from another. The only models consistent with transmembrane signaling through a single receptor subunit are those that involve motion perpendicular to the membrane by one or both of the transmembrane segments of the receptor (a "piston model") or those which involve lateral or rotational movement of the two transmembrane segments of one subunit relative to each other within the plane of the membrane.

The signaling mechanisms of the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors have been studied by testing the function of hybrid receptors containing truncated subunits (*12*). Transmembrane signaling by these receptor tyrosine kinases appears to require phosphorylation of one subunit by the neighboring subunit in a receptor dimer. Ligand binding leads to dimerization of these receptors, allowing intersubunit phosphorylation to occur and leading to activation of the kinase activity of the receptors (*13*). Consistent with this model, hybrid dimers of either the PDGF receptor or the EGF receptor with one active and one inactive subunit are not functional. Thus, when expressed at high levels, inactive subunits of these receptors act as dominant-negative mutations (*12*). Perhaps the differences observed between the growth factor receptors and the aspartate receptor stem from the differences in the transmembrane structures of the two receptor types. Each subunit of the aspartate receptor has two transmembrane segments, and each subunit of the dimer can signal independently. Each subunit of the EGF and PDGF receptors has only a single transmembrane-spanning segment, and two full-length subunits of these receptors are required to produce an activated receptor. The insulin receptor is composed of pre-existing disulfide-linked heterotetramers with two transmembrane segments per oligomer (*14*). A common organizational theme may exist in all of these receptors—two transmembrane segments in the signaling unit. The two transmembrane segments exist within a subunit of the aspartate receptor, are brought together by association of the EGF and PDGF receptors, or are held together by disulfide cross-links in the insulin receptor. It is possible that there is a variety of different mechanisms for transmembrane signaling, but a common mechanism may be exemplified by the aspartate receptor in which ligand binding induces a reorientation of two transmembrane segments to transmit a signal to the cytoplasmic domain of the receptor.

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8. Formation of the 1 + 2/3 hybrid receptor was achieved by incubating the partially purified Cys^{36} aspartate receptor (*4*) with a fragment of this receptor (residues 1 to 259) produced by limited trypsin treatment (*6*). First, exchange of subunits was allowed to occur by mixing equal amounts (as judged by Coomassie-stained SDS-polyacrylamide gels) of the two receptor types in a solution containing 50 mM tris-HCl (pH 7.4), 10% glycerol, 1% β-octylglucoside, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 10 mM dithiothreitol (DTT) at 37°C for 5 min. The reducing agent was removed by centrifugation of the mixture through a gel filtration column (Bio-Rad Bio-Spin 6) equilibrated in buffer A [50 mM sodium phosphate (pH 7.0), 10% glycerol, 1% β-octylglucoside, 1 mM PMSF]. Disulfide cross-linking was initiated by the addition of copper (1,10-phenanthroline)$_3$ to a final concentration of 1.5 mM from a 10× stock solution prepared in 1% β-octylglucoside. After incubation at 37°C for 10 min, the oxidizing agent was removed by centrifugation through another column equilibrated in buffer B (buffer A containing 1 mM EDTA).
9. The purified ligand-binding domain of the aspartate receptor (residues 25 to 188) containing the Cys^{36} mutation was reduced under nitrogen in a solution containing 50 mM tris-HCl (pH 8.0), 5 mM EDTA, and 50 mM DTT at 37°C for 30 min. Excess DTT was removed by gel filtration on a Bio-Rad 10 DG column equilibrated in N_2-saturated buffer containing 50 mM tris-HCl (pH 8.0), 5 mM EDTA, 0.5 mM DTT. Dithionitrobenzoate was added to a final concentration of 1.5 mM from a 100 mM stock in dimethylformamide. After 10 min at 23°C excess reagents were removed by gel filtration on another 10 DG column equilibrated in buffer B. The full-length Cys^{36} receptor was reduced by incubation in buffer B containing 10 mM DTT at 23°C for 15 min, and DTT was then removed by centrifugation of the sample through a column (Bio-Rad Bio-Spin 6) equilibrated in buffer B. The reduced full-length and activated truncated subunits were mixed and incubated at 23°C for 15 min so that approximately one-half of the full-length subunits were incorporated into hybrid dimers. Because no oxidizing agent was used in this procedure, the 1 + 1 receptors in this experiment were not disulfide-linked.
10. Mixtures of full-length and hybrid receptor dimers were reconstituted into phospholipid vesicles. The receptor extracts were mixed with an equal volume of a buffer containing 50 mM sodium phosphate

(pH 7.0), 70% glycerol, phospholipids from *E. coli* (4 mg/ml) (Avanti Polar Lipids), 1 mM PMSF, and 1 mM EDTA. After incubation at 23°C for 30 min, the solution was diluted with 25 volumes of ice-cold 50 mM sodium phosphate (pH 7.0), 10% glycerol, 1 mM PMSF, 1 mM EDTA. Membrane vesicles were collected by centrifugation at 400,000*g* for 30 min, and the vesicles were washed to remove residual detergent by two cycles of resuspension and centrifugation. The membrane vesicles were resuspended in the same buffer, frozen in liquid nitrogen, and stored at −70°C.

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Intracellular Signaling by 14-Hydroxy-4,14-*Retro*-Retinol

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In mammals, retinol is the precursor for retinoids, which affect various aspects of morphogenesis and development. However, B lymphocytes, although retinol-dependent, do not use retinoic acid as mediator. Retinol is metabolized by B lymphocytes and other cell lines to optically active 14-hydroxy-4,14-*retro*-retinol; it is this compound that mediates the growth control. Thus another second messenger molecule, in addition to retinoic acid and retinal, is derived from retinol.

IN VERTEBRATES, PROCESSES AS DIverse as growth, vision, and reproduction depend on the presence of retinol (vitamin A) (*1*). Retinol serves as a source for a variety of derivatives adapted to specialized functions, such as 11-*cis* retinal, that constitutes the chromophore of the visual pigment rhodopsin (*2*). Furthermore, retinoic acid induces differentiation in many in vivo and in vitro systems (*3*) by binding to a nuclear receptor and subsequently enhancing transcription of specific genes (*4*). Retinol is essential for the growth of a variety of cell types in culture, notably lymphocytes (*5*). However, retinoic acid cannot substitute for retinol in preventing necrotic cell death of activated immune cells (*5–7*). We have identified a retinol derivative on the basis of its growth-promoting properties and have characterized it as 14-hydroxy-4,14-*retro*-retinol (14-HRR).

Cells of the lymphoblastoid line 5/2 were grown in the presence of ^{3}H-labeled retinol–retinol binding protein complex (*8*), and the lipids were extracted from the cell pellet (*9*). High-pressure liquid chromatography (HPLC) on a reversed-phase C_{18} column yielded hitherto undescribed retinoids with retention times of 16 to 18 min, amounting from 4 to 9% of the total cellular retinoids (Fig. 1). These retinoids comprise at least four compounds with similar ultraviolet (UV) absorption spectra, and they are most likely *cis/trans* isomers of the same molecule. The most abundant of these, P3, was purified from lymphoblastoid or HeLa cells by means of a series of reversed-phase columns [a preparative C_{18} column of 250 by 22 mm internal diameter (ID) with water-methanol at 17/83 v/v; a semipreparative C_{18} column of 250 by 10 mm ID with water-acetonitrile at 25/75 v/v; and an analytical C_4 column of 250 by 4.6 mm ID with water-methanol at 78/72 v/v]. From 80 liters of HeLa cells, 30 absorption units at 348 nm (168 μg) of pure P3 were obtained. This compound was unstable on normal-phase columns and in chloroform but tolerated methanol, butanol, benzene, and acetonitrile and remained intact after storage for several months at −80°C.

The characteristic vibronic fine structure of the UV spectrum of P3 (Fig. 2A) suggested a *retro*-retinoid skeleton (*10*). The high-resolution electron ionization (EI) mass spectrum [(matrix perfluoro-kerosine (PFK)] gave an observed value of 302.2265 calculated for $C_{20}H_{30}O_2$ = 302.2246), indicating that P3 has one more oxygen than its precursor retinol, $C_{20}H_{30}O$. The low-resolution EI mass spectrum of P3 is described in (*11*). Proton nuclear magnetic resonance (NMR) measurements (Fig. 3) established that P3 is 14-HRR. The double-bond configuration between positions 6 and 7 was established as E by comparisons with the chemical shifts of 1,1-Me_2 (1.30) and 4-H (5.79) with those reported for 6-E-4,14-*retro*-retinyl acetate (*12, 13*), 1.28 and 5.76 ppm, respectively; the corresponding values for 6-Z-4,14-*retro*-retinyl acetate are 1.11 and 5.63 ppm. Furthermore, the 6-E configuration was confirmed by observation of an approximately 4% nuclear Overhauser effect between 1,1-Me_2 and 8-H. The configuration of the double bond between po-

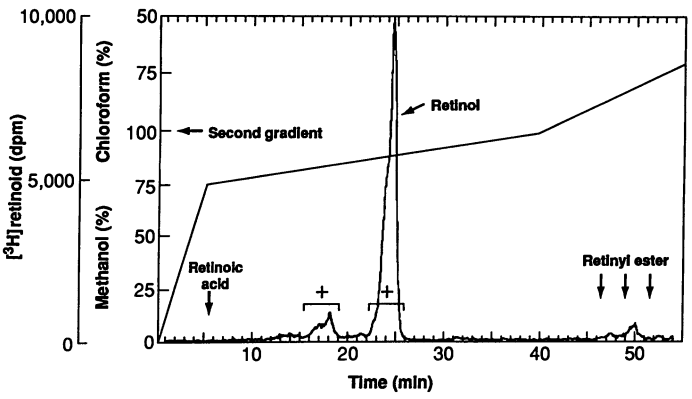

Fig. 1. High-pressure liquid chromatography of retinoids from lymphoblastoid 5/2 cells (Waters, Milford, MA), analytical C_{18} reversed-phase column (Vydac, Hesperia, CA); water-methanol-chloroform gradient; flow rate, 0.5 ml/min; photodiode array detection. Disintegrations per minute were determined with an on-line scintillation counter (Radiomatic, Tampa, FL). Fractions eluting at 16 to 18 min, as well as the original retinol peak, are bioactive in a bioassay performed as described in Fig. 4. The 5/2 cells [1.8 × 10^7 cells in 30 ml of RPMI/0.9% fetal calf serum (FCS) medium] were incubated with 2 × 10^{-6} M holo-retinol–binding protein to which were bound 30 μCi [^{3}H]retinol; specific activity, 49.3 Ci/mmol. Retinol-binding protein was purified from human serum by means of a transthyretin column (*21*). The absorption ratio (280 nm to 324 nm) of the retinol-binding protein–retinol complex used was 0.85. After 24 hours, retinoids were extracted from the washed cell pellet according to the procedure of McLean *et al.* (*9*). Unlabeled reference retinoids (arrows) were used to calibrate the column.

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