Reactivity of vitamin A derivatives and analogues with vitamin A antibodies

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Summary Vitamin A antibodies were obtained using retinoic acid conjugated to human serum albumin as an immunogen. The following constraints governed the reactivity of vitamin A analogues with such an anti-serum. The stereochemistry of the side chain is relatively unimportant, and 9- and 13-*cis* retinal react almost as well as all-*trans* retinal. The nature of the ring is important; all of the compounds that react readily carry a β-ionone ring; all of the compounds bearing an aromatic ring react poorly; the two compounds that display intermediate reactivity have non-aromatic 6- and 5-membered rings, respectively.—**Wirtz, G. H., and S. S. Westfall.** Reactivity of vitamin A derivatives and analogues with vitamin A antibodies. *J. Lipid Res.* 1981. **22:** 869–871.

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In 1973, Conrad and Wirtz (1) reported the preparation of antisera against vitamin A, using as the immunogen retinoic acid conjugated to human serum albumin. The potential utility of such antibodies includes their use as a histological stain for vitamin A (by means of a fluorescent tag) and their application to the radioimmunoassay (RIA) of vitamin A; an RIA for vitamin A has recently been described (2). In using these antibodies for such applications, their reactivity with various derivatives and analogues of vitamin A is relevant. In this communication, a survey of such cross-reactivity is reported. These compounds were evaluated by subjecting them, in known quantities, to the RIA protocol (2). The compounds are characterized by their ability to compete with [^{3}H]retinol for the binding site of the vitamin A antibody.

MATERIALS AND METHODS

Retinol, retinal, retinoic acid, 9-*cis*-retinal, and 13-*cis*-retinal were obtained from Eastman Organic Chemicals; retinyl acetate was purchased from Sigma Chemical Co.; 5,6-epoxy retinoic acid was a gift from Dr. O. Wiss of F. Hoffmann-La Roche & Co., Basel, Switzerland; retinyl phosphate was a gift from Dr. Luigi De Luca of the National Cancer Institute, Bethesda, MD. A series of retinoid derivatives was prepared by F. Hoffmann-La Roche & Co. and made available through Dr. Luigi De Luca; the manufacturer assigned each of these compounds a number bearing the prefix Ro and this designation will be used when referring to these compounds in Table 1.

Abbreviation: RIA, radioimmunoassay.

[^{3}H]Retinol (2.5 Ci/mmol) was supplied by New England Nuclear.

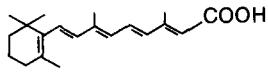
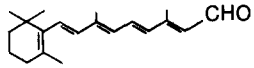
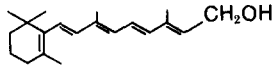
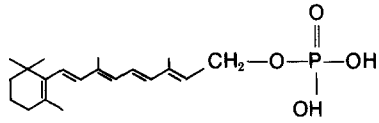
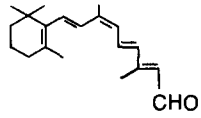
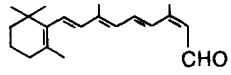
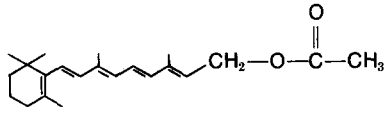
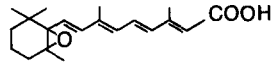
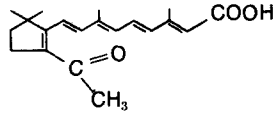
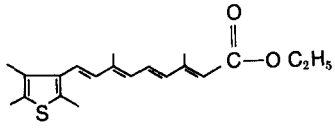
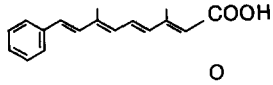
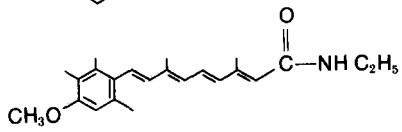
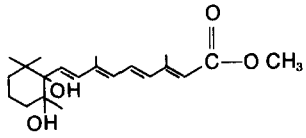
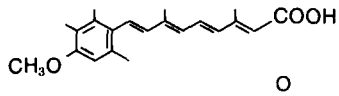
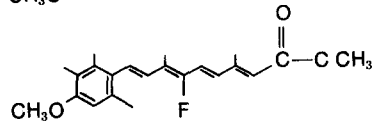
The antiserum used in this work displayed an affinity constant, in its reaction with retinol, of 2×10^8 1/M (2). For use in the RIA protocol, it was diluted 1/805. Based on the displacement curve, each ml of antiserum could potentially bind 6 μg of retinol.

Each compound to be studied was dissolved at a known concentration in absolute ethanol. This stock solution was used to prepare a working solution in 66.6% ethanol at a concentration of 50–100 μg of the compound per ml. A series of dilutions of the working solution was made, and these dilutions were subjected to the RIA protocol; at the concentrations used, these compounds were soluble in the RIA mixtures.

Briefly stated, the RIA method consisted of the following operations (complete details are presented by Westfall and Wirtz, reference (2). Each dilution of the compound was treated with the antiserum and a standard quantity of [^{3}H]retinol. After 1 hr, the unbound [^{3}H]retinol was removed from the RIA solution with dextran-coated charcoal. The antibody-bound [^{3}H]retinol that was left behind in the RIA solution was then counted in a scintillation counter. For each quantity (or dilution) of the compound, the ratio B/Bo was calculated. B is the number of antibody-bound counts in the RIA solution in the presence of the compound; Bo is a control that consists of the number of antibody-bound counts in the absence of the compound. The B/Bo ratios determined for seven concentrations of the compound were then used to draw the displacement curve by plotting the logit (3) of B/Bo versus the log of the nanograms of the compound in the assay tube. Two pieces of information were obtained from the displacement curve: the quantity of compound required to displace 50% of the [^{3}H]retinol from the antibody and the slope of the curve. Rodbard (3) has pointed out that in an ideal system operating at optimal efficiency (e.g., vitamin A displacing tritiated vitamin A from vitamin A antibodies) the slope should be -2.3; we typically found a value of -2.2. Compounds less able to react with the antibodies will have smaller slopes. Thus the logit plot (3) provides a convenient means of assessing the suitability of the reagents used in RIA. One would expect marked deviations from a slope of -2.3 if there were significant deterioration, such as oxidations, in either the labeled or unlabeled ligand (e.g., see 5,6-epoxy retinoic acid, Table 1).

As discussed in reference 2, a measurable portion of the tritium counts in the [^{3}H]retinol was found to bind neither antibody nor dextran-coated charcoal. The behavior of these counts suggests that they could be due to tritiated water. Such counts could be reduced by subjecting the [^{3}H]retinol to chromatog-

TABLE 1. Comparative reactivity of various retinoid derivatives with vitamin A antiserum

Compound	Structure	Picomoles for 50% Displacement	Slope of Displacement Curve
Group I			
Retinoic acid		7.3	−2.2
Retinal		12.0	−2.2
Retinol		13.1	−2.2
Retinyl phosphate		10.9	−1.65
9-*cis* Retinal		21.0	−2.2
13-*cis* Retinal		35.0	−2.2
Retinyl acetate		36.6	−1.7
Group II			
5,6-epoxy Retinoic acid		222	−1.0
Ro 8-7699		318	−1.15
Group III			
Ro 216583		8×10^3	−1.2
Ro 8-8717		33×10^3	−0.75
Ro 111430		339×10^3	−0.55
Ro 110652		No displacement	
Ro 101670		No displacement	
Ro 217887		No displacement	

raphy. Routinely however, the [^{3}H]retinol supplied by the manufacturer was used without further manipulation because of the vulnerability of the retinol to oxidation. By using the [^{3}H]retinol within 6 months of receipt, such counts (binding neither antibody nor dextran-coated charcoal) were held within acceptable limits (less than 15% of the total).

RESULTS AND DISCUSSION

The variation inherent in the RIA protocol is illustrated by the following statistical parameters. In nine determinations, retinol displayed a mean 50% displacement of 13.08 pmol (S.D. ± 2.66) and a mean slope of −2.20 (S.D. ± 0.102); in four determinations, retinal displayed a mean 50% displacement of 11.97 pmol (S.D. ± 3.38) and a mean slope of −2.23 (S.D. ± 0.070).

Table 1 presents a comparison of the compounds tested. These compounds have been arbitarily divided into three groups based on their efficiency in reacting with the antibody. Group I compounds react most efficiently, Group II compounds react only moderately well, and Group III compounds react poorly or not at all. The most effective compounds (Group I) all possess the β-ionone ring. These compounds displace 50% of the [^{3}H]retinol from the antibody at quantities ranging from 7 to 37 pmol. The all-*trans* forms of retinoic acid, retinal, and retinol are statistically indistinguishable by the antibody, presumably because these compounds share the same immunogenic determinant in the cyclohexene ring.

It is interesting that both 9-*cis* and 13-*cis* retinal display such high affinity for the antibody. One might have predicted that such a bend in the side chain would introduce considerable steric incompatibility. But in fact these two *cis* compounds are nearly as efficient as their all-*trans* counterparts. Conrad and Wirtz (1) found that β-ionone was less than one-hundredth as effective as retinoic acid in displacing tritiated vitamin A from the antibody. These observations may mean that the stereochemistry of the chain is less important than its hydrophobic character; they may further suggest that if a polar group, such as the carbonyl in β-ionone, is positioned too close to the ring, reaction with the antibody will be inefficient.

Retinyl phosphate is a puzzling compound; the negative slope of the displacement curve was found in duplicate experiments to be significantly less than the optimum value of −2.3. On the other hand, the quantity of retinyl phosphate required to displace 50% of the tritium label (10.9 pmol) was in the range displayed by retinol (13.1 pmol). Thus, these two parameters provide conflicting indications of the efficiency of this ligand-antibody reaction. We have no explanation for this finding. Retinyl acetate also shows a low negative slope but this is consistent with the fact that it is the least efficient reactant in Group I.

Table 1 provides a comparison of several retinoic acid derivatives; retinoic acid, 5,6-epoxy retinoic acid, Ro 8-7699, Ro 8-8717, and Ro 101670. When the ring loses its β-ionone character, the efficiency of reaction, compared to the parent compound retinoic acid, is considerably reduced. The loss of reactivity is most severe when the ring becomes aromatic and therefore planar (Ro 8-8717 and Ro 101670 in Group III); both compounds in Group II (which react moderately well) as well as the parent compound retinoic acid have non-aromatic rings which are thus non-planar. These observations suggest that a planar ring will react very poorly or not at all with the antigen-combining site of the antibody. Since Ro 110652 (Group III) is unreactive, it seems that non-planarity may be a necessary, but not sufficient, condition for high affinity betweeen the antigen and antibody.

The cross-reactivity of the antibodies reported here would be relevant to the application of such antisera for the detection of vitamin A either quantitatively (by RIA) or histologically. All of the compounds in Group I react to a significant extent with the antiserum. Thus, if these antibodies were applied to a material in which more than one retinoid is present, it would not be possible to distinguish one form from another. For example, retinol and retinyl phosphate are constituents of liver cells (4), and their precise location would be of some interest; however, the antiserum presently available would not differentiate between these two forms. Plans are under way to prepare immunogens that will allow the production of antibodies specific for the side chain and its functional group.

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