The structure of plasmalogens: VII. Analysis of mammalian liver lipids and the interference of vitamin A in the analysis

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SUMMARY Low values of the molar ratio of α,β-unsaturated ether to aldehydogenic lipid found with lipid extracts of mouse and rat liver are shown to result solely from the presence in these extracts of vitamin A rather than of long-chain aldehydes or mixed acetals. Vitamin A interferes in the spectrophotometric determination of specific iodination of α,β-unsaturated ethers because it absorbs some light at 355 mμ. The correction is made simply by using an appropriate sample blank. Vitamin A interferes much more seriously with the estimation of aldehydogenic lipids as *p*-nitrophenylhydrazones because of its transformation under the reaction conditions to anhydro vitamin A. The correction for this interference may be made by independent estimation of the vitamin A content (Carr-Price reaction), determining a standard curve for vitamin A subjected to the *p*-nitrophenylhydrazone procedure, and subtracting the appropriate value.

The vitamin A content of liver is dependent on the species of animal as well as on diet. In the animals studied, the average level of interference of vitamin A in estimations of liver plasmalogen was 75% for the mouse, 50% for the rat, 8% for the rabbit, and negligible for guinea pig.

IDENTIFICATION OF THE α,β-unsaturated ether linkage in naturally occurring plasmalogens (1, 2) led to the introduction of specific iodination as a stoichiometric analytical method for determing the quantities of these constituents in total lipid extracts (3). A number of modifications of the iodination method have been published (3–7). The principle (addition of iodine to a specific double bond) on which this analysis is based differs completely from that of an alternative stoichiometric method for analysis of aldehydogenic lipids, namely, measurement of the *p*-nitrophenylhydrazones formed from the aldehydes generated by acid hydrolysis (8). Comparison of the analyses of total lipid extracts of various organs by these two methods demonstrated that almost all aldehydogenic lipids did indeed contain the α,β-unsaturated ether group, since the molar ratios of α,β-unsaturated ether to aldehydogenic lipid approximated unity (3). Whereas the average value of the molar ratio found with lipids of 10 tissues from rats, 9 from rabbits, and 17 from humans was slightly greater than unity (i.e., 1.07, 1.08, and 1.10, respectively), the value with rat liver was lower than unity (0.90). In a later study, Norton (5) reported a value for the ratio in rat liver lipids that was much lower (0.71), and we found subsequently that very low values of the ratio ranging from 0.12 to 0.42 were obtained in studies of lipids from mouse liver.

The reason for this low value might have provided a clue to the metabolism of plasmalogens. On the one hand, the addition of any of a number of compounds with a free alcoholic hydroxyl group to the α,β-unsaturated ether linkage would be expected to produce unsymmetrical acetals (9). Such compounds would retain the capacity to generate free aldehyde under acid hydrolytic conditions while losing the ability to add iodine. On the other hand, the presence of appreciable quantities of free, long-chain aldehydes in these extracts would also result in low values for the molar ratio of unsaturated ether to aldehyde. The studies reported in this paper show that neither of these alternatives is the correct explanation, and that the low value is caused by the presence in mouse and rat liver of vitamin A.[1] The quantity of vitamin A present is sufficient to account for the discrepant values. The dependency of vitamin A content of liver on the diet provides ready explanation for the differences obtained

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[1] The I.U.P.A.C. Commission on the Nomenclature of Biological Chemistry recommends the designation "retinol" for pure vitamin A_1, "dehydroretinol" for pure vitamin A_2, and "retinoic acid" for pure vitamin A acid (15). Inasmuch as a pure compound has not been identified, we retain the generic name vitamin A in this paper.

by various investigators (3, 5, 10) in studies of rat liver lipids.

METHODS AND MATERIALS

Animals. Liver was obtained from adult New Zealand white rabbits, Sprague-Dawley rats, CBA (Strong) mice, and an unspecified strain of guinea pigs. The mice were from an inbred strain maintained in this department and kindly supplied by Dr. Abraham White. The other animals were purchased from commercial sources.

Lipid Extracts. Samples of liver weighing 2.0 g were homogenized for 3 min in a glass homogenizer with 20 volumes of chloroform–methanol 2:1. The extract was centrifuged and washed according to Folch et al. (11). The upper phases were discarded, and the lower phase was brought to 25 ml and stored in the cold.

Long-Chain Aldehydes. The method of Wittenberg et al. (8) with the modification described by Rapport and Alonzo (4) was used. A sample of lipid containing 0.05–0.10 μmole of aldehydogenic lipid was dissolved in 1.6 ml of 95% ethanol. Then 0.2 ml of freshly prepared 0.02 M *p*-nitrophenylhydrazine in 95% ethanol was added, followed by 0.2 ml of 1.0 N sulfuric acid. The solution was heated at 70° for 20 min. After cooling, 1 ml of water was added, followed by 2.00 ml of *n*-hexane. After shaking several minutes, the aqueous layer was removed. The hexane layer was then washed twice with 2-ml portions of water. The hexane layer was centrifuged, and an aliquot of 0.50 ml was mixed with 3.5 ml of 95% ethanol. Absorbancy was measured in a Beckman model B spectrophotometer at 390 mμ. The molar extinction coefficient of higher fatty aldehyde *p*-nitrophenylhydrazones was 23,800. Sample blanks were run in which pure solvent was substituted for the 95% ethanol solution of *p*-nitrophenylhydrazine. A Cary recording spectrophotometer was used to determine the spectrum of the reaction products in the final ethanolic dilution of the hexane extracts.

Specific Iodine Addition. The spectrophotometric method of Gottfried and Rapport (6) was used, adding a sample blank in which 0.5 ml of 3% KI was substituted for the iodine reagent (10). A lipid sample containing about 0.05 μmole of α,β-unsaturated ether was dissolved or suspended in 0.5 ml of methanol. Then 0.50 ml of iodine reagent (6×10^{-4} N iodine in 3% aqueous KI) was added. The mixture was stirred vigorously for 1 min and left at room temperature for 10 min. After addition of 4.0 ml of 95% ethanol, the absorbancy was determined in a Beckman model B spectrophotometer at 355 mμ. The molar extinction coefficient of iodine was 27,500.

Vitamin A Determinations. The total vitamin A content of the lipid extracts was determined using the Carr-Price reaction with the modification described by Oser et al. (12). A test portion of the lipid solution was evaporated to dryness in a stream of nitrogen and redissolved in 4 ml of dried chloroform. An internal standard of synthetic vitamin A alcohol, run with each lipid extract, showed that the method was free of interference. In measuring the vitamin A content of the hexane extracts obtained by subjecting lipids to the acidic conditions of the *p*-nitrophenylhydrazone determination in the absence of *p*-nitrophenylhydrazine, the hexane was removed in a stream of nitrogen and the residue taken up either in dried chloroform for the Carr-Price reaction or in 95% ethanol for determination of the absorption spectrum. Spectra of the Carr-Price reaction products were obtained by placing chloroform solutions in the cuvettes of the recording spectrophotometer followed by a drop of acetic anhydride and then the antimony trichloride solution. The scanning rate was 5 mμ/sec.

In the above procedure, standards were prepared from crystalline vitamin A alcohol and vitamin A acetate (California Corp. for Biochemical Research).

Silicic Acid Column Fractionation. The method used was that described by Rapport and Alonzo (4), using Baker's silicic acid and discontinuous gradient elution with mixtures of hexane, ethanol, and methanol. The eluted lipids were examined by thin-layer chromatography on silicic acid using chloroform–methanol–water 75:25:4 as developing solvent and iodine vapor for detection.

Lipid Weight. Lipid solutions were evaporated to dryness under nitrogen and the residues were dried in vacuo over P_2O_5 for 24 hr at room temperature.

Solvents. All organic solvents used for extractions, solutions, fractionations, and analytical determinations were redistilled before use.

RESULTS

Table 1 presents a summary of the analyses for the contents of vitamin A, aldehydogenic lipid (NPH reaction), and α,β-unsaturated ether (iodine uptake) in four total lipid extracts of mouse liver, four of rat liver, two of rabbit liver, and two of guinea pig liver.

Species Differences in the Molar Ratio of Unsaturated Ether to Aldehydogenic Lipid in Liver. Examination of Table 1, column 7 shows that the uncorrected values of this ratio for the four mouse liver extracts and the four rat liver extracts depart markedly from unity, whereas values close to unity were obtained in the case of the rabbit liver and guinea pig liver extracts. The average values shown are 0.24 for the mouse, 0.54 for

TABLE 1 VITAMIN A, ALDEHYDOGENIC LIPID, AND α,β-UNSATURATED ETHER CONTENT OF LIPID EXTRACTS OF MOUSE, RAT, RABBIT, AND GUINEA PIG LIVER

1		2	3	4	5	6	7	8
				Aldehydogenic Lipid			Molar Ratio α,β-Unsaturated Ether to Aldehydogenic Lipid	
Species*		Lipid Weight	Vitamin A	Uncorr.	Corr.	α,β-Unsat. Ether	Uncorr.	Corr.
		mg/g Fresh Tissue	*μg/mg Lipid*	*μmole NPH/g Lipid*		*μmole Iodine/g Lipid*		
Mouse	(*a*)	58.1	4.09	33.9	10.3	10.8	0.32	1.05
"	(*b*)	56.2	3.62	27.1	5.6	5.6	0.21	1.00
"	(*c*)	45.0	4.27	28.7	7.5	8.4	0.29	1.12
"	(*d*)	42.5	13.00	83.3	10.6	9.7	0.12	0.91
Rat	(*a*)	36.8	2.10	20.0	9.6	12.6	0.63	1.18
"	(*b*)	38.7	2.30	22.5	10.8	13.5	0.60	1.25
"	(*c*)	46.9	3.10	25.5	9.7	11.4	0.45	1.17
"	(*d*)	46.2	2.90	26.2	11.3	12.4	0.47	1.10
Rabbit	(*a*)	33.1	0.78	18.4	14.8	17.2	0.94	1.16
"	(*b*)	27.5	0.92	23.2	19.0	21.0	0.91	1.10
Guinea pig	(*a*)	26.3	<0.20	41.5		45.3	1.09	
" "	(*b*)	32.5	<0.20	27.5		27.6	1.01	

* Liver from a single animal was used to prepare extracts from rat, rabbit, and guinea pig. Mouse tissue extracts were obtained by pooling liver from two animals.

the rat, 0.93 for the rabbit, and 1.05 for the guinea pig. It may also be seen that the content of aldehydogenic lipid as distinct from vitamin A (column 5) is very low in mouse and rat liver ranging from 5.6 to 11.3 μmole/g of lipid, or 0.4–0.8% of the total lipid weight assuming a molecular weight of 700 (phosphatidal ethanolamine).

Detection and Measurement of Vitamin A. It was initially observed that sample blanks in the specific iodination determination exhibited an appreciable absorption at 355 mμ, the wavelength used to determine the iodine in the spectrophotometric estimation. The sample blank absorption was proportional to the lipid content. It was then observed that both total lipid of mouse liver and especially the neutral lipid fraction obtained from this by silicic acid chromatography and elution with hexane + 10% ethanol in hexane had a broad absorption band between 280 and 370 mμ with a maximum at 331 mμ (ethanol). In contrast, the cephalin fraction (eluted with absolute ethanol) and choline phosphatides (eluted with methanol) had no absorption between 270 and 500 mμ. The observed absorption maximum was close to that reported for vitamin A compounds. A comparison of the spectra obtained with total mouse liver lipid and crystalline vitamin A acetate (in isopropanol) is shown in Fig. 1.

Quantitative determination of vitamin A content of the lipid extracts based on the Carr-Price reaction were in good agreement with the estimates based on direct comparison of the absorption of the total lipid with vitamin A acetate at 329 mμ.

Interference of Vitamin A in the p-Nitrophenylhydrazone Reaction. Subjecting vitamin A acetate to the *p*-nitrophenylhydrazone reaction showed that the color ob-

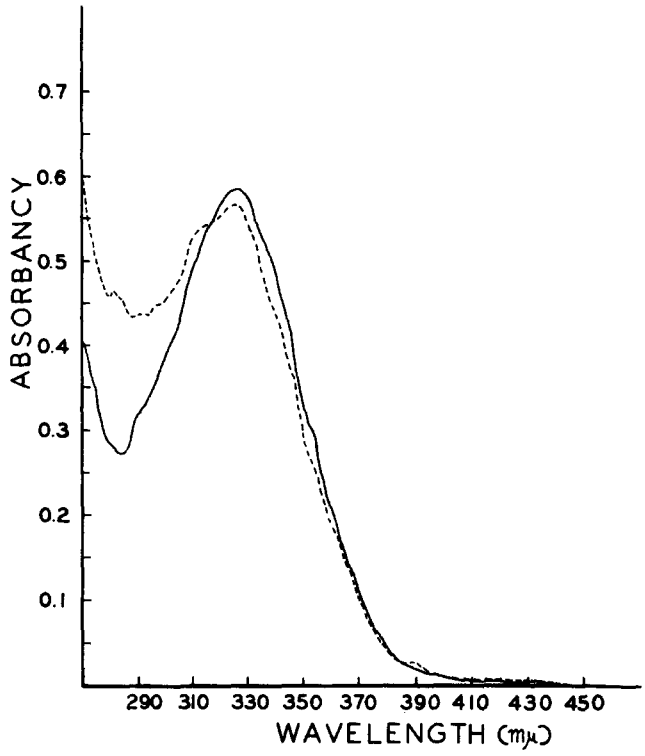

FIG. 1. Absorption spectra of mouse liver lipid (- - - -) and vitamin A acetate (——) in isopropanol. Concentrations: vitamin A acetate, 4.00 μg/ml ($E^{1\%}_{1cm}$ = 1,470); mouse liver lipid, 1.08 mg/ml containing 3.92 μg vitamin A (Carr-Price).

tained at 390 mμ was proportional to the quantity of vitamin A over the range 1–16 μg (Fig. 2). The color corresponded to 5.05 μmoles of long-chain aldehyde per mg of vitamin A acetate, and the slope of the line re-

mained constant when the vitamin A acetate was studied as an internal standard added to mouse, rat, or rabbit liver lipid (Fig. 2).

The spectra of hexane extracts obtained by subjecting mouse liver lipid, crystalline vitamin A alcohol, and crystalline vitamin A acetate to the reaction conditions of the *p*-nitrophenylhydrazone reaction in the absence of *p*-nitrophenylhydrazine are shown in Fig. 3B. All three samples showed maxima at 352, 370, and 392 mμ.

The absorption spectra shown in Fig. 3A were obtained in the absence of both sulfuric acid and *p*-nitrophenylhydrazine. These spectra have a maximum at 329 mμ.

Hexane extracts obtained by subjecting mouse liver lipid, vitamin A alcohol, or vitamin A acetate to the conditions of the *p*-nitrophenylhydrazone reaction in the absence of *p*-nitrophenylhydrazine were then studied using the Carr-Price reaction. All showed a single maximum at 620 mμ appearing 40 sec after addition of the antimony trichloride.

The concentrations of vitamin A in the various liver lipid extracts are shown in Table 1, column 3. It can be seen that the concentrations in mouse liver are considerably higher than those in rat liver, whereas the amounts found in rabbit liver and guinea pig liver are small. The values for aldehydogenic lipid (plasmalogen) corrected for the interference attributable to vitamin A are shown in Table 1, column 5.

Interference of Vitamin A in the Spectrophotometric Iodination Reaction. As mentioned above, and as can be seen from Fig. 1, the absorption of vitamin A compounds is appreciable at 355 mμ, the wavelength at which the iodine is measured in the spectrophotometric method for α,β-unsaturated ethers. The absorbancy of the sample blanks obtained by substituting 3% KI for the iodine reagent were compared with the quantity of vitamin A estimated by means of the Carr-Price reaction. The relative constancy of the ratio (averaging 0.011 ± 0.003 absorbancy units per μg vitamin A) shows that vitamin A accounts for the observed sample blank. Although this blank value may be large (27–58% of the observed decrease in iodine absorption for the rat liver extracts and 125–250% for the mouse liver extracts), the interference is adequately controlled by adding the blank to the absorption of the iodine in the absence of lipid.

Corrected Values of the Molar Ratio of α,β-Unsaturated Ether to Aldehydogenic Lipid. When corrections are made for the vitamin A content of liver lipids (Table 1, column 8), it is seen that the values for the ratio are no longer low. The average value for the four mouse extracts is 1.02 and for the four rat extracts is 1.17. The two rabbit liver

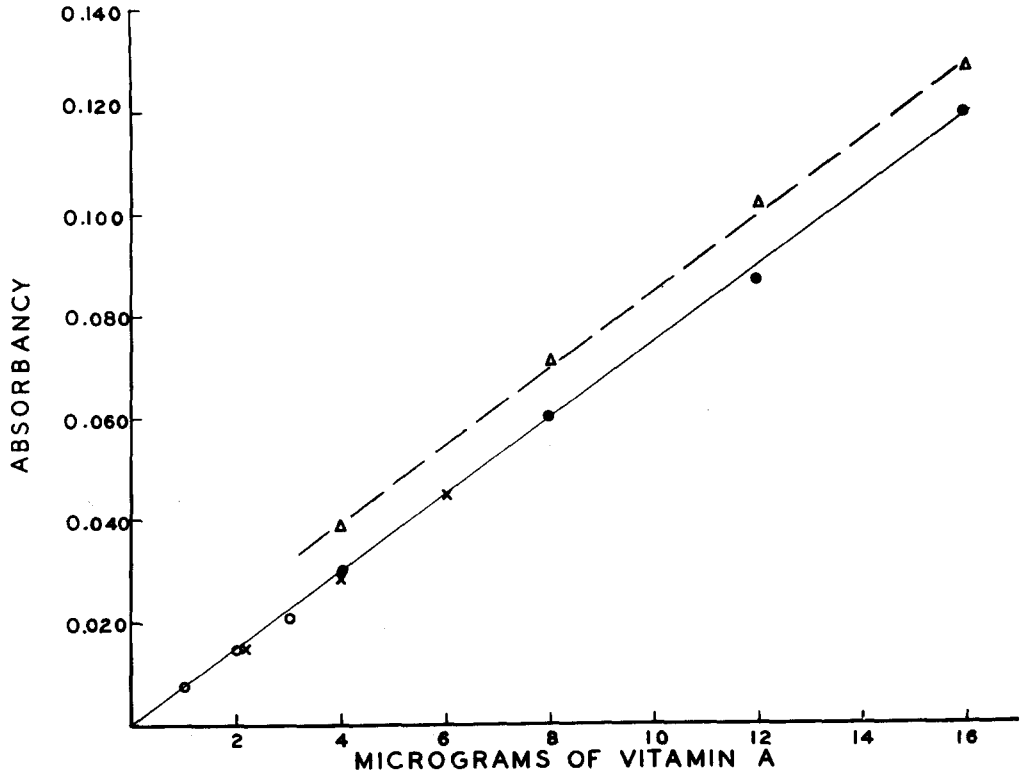

FIG. 2. Standard curves obtained with vitamin A acetate in the *p*-nitrophenylhydrazone reaction: external standard (●), internal standard in rat liver lipid (×), in rabbit liver lipid (○), in mouse liver lipid (Δ).

extracts average 1.13 in accord with the values recorded earlier (3).

DISCUSSION

The results of this study show that low values of the molar ratio of α,β-unsaturated ether to aldehydogenic lipid observed with lipid extracts of mouse and rat liver are attributable to the interference caused by vitamin A in the determination of aldehydogenic lipid and do not indicate the presence of either mixed acetals or free long-chain aldehydes. Although all-*trans* vitamin A alcohol and acetate do not absorb light at the wavelength used for the measurement of aldehyde *p*-nitrophenylhydrazones (390 mμ) and thus offer no direct interference, they are converted under the conditions of the method to substances that do absorb at 390 mμ. The spectral changes suggest that the product that forms on heating with sulfuric acid and alcohol is anhydro vitamin A, and this conclusion is supported by the spectra obtained for the Carr-Price reaction product (13, 14). Although it is not proved that an identical change occurs in the presence of *p*-nitrophenylhydrazine, the correspondence of external and internal standard curves strongly suggests that the changes are qualitatively and quantitatively the same.

Vitamin A interference occurs at a level approximating 75% of the uncorrected observed readings for the quantities of plasmalogen and vitamin A found in mouse liver. The corresponding level of interference for rat liver lipid is 50%, for rabbit liver lipid only 8%, and no interference was attributable to this source in guinea pig liver lipid. Aside from these species differences, the level of vitamin A present in liver is dependent on diet, and therefore no uniformity may be expected in the level of interference found by different investigators. It is possible to correct the observed results by determination of the quantity of vitamin A present. Although recorded studies of plasmalogen concentrations in mammalian organs other than liver do not appear to suffer from interference from this source, the measurement of aldehydogenic lipids by phenylhydrazone formation alone may yield unreliable results if inadvertently applied to specific cellular fractions in which vitamin A is concentrated or to tissues in which elevated concentrations of vitamin A may be present, such as intestine, adipose tissue, and retina.

The separation by silicic acid chromatography of the neutral lipids from the cephalin and choline phosphatide fractions showed that interference by vitamin A is restricted to determination of aldehydogenic neutral lipids.

Inasmuch as the analysis of aldehydogenic lipids in liver, the organ in which they are present in lowest concentration, actually involves very small measurements of absorbancy, an attempt to interpret the positive deviation from unity of the molar ratio of unsaturated ether to aldehydogenic lipid does not appear to be warranted. With the low concentrations encountered in liver and the consequent need for large amounts of lipid, emulsion formation produces a disproportionate increase in readings as the quantity of lipid is increased. As an upper limit, 3 mg of lipid containing 0.005–0.010 μmole of plasmalogen produces a 6% deviation from linearity in the *p*-nitrophenylhydrazone determination.

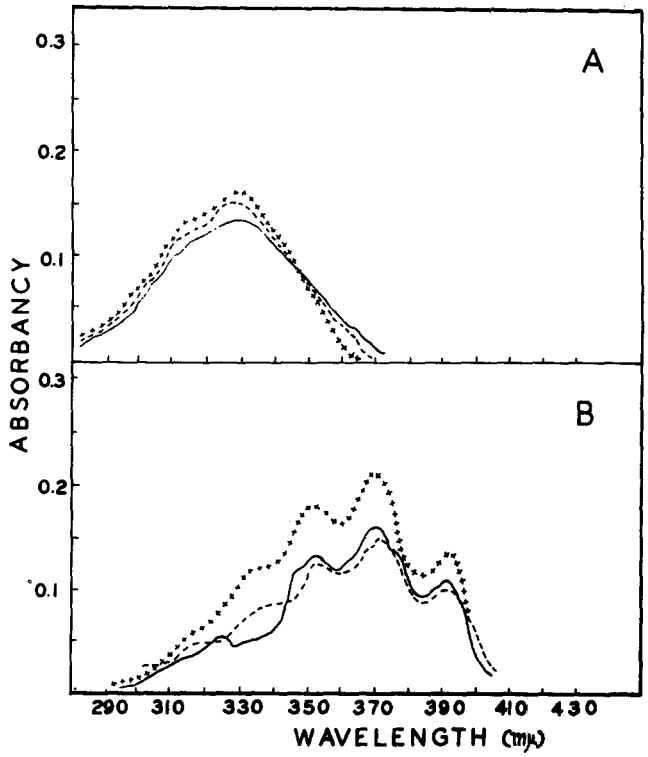

FIG. 3. Absorption spectra of hexane extracts of vitamin A alcohol (——), vitamin A acetate (- - - - -), and mouse liver lipid (+ + + + +) subjected to the conditions of the *p*-nitrophenylhydrazone reaction. A. In the absence of sulfuric acid and *p*-nitrophenylhydrazine; B. complete reaction.

The corrected value for the molar ratio thus shows that plasmalogens of mouse and rat liver conform in structure to those of other tissues in containing the α,β-unsaturated ether linkage.

We wish to thank the Venezuelan Council for Development of Scientific Research for the scholarship awarded to Mr. Camejo.

This work was supported by PHS Research Grant NB-01570-06, National Institutes of Health, U. S. Public Health Service.

Manuscript received July 3, 1963; accepted September 30, 1963.

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