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149. Makoto Hayashi, Komei Miyaki, and Tsutomu Unemoto: Studies on the Determination of Serine and 2-Aminoethanol in Phospholipid.

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There are two methods for quantitative determination of serine and 2-aminoethanol in phospholipids by hydrolysis. The one is to effect dinitrophenylation of serine and 2-aminoethanol, separation of respective products by the difference in their solubility, and colorimetric determination of each component.[1~3] The other method effects separation of serine and 2-aminoethanol first through adsorption on Permutit,[4,5] Zeo Karb,[6] or cation-exchange resin,[7] and colorimetric determination of each fraction with β-naphthoquinone-4-sulfonate,[5] or decomposition with periodic acid and titration of the ammonia liberated.[4,7] These determination methods should be specific to serine and 2-aminoethanol. In this sense, oxidation with periodic acid and determination of the ammonia or formaldehyde formed, according to the following formula, is the most suitable.

$$(1)\quad HO\text{-}CH_2\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{-}COOH \xrightarrow{HIO_4} HCHO + NH_3 + CO_2 + HCOOH$$

$$(2)\quad HO\text{-}CH_2\text{-}CH_2\text{-}NH_2 \xrightarrow{HIO_4} 2HCHO + NH_3$$

In this process, the ammonia formed must be isolated from the reaction mixture for determination. In the determination of a trace of 2-aminoethanol by hydrolysis of phospholipid, contamination of ammonia results in lower accuracy of the determination. On the other side, quantitative determination of formaldehyde does not require isolation of formaldehyde from the reaction mixture and colorimetry can be carried out directly by the use of chromotropic acid.[8] This coloration is highly sensitive and the color developed is extremely stable.

In the present series of experiments, quantitative determination of serine and 2-aminoethanol was successfully carried out by fractionating the hydrolyzate of lipid into glycerol, serine, and 2-aminoethanol fractions, decomposing the serine and 2-aminoethanol fractions with periodic acid, and determining the amount of formaldehyde produced. The analytical values obtained by this method on rat and mouse liver were compared with those given in past literature.

Experimental Method and Results

Determination of Formaldehyde produced by Periodate Oxidation—The method of Frisell[8] was simplified so as to carry out the whole procedure from oxidation to coloration in one test tube and to measure 0.1~0.3 μmoles of formaldehyde.

Reagents: 0.5*M* Na_2HPO_4, 0.05*M* HIO_4, 3% $NaHSO_3$, 2% chromotropic acid in 2*N* H_2SO_4 (treated with activated carbon after dissolution), conc. H_2SO_4, and 2.5% thiourea.

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Procedure: To 1.5 cc. of the sample (serine or 2-aminoethanol corresponding to 0.1~0.3 μmoles of formaldehyde), 0.5 cc. each of Na_2HPO_4 and HIO_4 were added and the mixture was allowed to stand for 5 min. at room temperature. To this mixture, 0.5 cc. of $NaHSO_3$ was added gently, followed by 0.5 cc. of chromotropic acid, and 4.0 cc. of conc. H_2SO_4 was superimposed on it. This was shaken vigorously and allowed to stand in a boiling water bath for 30 min. After cool, 2.5 cc. of thiourea was added to decolorize the red tint produced by iodine and the solution was colorimetrically determined at 570 mμ.

The results of colorimetry on serine and 2-aminoethanol using the Coleman Universal Spectrophotometer are shown in Fig. 1. As indicated in Fig. 2, velocity of periodate decomposition depended on pH and it was necessary to have the reaction mixture at above pH 6.5 in order to effect 100% decomposition of serine and 2-aminoethanol during 5 min. under the foregoing reaction conditions.

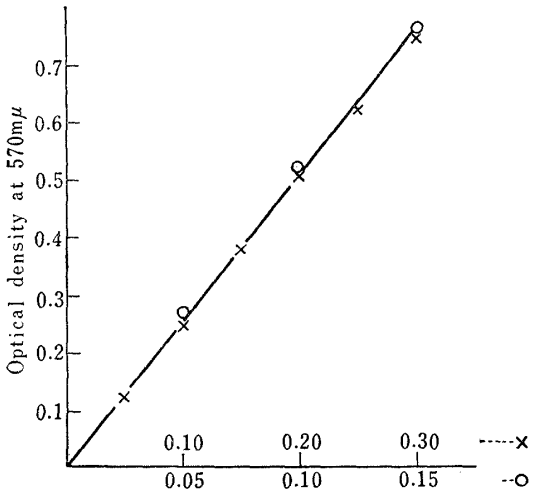

Fig. 1. Optical Density and Amounts of Serine and 2-Aminoethanol

×—× serine
o—o 2-aminoethanol

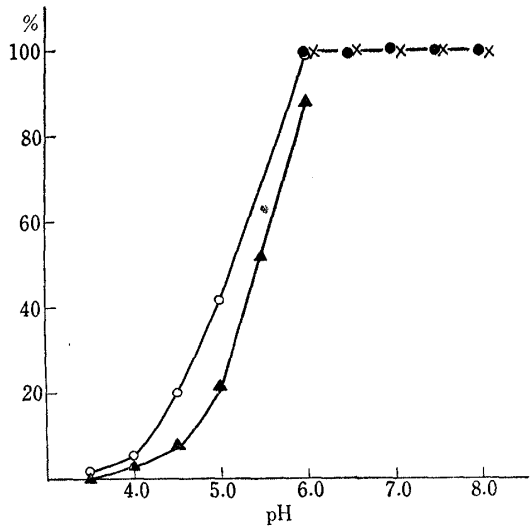

Fig. 2. Effect of pH on the Oxidation of Serine and 2-Aminoethanol

0.5 cc. of 0.5*M* buffer was used and formaldehyde produced by periodate oxidation was measured as described in the text. Oxidation time, 5 min.

o—o acetate buffer } serine
•—• phosphate buffer } (0.2 μmole)
▲—▲ acetate buffer } 2-aminoethanol
×—× phosphate buffer } (0.1 μmole)

Separation of Glycerol, Serine, and 2-Aminoethanol—Separation of glycerol was effected by the modified method of Dittmer and others.[7]

A column of about 10 mm. in internal diameter was filled with about 3 cc. of cation exchanger, Amberlite IR-112 (150~180 mesh), washed with 0.5*N* NaOH solution to change the resin into Na form, and washed with 0.2*M* AcOH until the effluent pH became below 3.0. The sample was adjusted to

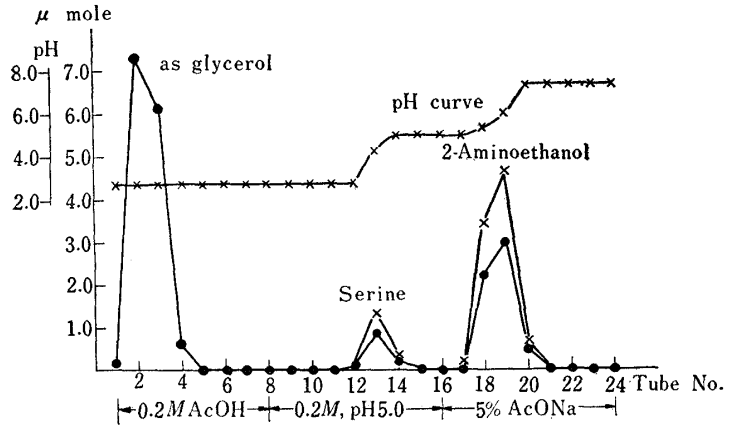

Fig. 3. Chromatography of Lipid Hydrolyzate from Mouse Liver on Amberlite IR-112

Lipid hydrolyzate corresponding to 21.4 μmoles of lipid P was used. For the determination of glycerol oxidation, time was prolonged to 30 min., during which glycerol decomposed completely.

×—× by Ninhydrin method
•—• by HIO_4 method

pH 2.8 (amount corresponding to 1~2 μmoles of serine or 2-aminoethanol), adsorbed on the resin, and the resin was eluted with three 5-cc. portions of 0.2*M* AcOH. The combined effluent was taken as the glycerol fraction. The column was then eluted with 15 cc. of 0.2*M* acetate buffer (pH 5.0) and the effluent therefrom was taken as the serine fraction. The final fraction obtained by elution with 15 cc. of 5% AcONa was taken as that of 2-aminoethanol.

The elution curve obtained by this procedure on the lipid hydrolyzate from mouse liver is shown in Fig. 3. The solutions used for development were taken as the control for colorimetry.

Application of the Present Procedure to Analysis of Liver Lipid—The liver (2~5 g.) obtained from rat or mouse was homogenized with 20 volumes of $CHCl_3$:MeOH (2:1), the homogenate was boiled in a water bath, and allowed to cool to room temperature. This was filtered, the filtrate was purified by diffusion according to the method of Sperry,[9] and concentrated in N_2 atmosphere. The residue was dissolved in $CHCl_3$, filtered, and $CHCl_3$ was evaporated. The residue was dried in a desiccator until loss in weight became constant to determine the amount of total lipid, and the residue was dissolved in a definite quantity of $CHCl_3$.

Total N was measured by the Unemoto method[10] after decomposition by the Kjeldahl procedure, total P by the Allen method,[11] and total choline by the Appleton method[12] after decomposition of the lipid with saturated $Ba(OH)_2$ solution for 3 hr. and neutralization with H_2SO_4. For serine and 2-aminoethanol, the amount of lipid corresponding to 50~100 μmoles of lipid-P was decomposed with 10 cc. of 6*N* HCl at 100° for 3 hr. As indicated in Table I, the serine and 2-aminoethanol added were quantitatively recovered under these conditions. The hydrolyzate was treated with three 5-cc.

Table I. Recovery of Serine and 2-Aminoethanol added to a Lipid before Hydrolysis by Various Methods

		Analytical method					
		DNFB		HIO_4		Ninhydrin	
		S	A	S	A	S	A
Lipid* from rat liver		2.04	3.40	0.52	2.99	1.01	5.25
Lipid* +	2-aminoethanol 2.0	2.40	5.52	0.93	4.94	1.45	8.45
	serine 0.4						

* Lipid from rat liver corresponding to 73.0 μmoles of lipid P was used and 1/5 of the hydrolyzate was analysed by Nojima's method (DNFB-method) or by the method described in the text (HIO_4 method). Serine and 2-aminoethanol fractions were also analysed by Moore's method (Ninhydrin method) and in this case, serine and 2-aminoethanol were used as a standard substance. All figures are in μmole.

portions of Et_2O to extract Et_2O-soluble material, the combined Et_2O layer was washed with 5 cc. of water, and the washing was combined with the original aqueous layer. This aqueous solution was evaporated in N_2 stream under a reduced pressure and the residue was dried in a desiccator. The dried residue was dissolved in a definite quantity of water, divided into two parts; one part was analyzed as described above, and the other part was analyzed by the dinitrofluorobenzene (DNFB) method.

Table II gives the mean values of three experiments carried out on the lipids from rat and mouse liver.

Table II. Analytical Data of Mouse and Rat Liver Lipid

	Mouse liver	Rat liver	Rat liver (Artom[4])
Total lipid (mg.)	59.8	50.6	
Total P (μmole/g.)	32.5	42.0	39.0
Total N (μmole/g.)	40.5	45.3	
Phosphatides containing			
choline (μmole)	21.8	23.4	23.4
2-aminoethanol (μmole)	7.6	8.8	11.3
serine (μmole)	1.6	1.4	3.8

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Discussion

As indicated in Table I, recovery of serine and 2-aminoethanol added from the lipid hydrolyzate was quantitative both by the present method and by the dinitrofluorobenzene method. Nojima and others[2] reported that the ratio of serine to 2-aminoethanol was 1:1.6 to 1:2.6 in the rat liver lipid by NaOH decomposition and analysis by the dinitrofluorobenzene method. In the present series of experiments, the lipid was decomposed with hydrochloric acid and determination was made by the periodic acid-formaldehyde method, and the ratio of these two substances was 1:5.7 to 1:6.7. The values agreed with those of Nojima when the determination was made by the dinitrofluorobenzene method. The large value of serine by the latter method may be due to contamination of some other material. This was actually confirmed by paper chromatography. The serine fraction obtained by the Nojima method from the lipid of rat and mouse liver was concentrated under a reduced pressure and submitted to paper chromatography. Development with butanol saturated with 2*N* ammonia showed, besides the spot of dinitrophenylserine, a bright yellow spot at around Rf 0.7 and a faint yellow spot near the original point. These are clearly different substances from 2,4-dinitrophenol.

Dittmer and others[7] reported that the unknown nitrogenous compound in the hydrolyzate of lipid rich in phosphatidylethanolamine is 2-aminoethyl dihydrogenphosphate formed during hydrolysis. 2-aminoethyl dihydrogenphosphate should naturally be determined as serine by the dinitrofluorobenzene method. The spot near the original point on the paper chromatogram in the present experiment probably corresponds to this substance but the amount is so minute that it could not have caused any variation in the value of serine. It would be more natural to assume that the presence of an unknown substance, appearing at Rf 0.7 on the said paper chromatogram, is the cause of such variation.

The determined value of 2-aminoethanol from the fraction obtained through cation exchanger fractionation, detected by the Ninhydrin method, is too high and this is chiefly due to the contamination of ammonia in the hydrolyzate and during fractionation procedure. It has been found that the 2-aminoethanol fraction contained ammonia in an amount around 0.5~1.0 μmole by the fractionation procedure and that the 3-hour hydrolyzate with 6*N* hydrochloric acid contained ammonia corresponding to 2~4% of total nitrogen.

The large value obtained on determination of serine by the Ninhydrin method is not due to ammonia. The values listed in Table I seem to suggest the presence of substances other than serine positive to Ninhydrin reaction. As indicated in Table II, the values of serine obtained by the present method of determination seem to be rather small compared to the values given in past literature and the reason for it must be the same as iterated above.

Summary

As a new method for determination of serine and 2-aminoethanol in phospholipid, the lipid hydrolyzate was fractionated by cation exchange resin, each fraction was oxidized with periodic acid to produce formaldehyde, and the color developed by chromotropic acid was submitted to colorimetry. This method is more simple and accurate than the existing methods and is thought to be suitable for micro-determination.

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