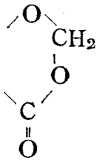
Summary

The nature of all six oxygen atoms in enmein, $C_{20}H_{26(28)}O_6$, were clarified as two of hydroxyls, one of inert ketone, and one of methylene ether ester of hydroxy acid. The discovery of a new group, methylene ether ester of hydroxy acid  seemed of great interest.

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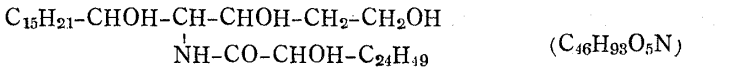
UDC 547.953 : 582.282.23

137. Takeshi Oda and Hiroko Kamiya: On the Complex Lipid, Cerebrine Phosphate, of Yeast.

(*Shizuoka College of Pharmacy**)

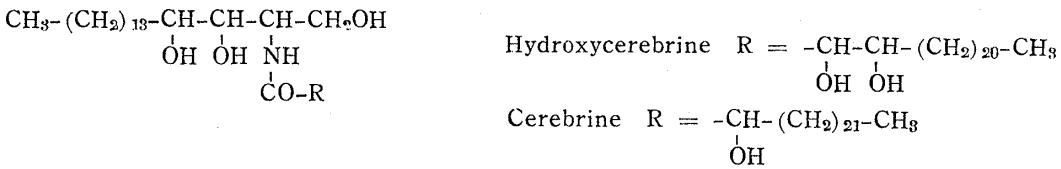
Reindel and others[1,2] made detailed studies on the complex lipid of yeast, cerebrine, and proposed the following structure for it.



$(C_{46}H_{93}O_5N)$

On the other hand, Ruppol[3] isolated a complex lipid corresponding to the formula $C_{46}H_{91}O_4N$ from beer yeast and reported that its hydrolysis afforded 2-hydroxy acid of C_{28} and sphingosine, but validity of the latter is questionable.

One of the writers (T.O.) obtained cerebrine from the mycelium of surface-cultivated penicillin-producing fungus and corrected Reindel's structural formula, establishing its chemical structure to be as follows:



Carter and others[5] isolated a long-chain base by hydrolysis of the complex lipid fraction, which had heretofore been called inositol lipid, from soybean and corn (*Zea Mays* L.) and proved that it was identical with the cerebrine base obtained from the above-mentioned *Penicillium* sp. They named this phytosphingosine, as against sphingosine isolated from animals. Cerebrine had been isolated from the mycelium of surface-cultivated penicillin-producing fungus and from *Aspergillus sydowi*,[6] as a product of autolysis, and yeast cere-

* Oshika, Shizuoka (小田 武, 神谷弘子).

1) F. Reindel: Ann., **480**, 76 (1930).
2) F. Reindel, A. Weickmann, S. Picard, K. Luber, P. Tulula: *Ibid.*, **544**, 116 (1940).
3) E. Ruppol: Bull. soc. chim. biol., **19**, 1164 (1937).
4) T. Oda: Yakugaku Zasshi, **72**, 136 (1952).
5) H. E. Carter, W. D. Celmer, M. L. Willaim, L. M. Catherine, H. H. Tomizawa: J. Biol. Chem., **206**, 613 (1954).
6) N. Bohonos, W. H. Peterson: *Ibid*, **149**, 295 (1943).

brine of Reindel had also been obtained from the saponified matter of yeast fatty oil, showing that it is not contained in the living organism as such.

In order to find out in what form this cerebrine fraction is present in the living organisms of Aspergillus and yeast, the present series of studies was carried out using comparatively easily available baker's yeast.

The baker's yeast was dehydrated with acetone and extracted with hot ethanol and hot chloroform-methanol (1 : 1) mixture. Cerebrine or lipids containing it was not obtained by this extraction but extraction with hot glacial acetic acid afforded a cerebrine-like subtance, somewhat more sparingly soluble in dehyd. ethanol and ether than cerebrine. This substance was slightly hygroscopic and repeated recrystallization from ethanol afforded a white powder of m.p. 143~144°. It contained, on an average, 4.2% of phosphoric acid and it showed no reaction for sugars. The analytical values of this substance corresponded approximately to $C_{42}H_{86}O_8N$. This was found by later studies to be cerebrine bonded to phosphoric acid and was therefore named cerebrine phosphate. Cerebrine phosphate can be obtained in a better yield by extraction with hot ethanol from yeast cells after a slight autolysis, rather than by direct extraction with glacial acetic acid. It is assumed that cerebrine is bonded to other macromolecular substance through phosphate bonding in the yeast cells.

Hydrolysis of cerebrine phosphate with 10% methanolic sulfuric acid results in breakdown into fatty acid, long-chain base, and phosphoric acid. The elementary analytical values of the fatty acid, m.p. 94~96°, agree with those of tricosanoic acid, $C_{23}H_{46}O_2$. The reverse-phase chromatography of Howard and Martin[7] was used for the analysis of fatty acid, but this procedure is not suitable to the analysis of hydroxy acid, as indicated in Fig. 1. Therefore, the fatty acid obtained by hydrolysis was oxidized with chromium trioxide and derived to a straight-chain fatty acid with one less carbon, and this acid was submitted to the foregoing chromatography. This indicated the presence of straight-chain fatty acids of C_{23} and C_{25}. Therefore, the componental fatty acid in cerebrine phosphate is a mixture in 3 : 1 ratio of cerebronic acid (C_{24}-2-hydroxy acid) and C_{26}-2-hydroxy acid. The presence of a C_{26}-2-hydroxy acid reported by Reindel and of C_{28}-2-hydroxy acid reported by Ruppole in yeast cerebrine can probably be ascribed to their inability to separate the mixture.

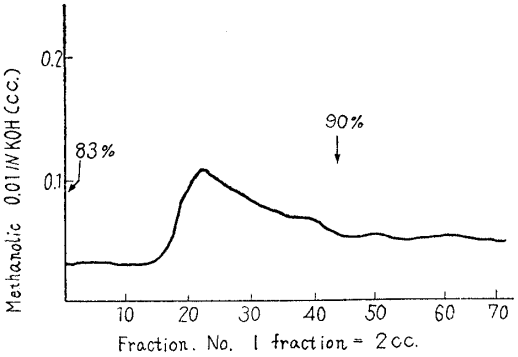


Fig. 1. Reverse-phase Chromatogram of Standard Cerebronic Acid

(Standard cerebronic acid, 10 mg.)

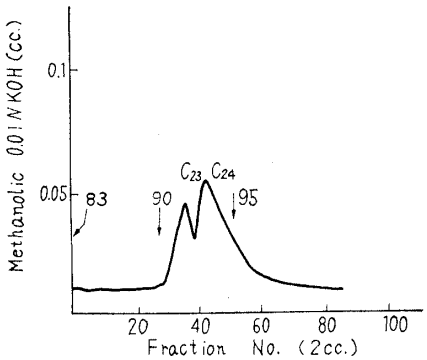


Fig. 2. Reverse-phase Chromatogram of Standard Fatty Acid

(Standard saturated acids, C_{23} 5 mg. + C_{24} 5 mg.)

For the sake of comparison, the three kinds of fatty acid samples obtained from cerebrine of penicillin-producing fungi were submitted to the same chromatography, lignoceric acid *per se*, and cerebronic acid and 2,3-dihydroxytetracosanoic acid after oxidation with

7) G. A. Howard, A. J. P. Martin: Biochem. J., **46**, 532 (1950).

chromium trioxide. The sample did not contain any fatty acids other than C_{24}-acid. While the fatty acids from cerebrine obtained from penicillin-producing fungus are all C_{24}-acids with different number of hydroxyl groups, those from yeast were found to be composed of cerebronic acid and C_{26}-acid homologs.

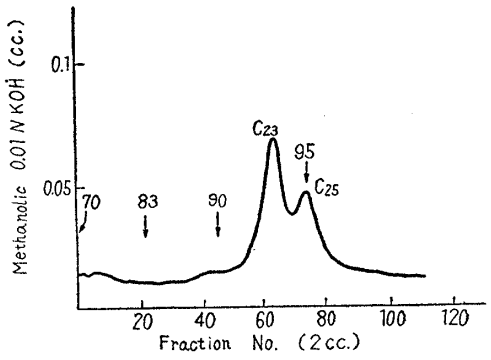


Fig. 3. Chromatogram of Oxidation Products
(Fatty acids (10 mg.) from yeast cerebrine phosphate oxidized with CrO_3)

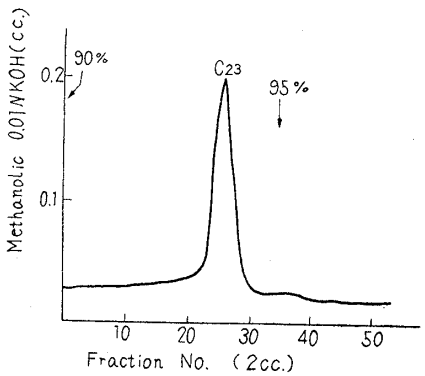


Fig. 4. Chromatogram of Oxidation Product
(Cerebronic acid from cerebrine of penicillin-producing fungus oxidized with CrO_3. Standard acid of cerebrine, 10 mg.)

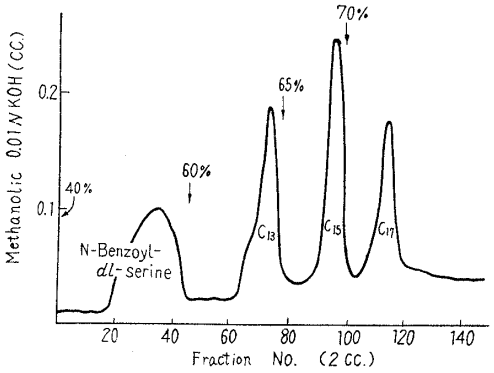


Fig. 5. Chromatogram of Standard Fatty Acids

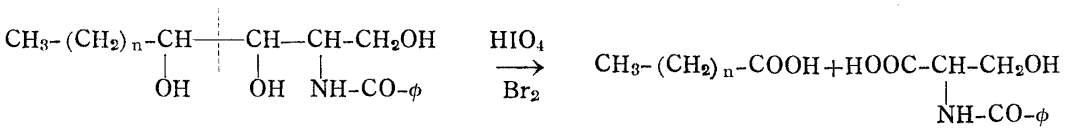
Standard fatty acids { N-Bezoyl-*dl*-serine 4 mg.; C_{13} 3 mg.; C_{15} 〃; C_{17} 〃

The elemental analytical values of the N-benzoylated base, derived from the base obtained from cerebrine phosphate, agreed with $C_{25}H_{43}O_4N$. The melting point of this base, 131~132°, did not rise by further recrystallization. This was in approximate agreement with the N-benzoylated base obtained by Reindel from yeast cerebrine but lower by 3~5° than that of the base obtained from the penicillin-producing fungi or from soybeans and corn.

TABLE I.

Source	N-Benzoylated base
Cerebrine from	m.p. (°C)
Penicillin-producing fungi[4]	137
Soybean and corn[5]	135~136
Yeast[1]	130
Cerebrine phosphate	131~132

Glycolysis of this N-benzoylated base with lead tetraacetate or periodic acid, and further oxidation with bromine water resulted in the formation of a fatty acid and N-benzoylserine.



Reverse-phase chromatography of the acid products indicated the presence of C_{15}- and C_{17}-straight-chain fatty acids besides N-benzoylserine. C_{15}- and C_{17}-acids were present in 2:1 ratio and this fact indicates the presence of two kinds of straight-chain bases of different number of carbon atoms. This mixture is responsible for the failure of the melting point of the N-benzoylated base to go beyond 132°. The fact that Reindel obtained an acid of $C_{16}H_{32}O_2$ or $C_{17}H_{34}O_2$ on oxidation of N-benzoylated base of yeast cerebrine with chromium trioxide may also be due to the presence of two kinds of acids, $C_{15}H_{30}O_2$ and $C_{17}H_{34}O_2$.

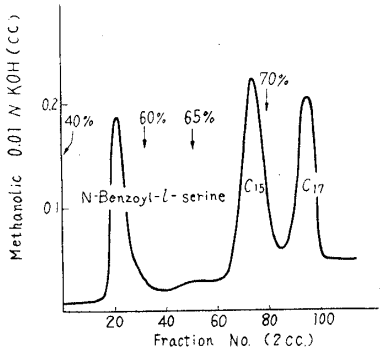


Fig. 6. Chromatogram of Oxidation Product

(N-Benzoylated base of yeast cerebrine phosphate oxidized with HIO_4. Oxidation product of cerebrine phosphate N-benzoate, 10 mg.)

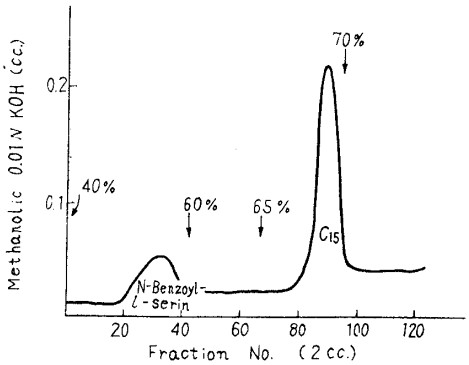
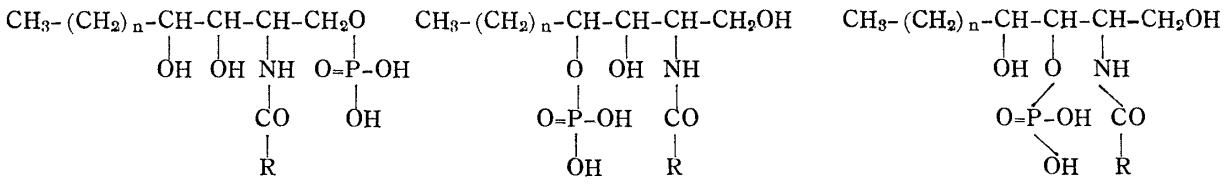


Fig. 7. Chromatogram of Oxidation Product

(N-Benzoylated base of cerebrine from penicillin-producing fungus oxidized with HIO_4. Oxidation product of cerebrine N-benzoate, 10 mg.)

The foregoing facts indicate that the yeast cerebrine base contains a mixture of CH_3-$(CH_2)_{13}$-CHOH-CHOH-CH(NH_2)-CH_2OH and its higher homolog, CH_3-$(CH_2)_{15}$-CHOH-CHOH-CH(NH_2)-CH_2OH. Sphingosine and phytosphingosine are C_{18}-compounds and presence of other homologs has not been proved as yet. It is interesting that the present series of work has shown the yeast cerebrine base to be a mixture of C_{18}- and C_{20}-compounds in 2:1 ratio.

In order to determine the position at which phosphoric acid is bonded to cerebrine in cerebrine phosphate, periodic acid was applied to cerebrine phosphate in glacial acetic acid and about 1 mole of the acid was consumed during 24~36 hours. The bonding position of phosphoric acid in cerebrine phosphate offers following three possibilities, (a), (b), and (c):



(a) (n=13 and 15) (b) (c)

In order to satisfy the consumption of 1 mole of periodic acid, formula (a) is the most appropriate, and formulae (b) and (c) should not consume periodic acid. Therefore, formula (a) is forwarded as the chemical structure of cerebrine phosphate.

Experimental

Preparation of Cerebrine Phosphate—A mixture of 10 lbs. of fresh, commercial baker's yeast and 10 L. of water was maintained at 37° for 4 days and filtered. The residue was added to 10 L. of 95% EtOH and the mixture was refluxed in a boiling water bath for 5 hrs. This mixture was filtered and the residue was again treated in the same way with 5 L. of 95% EtOH. This was filtered and cooling of the filtrate afforded a mixture of sterols, cerebrine, and a salt of cerebrine phosphate. Sterols and cerebrine were removed completely by washing with ether. The purified salt of cerebrine phosphate was obtained as a white powder, m.p. 205° (decomp.). This salt was recrystallized twice from glacial AcOH and twice from hot EtOH to give the free cerebrine phosphate, m.p. 143~144°. Yield, ca. 0.5 g. *Anal.* Calcd. for $C_{42}H_{86}O_8NP$: C, 66.05; H, 11.27; N, 1.84; P, 4.06. Found: C, 66.59; H, 11.26; N, 1.95; P, 4.20.

Hydrolysis of Cerebrine Phosphate with 10% MeOH-H_2SO_4—A mixture of 0.4 g. of crude cerebrine phosphate in a solution of 4 g. of conc. H_2SO_4 in 40 cc. of dehyd. MeOH was refluxed for 6 hrs., the solution was cooled to room temperature, and the precipitate formed was filtered off. The filtrate was added to a solution of 0.8 g. of KOH in 5 cc. of dehyd. MeOH, the mixture was refluxed for 2 hrs., and allowed to cool to room temperature. Potassium salt of fatty acid was collected by filtration and recrystallized from glacial AcOH-acetone to the purified fatty acid, m.p. 94~96°. *Anal.* Calcd. for $C_{24}H_{48}O_3$: C, 74.92; H, 12.59. Calcd. for $C_{26}H_{52}O_3$: C, 75.67; H, 12.70. Found: C, 74.80; H, 12.66.

Oxidation of the Fatty Acid with Chromium Trioxide—A solution of 50 mg. of the fatty acid described above dissolved in 3 cc. of glacial AcOH at 60° was added to 0.5 cc. of 10% CrO_3-AcOH solution, cooled, and poured into 50 cc. of water. The precipitate was collected, dissolved in 30 cc. of dehyd. Et_2O, the solution was washed with water, and evaporated to dryness. The residue was recrystallized from glacial AcOH to 20 mg. of a fatty acid, m.p. 78~79°. *Anal.* Calcd. for $C_{22}H_{46}O_2$: C, 78.00; H, 12.80. Calcd. for $C_{25}H_{50}O_2$: C, 78.53; H, 13.06. Found: C, 78.20; H, 13.29.

N-Benzoylcerebrine—The solution of cerebrine phosphate hydrolyzed with methanolic H_2SO_4 was concentrated to about 10 cc. *in vacuo* and poured into a large quantity of water. The mixture was allowed to stand at room temperature for 12 hrs., the precipitate formed was collected by filtration, and washed with hot acetone. The precipitate was added to 6 cc. of 5% methanolic KOH solution, and the mixture was refluxed for 1 hr. This was poured into a large quantity of water, the aqueous solution was shaken three times with 30-cc. portions of dehyd. Et_2O, and Et_2O extract was washed with water. The residue obtained on evaporation of Et_2O was dissolved in a mixture of 1 cc. of pyridine and 1 g. of BzCl, the mixture was allowed to stand at room temperature for 24 hrs., and poured into a large quantity of water. The aqueous solution was again extracted with three 30-cc. portions of Et_2O and the Et_2O extract was washed consecutively with $NaHCO_3$ solution, dil. HCl, and water. Et_2O was then evaporated, the residue was refluxed in 5 cc. of 0.5*N* methanolic NaOH for 1 hr., and the mixture was poured into 30 cc. of water. The aqueous solution was shaken with three 10-cc. portions of Et_2O, which was washed with water and evaporated to dryness. The residue was crystallized 5 times from benzene-acetone mixture to 50 mg. of pure N-benzoylcerebrine, m.p. 131~132°. *Anal.* Calcd. for $C_{25}H_{43}O_4N$: C, 71.22; H, 10.28; N, 3.32. Found: C, 71.48; H, 10.12; N, 3.26.

Oxidaton of N-Benzoylcerebrine with Lead Tetraacetate—A solution of 110 mg. of N-benzoylcerebrine dissolved in 6 cc. of 0.1*N* $Pb(OAc)_4$ solution (f=0.86, 1.1 moles) was heated at 50° for 2 hrs., poured into 3 cc. of AcOH, and H_2S gas was bubbled through the solution. The precipitated PbS was filtered off, the filtrate was added to a solution of 0.1 g. of Br_2 in 1 cc. of water, and the mixture was allowed to stand over night at room temperature. A slight excess of $Na_2S_2O_3$ solution was added to this mixture, the solvent was evaporated to dryness, water was added to the residue, and the solvent was again evaporated to dryness in oder to free AcOH. The residue was then dissolved in 20 cc. of AcOEt, the solution was filtered, and the filtrate was evaporated to dryness. The residue was submitted to column chromatography.

Reversed-phase Column Chromatography of Fatty Acids—A standard column (0.8×90 cm), packed with non-wetting kieselguhr impregnated with liquid paraffin, and hydrous acetone-medicinal paraffin system, were used throughout the present series of experiments. The column was maintained at 37° throughout the experiment.

Identification of Serine—The fraction of N-benzoylserine from the column chromatography was decolorized with carbon and evaporated to dryness *in vacuo*. The residue was hydrolyzed with 23 cc. of 20% HCl by boiling for 3 hrs. and the solution was shaken twice with 30 cc. of petr. ether. The residual aqueous solution was neutralized with dil. NaOH solution and submitted to paper chromatography. The paper chromatogram was run side by side with *dl*-serine, using 80% phenol as a solvent system, and a single spot positive to ninhydrin was identified as that of serine (Rf 0.29).

The petr. ether solution was shaken with dil. NaOH solution, the alkali solution was acidified with HCl, and extracted with 30 cc. of Et_2O. The residue obtained on evaporation of Et_2O melted at 120° and was identified as benzoic acid.

Periodate Oxidation of Cerebrine Phosphate—A suspension of 38.2 mg. (0.05 millimole) of cerebrine phosphate in 10 cc. of 0.2*M* aq. HIO_4 solution was diluted with glacial AcOH to 50 cc. and the mixture was allowed to stand at room temperature, with occasional shaking. The same treatment was made with 34.2 mg. (0.05 millimole) of cerebrine as a control. The excess HIO_4 remaining after a definite period of time was determined by titration with $Na_2S_2O_3$ in the presence of an iodate. Result is given in Table II.

TABLE II.

Period (hrs.)	HIO_4 consumed (mole)	
	Cerebrine	Cerebrine phosphate
2	0.32	0.14
6	0.51	0.24
24	0.99	0.91
48	1.01	1.05

Summary

Lightly autolyzed baker's yeast was extracted with hot ethanol and cerebrine phosphate was isolated from the extract. This ester was compared with cerebrine obtained from penicillin-producing molds to elucidate the chemical structure of cerebrine phosphate. The two substances were similar but the present one extracted from yeast differed from that from the penicillin-producing molds in that it was accompanied by C_{18}-base and C_{20}-long chain base, and that its componental fatty acid is a mixture of cerebronic acid (2-hydroxy-C_{24}-acid) and its homologous 2-hydroxy-C_{26}-acid. The presence of phosphoric acid is rather interesting.

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