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# A sensitive and specific method for plasmalogens and other enol ethers*

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## SUMMARY

A spectrophotometric procedure for estimating plasmalogens and other enol ethers, based upon the specific reaction of the enol ether group with iodine, is presented. Optimum conditions for iodination with respect to methanol and KI concentration, pH, and time of incubation have been defined. After evaporating the solvent from the lipid extract containing 0.02 to 0.125 μmole plasmalogen, 0.9 ml methanol, 3.2 ml 0.094 M Na citrate (pH 5.5), 0.4 ml 3 M KI, and 0.5 ml 0.0005 M $I_2$ in 3 M KI are added and mixed. After standing for 40 min at room temperature, the mixture is extracted with 5 ml *n*-butyl acetate. Absorbancy of the butyl acetate layer is read at 363 mμ. Lipid and reagent blanks and an $I_2$ standard containing 0.25 μmole $I_2$ are treated similarly except that 0.9 ml 3 M KI is added to both blanks in place of $I_2$. Reproducibility is ±3.6% with 0.1 μmole levels of plasmalogen in lipid extracts. The range of enol ether estimated by this procedure is 0.02–0.125 μmole.

In 1948, Siggia and Edsberg (1) reported that vinyl ethers may be determined specifically by measuring the uptake of iodine in 50% methanol. Their procedure suggested the use of 1,000 to 2,000 μmoles of vinyl ether per sample, which in many biological studies is prohibitively large. Thus far, the enol ethers encountered in biological materials are the plasmalogens, which are usually found in the order of 1 to 10 μmoles/g of fresh tissue. The sample size used in the procedure of Siggia and Edsberg was scaled down to 10 μmoles by Rapport and Franzl (2) and Rapport and Lerner (3). In both procedures, however, a titrimetric analysis for the iodine remaining after reaction was employed, which is quite laborious if large numbers of samples are employed. Norton (4) modified the iodination procedure by using a potentiometric titration. He states that 0.5 μmole of plasmalogen can be determined by his procedure. In the present paper, we wish to report a procedure in which as little as 0.01 to 0.02 μmole of enol ether can be determined accurately and rapidly by using the iodination reaction coupled with a spectrophotometric measurement of the iodine remaining after reaction. Sloane-Stanley (5) has suggested the use of spectrophotometric iodimetry in the estimation of plasmalogens. Our procedure, however, utilizes a different approach in that iodine is extracted into a suitable solvent under standardized conditions and then read spectrophotometrically.

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### REAGENTS

*Methanol*, absolute, reagent grade.

*Sodium Citrate*, 0.094 M, adjusted to pH 5.5. Kept at 3°, this reagent is stable indefinitely.

*Potassium Iodide*, 3 M, reagent grade. Kept at 3° and in a dark bottle, this reagent is stable for about 1 week.

*Iodine*, 0.0005 M, reagent grade, dissolved in 3 M KI. Kept at room temperature in an aluminum foil-covered bottle or otherwise protected from light, the reagent is stable for at least a month.

*n-Butyl acetate*, reagent grade (Eastman).

## PROCEDURE

Triplicate lipid samples prepared by the procedure of Folch et al. (6) and containing from 0.02–0.125 μmole of plasmalogen are added to test tubes that can be centrifuged. A glass bead is added to each tube, and the solvent is evaporated under nitrogen at 70°. One of the tubes serves as a sample blank and the other two for measuring iodine uptake by the plasmalogen in the lipid. After evaporation of the solvent, 0.9 ml methanol is added and the contents are mixed. The tubes are allowed to stand for 5 min to dissolve the lipid residue and mixed again. If the glass bead is not loosened from the lipid sample after this time, the tube is warmed in a water bath for a few seconds at 70° to expedite solution. Then 3.2 ml of 0.094 sodium citrate (pH 5.5) are added and the tube contents mixed well. To the sample blank tube is added 0.9 ml of 3 M KI and to the other two tubes are added 0.4 ml of 3 M KI and 0.5 of 0.0005 M $I_2$ in 3 M KI. The tubes are shaken vigorously and allowed to stand for 40 min at room temperature. To each tube is added 5 ml of *n*-butyl acetate, the tubes are shaken vigorously for 10 sec and then centrifuged for 10 min. Aliquots of the *n*-butyl acetate layer (the upper layer) are transferred to quartz cuvettes by pipette. The samples are read at 363 mμ against a reagent blank. A standard for iodine is set up as above in each analysis. One level of iodine (0.5 ml of 0.0005 M $I_2$ in 3 M KI, or 0.250 μmole $I_2$) will suffice since the curve is linear up to that level of iodine.

## RESULTS

*Iodine Standard Curve.* Iodine extracted into *n*-butyl acetate was found to give a sharp absorption maximum at 363 mμ and followed Beer's law up to 0.250 μmole of iodine/5 ml of *n*-butyl acetate. The molecular extinction coefficient of iodine under these conditions is $2.12 \times 10^4$. The presence of the excess 3 M KI in all tubes before extraction was necessary to give a linear curve that passed through the origin. If the KI was not included in the reagent blank, the curve was linear but crossed the ordinate slightly below the origin.

*Linearity of Iodine Uptake as a Function of Amount of Lipid Added.* In Figure 1 are shown results demonstrating the linear relationship between iodine uptake and sample size for several lipid preparations. The

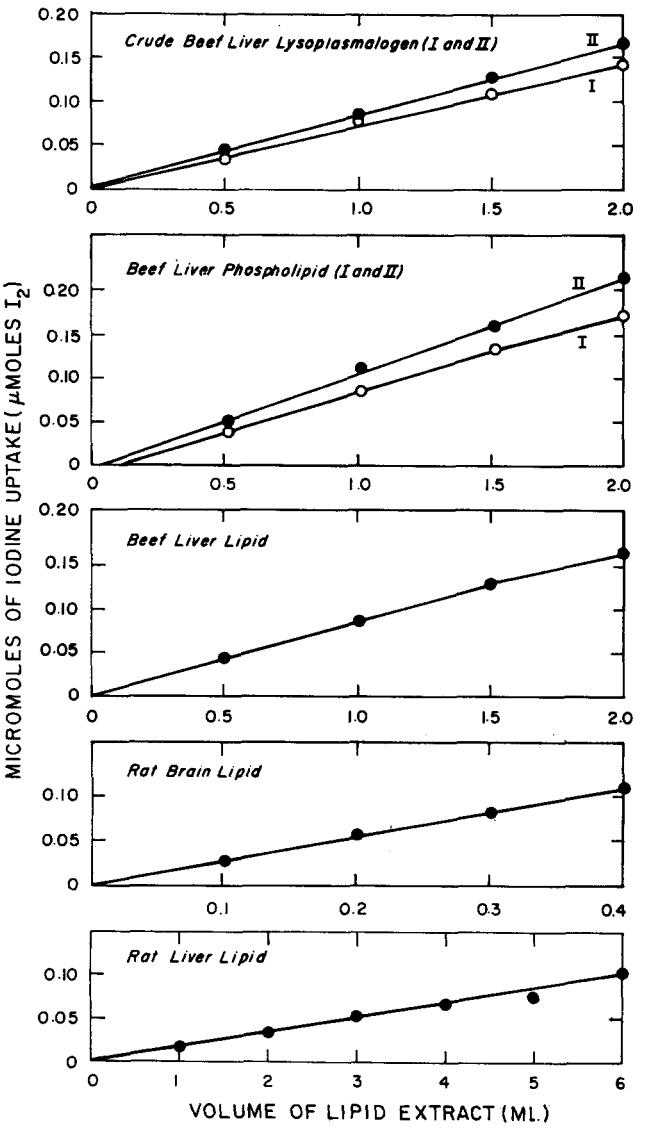


FIG. 1. Iodine uptake by plasmalogen *vs.* lipid extract volume. The extracts were made so that the tissue represented in each (milligrams of fresh organ/ml of extract) was as follows: rat liver lipid, 32; rat brain lipid, 21.4; beef liver lipid, 22.7; beef liver phospholipid preparations I and II and beef liver lysoplasmalogen preparation I and II, 36.3.

lipid preparations studied were rat liver lipid, rat brain lipid, beef liver lipid, two preparations of beef liver phospholipid, and two crude lysoplasmalogen preparations. The first three extracts were prepared by the method of Folch et al. (6). The two phospholipid preparations were obtained after adsorption of whole beef liver lipid on a silicic acid column, washing exhaustively with chloroform and eluting with 2:1 chloroform-methanol followed by methanol. The crude lysoplasmalogen was prepared from a portion of the beef liver phospholipid by a modification of the method

of Dawson (7). In every case, with the exception of phospholipid preparation I, the response was linear and passed through the origin. With phospholipid preparation I, the error due to the curve not passing through the origin, when 0.1 μmole of iodine uptake was used to base the calculation, was −6.8%. The average error from all of the curves due to deviation from zero at the origin was −0.7%. With beef liver lipid and beef liver phospholipid preparation I, a break in the curves began beyond 0.125 μmole of iodine uptake. Below this point in every case, the curves were linear.

TABLE 1. A COMPARISON OF RESULTS BY OUR METHOD AND BY THE *p*-NITROPHENYLHYDRAZONE (NPH) PROCEDURE OF WITTENBERG ET AL. (8), USING LIPID EXTRACTS FROM REPRESENTATIVE RAT ORGANS AND BEEF LIVER PREPARATIONS

| Organ or Preparation | Number of Extracts Analyzed[1] | Plasmalogen Content/ml Extract | | μmoles $I_2$ Uptake / μmoles NPH Formed |
|---|---|---|---|---|
| | | By $I_2$ Uptake | By NPH Formation | |
| | | *μmoles* | | |
| Rat brain | 4 (12)[2] | 0.272 | 0.246 | 1.09 |
| Rat liver | 9 (13) | 0.027 | 0.030 | 0.95 |
| Rat muscle | 3 (9) | 0.037 | 0.033 | 1.16 |
| Rat lung | 3 (9) | 0.118 | 0.114 | 1.03 |
| Beef liver | 1 (1) | 0.073 | 0.081 | 0.91 |
| Beef liver lysoplasmalogen | 1 (1) | 0.090 | 0.090 | 1.00 |

[1] The extracts were made so that the tissue represented in each (milligrams of fresh organ/ml of extract) was as follows: rat brain lipid, 20.5; rat liver lipid, 40.0; rat muscle lipid, 30.8; rat lung lipid, 34.3; beef liver lipid, 22.7; beef liver crude lysoplasmalogen, 36.3.

[2] The numbers in parentheses refer to the number of animals used to prepare the extracts.

*Effect of KI.* The high level of KI is absolutely essential to obtain linearity of the iodine standard curve and of the curve for uptake of iodine *vs.* lipid concentration. The excess KI also appears to be necessary to obtain complete extraction of the iodine from the aqueous phase by the *n*-butyl acetate. The reasons for these effects are not known.

*Recovery of a Synthetic Enol Ether and Other Unsaturated Compounds.* When synthetic vinyl 2-*n*-3-(1′-octenyloxy)-1,2-proponidial[1] was added to beef liver lipid, analysis indicated an increment in enol ether content equal to 94% of the theoretical increment expected. Also, when this compound was used as a standard and μmoles of iodine taken up were plotted *vs.* the μmoles of compound added, the curve obtained was linear to 0.4 μmole of compound and passed through the origin. When oleic and linoleic acids were used as substrates in the reaction, each acid took up only 0.0001 μmole of iodine/μmole of acid added.

*Effect of pH, Methanol Concentration, and Time of Incubation.* Early in the development of the procedure, when no buffer was included, the iodine uptake by beef liver lipid was too great. Buffer of pH 5.5 was found to give the best results. Although enol ethers are hydrolyzed in acid solution, this pH was not low enough to hydrolyze the enol ether linkage but was low enough to prevent loss of iodine by what the authors believe are the following reactions: $I_2 + H_2O \rightleftarrows H^+ + I^- + HOI$; then, $3\ HOI \rightarrow 2\ I^- + IO_2^- + 3\ H^+$. Suppression of hydroxyl ion concentration would shift the equilibrium of the first reaction far to the left and thus prevent the second irreversible reaction from taking place.

A study of the effects of methanol concentration, by using citrate buffer of pH 5.5, showed that the maximum iodination of the plasmalogen in beef liver lipid occurred with 18% methanol in the final reaction mixture. The curve for iodine uptake *vs.* methanol concentration gave an increase in iodination up to 18% methanol followed by a falling off of iodination at higher methanol concentrations.

A study of the time of incubation necessary for maximum reaction to take place, by using optimal KI concentration, methanol concentration, and pH, showed that the reaction was not complete until 30 min followed by a plateau for up to 50 min. Thus, we chose a 40-min incubation time.

*Stability of the Reaction.* The reaction may be run under fluorescent lights with no attempt made to keep the tubes dark. Once the iodine has been extracted into *n*-butyl acetate, the iodine absorption at 363 mμ is stable at least for 24 hr.

*Comparison with Other Methods.* Representative rat organs were extracted by the procedure of Folch et al. (6) and analyzed simultaneously by our procedure and that of Wittenberg et al. (8). The beef liver extract and crude lysoplasmalogen preparation, used to prepare the curves in Figure 1, were also analyzed by both methods. The results are presented in Table 1. The variation in the ratios of the results of the two methods is quite similar to that reported by Rapport and Lerner (3) and Norton (4). The over-all average for all extracts in the table is 1.02. Norton (4) obtained 0.96 (exclusive of liver), and Rapport and Lerner (3) 1.08 for the

[1] Prepared and kindly supplied by Dr. Claude Piantidosi, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina.

over-all average of this ratio.

*Reproducibility.* Analysis of 28 different samples of a single beef liver lipid extract gave a variation of ± 3.6% among the different samples.

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# Erratum

In the paper by J. N. Williams, Jr., Carl E. Anderson, and Alice D. Jasik, on page 380, Volume 3, Number 3, July, 1962, left column, 4 lines from the bottom, the name of the synthetic enol ether should have read:

3-(1′octenyloxy)-1,2-propanediol