# Lipid abnormalities in foam cell reticulosis of mice, an analogue of human sphingomyelin lipidosis

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ABSTRACT The lipid changes in the inheritable foam cell reticulosis of mice discovered by Lyons, Hulse, and Rowe have been reexamined. The major abnormality in thymuses from homozygous-abnormal animals has been identified as an increase in the concentration (per milligram of protein) of sphingomyelin and cholesterol. This increase is associated with normal sphingomyelin-cleaving activity. The lipid compositions of the liver and spleen in the homozygous-abnormal animal and of the thymus in the heterozygous-abnormal mouse are normal.

The disorder appears to be chemically analogous to those forms of human sphingomyelin lipidosis (Niemann–Pick disease) that are not accompanied by a decrease in tissue sphingomyelinase.

SUPPLEMENTARY KEY WORDS inherited · lipid-storage disease · sphingomyelin · cholesterol · thymus · sphingomyelin-cleaving enzyme · lysolecithin · Niemann–Pick disease

In 1965 Lyon, Hulse, and Rowe discovered an inheritable foam cell reticulosis in CBA mice (1). Animals homozygous for the *fm* gene invariably died within 100–180 days. The postmortem appearance was characterized by severe inanition, and enlargement and opaque-white coloration of the thymus and Peyer's patches. These organs, and occasionally the lymph nodes, uterus, lungs, liver, spleen, and muscles, contained foam cell infiltrates. The authors reported that the cholesterol content of the thymus was increased and that, whereas the total amount of phospholipid was normal, the proportion of lysolecithin was abnormally high.

This report describes further studies of this disorder, which indicate that the major change in tissue phospholipids is an increase in the content of sphingomyelin, and suggests that the disease may be a biochemical analogue of the form of Niemann–Pick disease that is not accompanied by sphingomyelinase deficiency.

## MATERIALS AND METHODS

### *Mice*

Original *fm* stock was generously supplied by Dr. Mary Lyon. The first chemical studies were performed on affected homozygotes received from the Atomic Energy Research Establishment, Harwell, England. All subsequent determinations have been made in members of $F_2$–$F_{11}$ generations bred at Bethesda. Potentially affected animals were segregated at 90 days; those showing signs of this disease were killed by ether anesthesia at 110–130 days. Phenotype was assigned in the following manner. Animals were judged to be homozygous-abnormals (*fmfm*) from the macroscopic pre- and postmortem changes previously described as typical (1). Heterozygous-abnormals (*fm* +) were parents of at least one *fmfm* offspring, and normals (++) were animals from collateral sublines maintained free of the disease.

### *Tissues*

Organs were removed immediately after the mice had been killed, washed in isotonic saline, and either stored at −20°C or homogenized immediately for enzyme assays (2). Weights were affected by dehydration during storage and all values were referred to tissue protein content.

*Chemicals*

Sphingomyelins employed for chromatographic standards included bovine spinal cord sphingomyelins obtained from Applied Science Laboratories, Inc., State College, Pa. (lot No. 3, 67-40) and from Pierce Chemical Co., Rockford, Ill.; (methyl-$^{14}$C)-sphingomyelins isolated from tissue cultures of fibroblasts from a patient with Niemann–Pick disease which were incubated with methionine-methyl-$^{14}$C and choline-methyl-$^{14}$C; and both unlabeled and methyl-$^{14}$C-*N*-palmitoylsphingosyl phosphorylcholine, synthesized and kindly supplied by Dr. Julian Kanfer. The synthetic sphingomyelin-$^{14}$C had a specific activity of 0.16 mc/mmole and was also used as the substrate for enzyme assays. On thin-layer plates the mixed sphingomyelin standards formed two closely adjacent bands, the lower of the two showing the same $R_f$ as the synthetic palmitoyl sphingomyelin. Lysophosphatidyl choline was obtained from Pierce Chemical Co. (batch No. 13558).

*Chemical Analyses*

20–50 mg of tissue was homogenized in 3 ml of ice-cold saline for 10 min in a Lourdes homogenizer with microattachment, and then for 5 min in a hand-driven glass–Teflon homogenizer. The homogenate was made to a volume of 5 ml and a 0.5 ml aliquot was taken for protein analysis (3). The remaining 4.5 ml was shaken with 95 ml of chloroform–methanol 2:1 for 5 min. 20 ml of 0.003 N $CaCl_2$ solution was added and the phases were separated overnight at room temperature. The chloroform phase was removed, dried under nitrogen at 37°C, taken up in chloroform, and passed through a fine sintered-glass filter. The extracts were stored at −20°C.

*Phospholipids.* One-dimensional thin-layer chromatography was carried out on layers of Silica Gel G, 500 nm thick. For quantitative two-dimensional separation of phospholipids, plates coated with Silica Gel F/254 (250 nm thick) from Brinkman Instruments, Inc., Westbury, N.Y. were employed. These plates were washed with developing solvents and activated at 110°C for 40 min immediately before application of the sample. Extracts containing 125–250 μg of lipid were applied and the plates were sequentially developed in chloroform–methanol–15 N ammonium hydroxide–water, 180:105:7.5:7.5 and 120:160:5:5 (v/v). After they had dried in air, the plates were sprayed with anisaldehyde (1 ml in 2 ml of concentrated $H_2SO_4$ and 97 ml of glacial acetic acid) (4). The zones of phospholipids were scraped from the plate and their phosphorus content was determined by Bartlett's procedure (5).

*Other Lipids.* The composition of less polar lipid components was examined qualitatively by one-dimensional thin-layer chromatography. After development the plates were dried and sprayed with the anisaldehyde solution (4). Cholesterol in tissue extracts was determined by the AutoAnalyzer (6). Total neutral glycolipids were isolated from the chloroform phase of the lipid extracts by the method of Vance and Sweeley (7) and the total reducing sugars were determined by the phenol–sulfuric acid method of Dubois, Gilles, Hamilton, Rebers, and Smith (8). The results were expressed as μg of galactose/100 mg of protein. Triglycerides were determined by the fluorometric AutoAnalyzer method (9) on the chloroform eluates from the chromatographic separation of Vance and Sweeley.

*Enzyme Assays*

Sphingomyelin-cleaving activity was determined by the method of Brady, Kanfer, Mock, and Fredrickson (2) with sphingomyelin-$^{14}$C as substrate.

*Radioactivity*

Samples were assayed for radioactivity in a Packard scintillation spectrometer; quench correction was made by the automatic external standard technique with the channels ratio method.

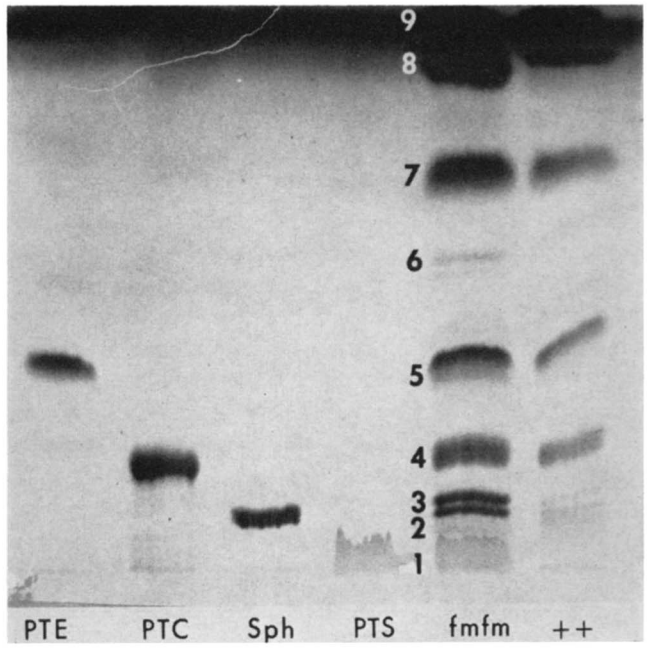


FIG. 1. Thin-layer chromatogram of polar lipids in thymus glands from homozygous-abnormal (*fmfm*) and normal (++) mice. The loads represent extracts equivalent to identical amounts (0.50 mg) of tissue protein. Solvent, chloroform–methanol–water 195:75:12. The standards are: PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; Sph, sphingomyelin; PS, phosphatidyl serine. The compounds in the tissue extracts are identified as: (*1*) PS; (*2*) lysophosphatidyl choline and phosphatidyl inositol; (*3*) sphingomyelins; (*4*) PC; (*5*) PE; (*6*) cerebrosides; (*7*) free fatty acids; (*8*) cholesterol; and (*9*) cholesteryl esters and triglycerides. The solvent front was 15 cm from the origin. The plate was stained with anisaldehyde reagent (4).

TABLE 1 THYMUS PHOSPHOLIPIDS IN DISEASED (*fmfm*) AND NORMAL (++) MICE

| Phospholipid Class | *fmfm* % of total P* A | B | C | *fmfm* Mean | ++ % of total P* D | E | F | ++ Mean |
|---|---|---|---|---|---|---|---|---|
| | | | | *μg/mg protein* | | | | *μg/mg protein* |
| Sphingomyelin | 22.1 | 28.2 | 16.4 | 66.8 | 5.9 | 5.9 | 6.0 | 9.4 |
| Phosphatidyl choline | 37.5 | 21.9 | 29.8 | 89.4 | 37.8 | 30.7 | 36.2 | 55.4 |
| Phosphatidyl ethanolamine | 23.3 | 11.3 | 18.2 | 53.0 | 24.9 | 19.5 | 21.5 | 35.0 |
| Phosphatidyl serine | 9.1 | 10.3 | 9.8 | 29.2 | 10.6 | 12.1 | 10.3 | 17.5 |
| Phosphatidyl inositol | 3.5 | 11.3 | 7.5 | 22.3 | 7.9 | 10.7 | 10.9 | 15.6 |
| Lysophosphatidyl choline | 0.0 | 3.4 | 2.3 | 5.7 | 1.5 | 3.4 | 2.4 | 3.8 |
| Other† | 4.3 | 12.3 | 10.3 | 34.3 | 13.3 | 17.6 | 13.7 | 22.1 |
| Total | | | | 300.7 | | | | 158.8 |

Samples A–F each represent extracts of two thymuses from male or female mice 113–119 days old.
* Per cent of phosphorus recovered from plates (average recovery = 90% of load).
† Includes P at origin and front and unidentified spots.

## RESULTS

### LIPID ANALYSES

#### *Thymus*

*Homozygotes.* One-dimensional chromatography of thymus lipids revealed three major differences in tissues from the homozygous abnormal (*fmfm*) mice compared to normals (++). These were apparent increases in total phospholipids and cholesterol per unit weight of protein, and in the relative content of sphingomyelins (Figs. 1 and 2). As judged from the appearance of the plates, the amounts of triglycerides were variable; but they appeared to be lower in the *fmfm* glands (Fig. 2), which perhaps accounts for the firmer and less fatty appearance of these glands.

*Phospholipids.* The quantities of total phospholipids and of the several phospholipid classes found in three separate analyses of a total of six thymuses from *fmfm* and ++ mice are shown in Table 1. Sphingomyelin comprised an average of 22% of the total phospholipids recovered from *fmfm* tissues compared to 6% in the controls. When the sphingomyelin content was compared for tissue protein, the difference was sevenfold. Variance between the small number of mean values in each group was considerable, but the difference between the means was nevertheless significant at the level of $P < 0.05$.

The relative concentrations of all other phospholipid classes, based on glandular protein content, were modestly increased in the *fmfm* tissue as well. These increases were internally proportional and probably related to a general change in lipid content relative to protein in the *fmfm* glands compared to the normals.

*Further Identification of Sphingomyelin.* The material in the *fmfm* tissues identified as sphingomyelin in the thin-layer chromatographic separations was invariably stained by anisaldehyde in the same manner as the sphingomyelin standards; it gave a blue color unlike that for any of the other phospholipids. Several further tests were applied to confirm the identity of this material.

Firstly, lipids from several *fmfm* thymuses were chromatographed and the spots identified as sphingomyelins, lecithins, and lysolecithins were eluted. Their concentrations were separately determined and then the samples were combined. The mixture contained 2.76, 3.29, and 0.22 μg P attributed to sphingomyelins, lecithins, and lysolecithins, respectively. After hydrolysis in 1 N KOH at 37°C for 24 hr, 2.32 μg of lipid P remained. On rechromatography only a sphingomyelin spot was visible; it gave the characteristic blue color with anisaldehyde.

Secondly, the sphingomyelin spot from a similar thin-layer separation of *fmfm* thymus lipids was eluted and an aliquot was used for determination of sphingosine by the Sweeley modification (10) of the method of Lauter and Trams (11). The theoretical amount of sphingosine in the *fmfm* sample, based on initial phosphorus determinations, was 0.021 μmole. The yield was 0.019 μmole.

Thirdly, the sphingomyelin from several similar thin-layer separations of *fmfm* thymus lipids was eluted and its infrared spectrum was obtained in a Perkin–Elmer model 421 Infrared spectrophotometer. A Perkin–Elmer micro cell (0.454-mm path length) contained the sample dissolved in chloroform; the reference cell was a macro cell (0.455-mm path length). Spectra were also obtained in potassium bromide (KRr) pellets. The IR spectrum of the material in the *fmfm* tissues that was presumed to be sphingomyelin was identical to that of standard sphingomyelin. Specifically, the characteristic 1640 $cm^{-1}$ amide I band was present and the 1735 $cm^{-1}$ ester band was absent.

Finally, a sample of the lysolecithin standard was

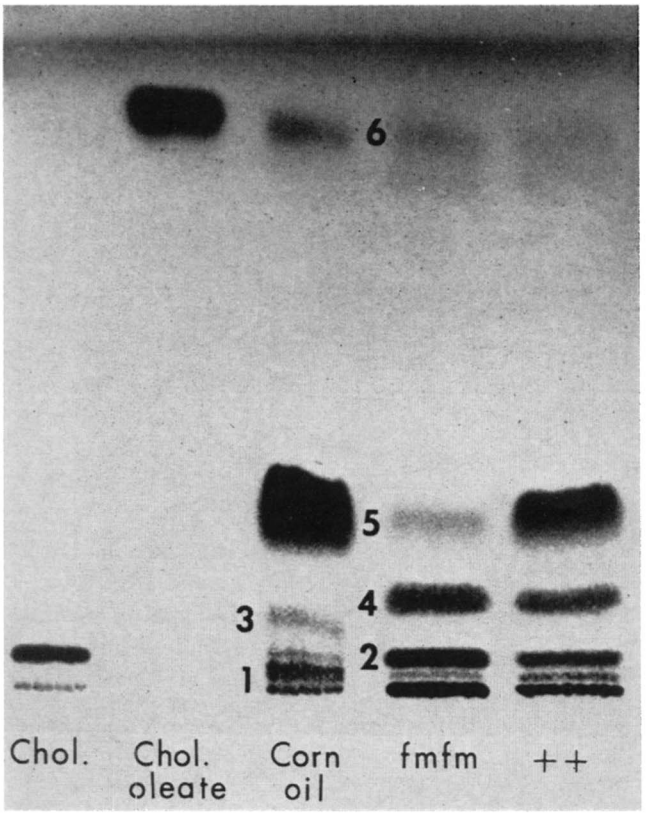


FIG. 2. Thin-layer chromatogram of less polar lipids in thymus glands from homozygous-abnormal (*fmfm*) and normal (++) mice; the amounts of extract applied each corresponded to 0.5 mg of tissue protein. Solvent, petroleum ether–diethyl ether–glacial acetic acid 90:10:1. The standards are: chol., cholesterol; cholesteryl oleate; and corn oil. The compounds in the tissue extracts and corn oil are identified as (*1*) monoglycerides; (*2*) cholesterol; (*3*) diglycerides; (*4*) free fatty acids; (*5*) triglycerides; and (*6*) cholesteryl esters. The solvent front was 14 cm from the origin. The plate was stained with anisaldehyde (4).

cochromatographed with synthetic sphingomyelin-$^{14}$C on a two-dimensional thin-layer plate. The lysolecithin and sphingomyelin spots were identified, separately scraped off and eluted, and assayed for radioactivity. Of the total radioactivity recovered, 99% was in sphingomyelin and 0.5% in lysolecithin.

These results indicated that most, if not all, of the phospholipid in the *fmfm* tissues having an $R_f$ like that of several sphingomyelin standards was sphingomyelin, without significant contamination by lysolecithin.

*Other Lipids.* Total cholesterol was determined only in the extracts A and D in Table 1. In the *fmfm* animals the content was 150 μg/mg of protein and in the controls, 51 μg/mg. Total neutral glycolipids were determined on extracts of four pools of thymus tissue from *fmfm* animals and four pools from ++ mice. Each pool contained approximately 100 mg wet weight of thymus. The *fmfm* thymuses contained 7.7 μg of galactose/mg of protein (range, 5.7–9.1) and the ++ thymuses 4.5 (range, 2.9–6.1). The modest increase in the content of total neutral glycolipids, referred to tissue protein, in the *fmfm* mice was similar to the increases in phospholipids other than sphingomyelin (Table 1); we believe it to be nonspecific. Triglycerides were measured on the same pools. The mean concentrations, in μg/mg of protein, were *fmfm*, 45 and ++, 210; this confirmed the impression from thin-layer chromatography of a higher glyceride content in the normals.

*Heterozygotes.* Phospholipids in thymus glands from heterozygotes were also analyzed in the same manner as for the homozygotes for which the results are shown in Table 1. The heterozygotes, however, were 213–348 days old both males and females, and the controls were selected from the same age range. The mean values obtained on separate analyses of paired organs from these *fm* + and ++ animals, respectively, were as follows: the percentages of the total phospholipids represented by sphingomyelin, 5.0 and 6.3; the concentrations (in μg/mg of protein) of sphingomyelin, 9.4 and 11.6, of total phospholipids, 188 and 184, and total cholesterol, 58 and 84. It was concluded that considerably larger numbers of animals would be required to demonstrate in the older heterozygotes any significant departure from the normal content of thymus phospholipids. These data do not exclude the possibility that a more readily detectable abnormality might be present in younger heterozygotes.

### *Liver and Spleen*

These organs are not enlarged in the *fmfm* animals and are not always infiltrated with foam cells. This is in contrast to Niemann–Pick disease and similar disorders in man in which the spleen, at least, is invariably enlarged. There were no significant differences in the content of major lipid classes in the livers and spleens from affected and control animals (Table 2). The total neutral glycolipids were determined on other samples of liver from five *fmfm* and five ++ mice. The mean values and range for the *fmfm* were 2.7 (2.3–3.1) μg of galactose/mg of protein and for ++, 2.0 (1.9–2.2).

## ENZYME ANALYSES

Sphingomyelin-cleaving activity was determined in the thymus, liver, and spleen. The mean values for duplicate determinations on each organ from three *fmfm* and three ++ animals, respectively, were as follows: thymus, 8.2 and 8.5; liver, 25.8 and 23.4; and spleen, 11.2 and 10.7 (mμmoles of sphingomyelin cleaved/mg of protein per hr). There was therefore no evidence of deficient activity of the enzyme in the thymus, which is chemically the most affected tissue, or in two other organs that are occasionally affected.

TABLE 2 LIPIDS IN LIVER AND SPLEENS OF *fmfm* AND CONTROL MICE

| Lipid | Liver* | | | | Spleen† | | | |
|---|---|---|---|---|---|---|---|---|
| | *fmfm* | ++ | *fmfm* | ++ | *fmfm* | ++ | *fmfm* | ++ |
| | *% of total phospholipids* | | *μg/ml protein* | | *% of total phospholipids* | | *μg/ml protein* | |
| Cholesterol | | | 53 | 49 | | | 57 | 49 |
| Total phospholipids | | | 190 | 238 | | | 68 | 91 |
| Sphingomyelin | 8.0 | 5.5 | 148 | 131 | 12.9 | 11.0 | 87 | 98 |
| Phosphatidyl choline | 27.3 | 34.5 | | | 30.4 | 27.5 | | |
| Phosphatidyl ethanolamine | 10.4 | 12.1 | | | 14.8 | 16.0 | | |
| Phosphatidyl serine | 9.9 | 9.3 | | | 12.9 | 16.7 | | |
| Phosphatidyl inositol | 8.0 | 8.2 | | | 10.1 | 9.1 | | |
| Lysophosphatidyl choline | 12.3 | 10.6 | | | 1.8 | 1.7 | | |
| Other‡ | 25.2 | 19.9 | | | 17.2 | 18.2 | | |

* Mean of values from five mice of both sexes 113–119 days old.
† Mean of values from four mice of both sexes 113–119 days old.
‡ Includes P at origin and front and unidentified spots on thin-layer chromatogram.

### PLASMA LIPOPROTEINS

Pooled samples of blood were collected from *fmfm* and control mice, with EDTA (1 mg/ml of blood) as anticoagulant. The paper electrophoretic patterns of plasma lipoproteins were not grossly different. Each pool was also subjected to immunoelectrophoresis (12), against human anti-$\alpha$ and anti-$\beta$ sera, which cross-react with mouse lipoproteins. The *fmfm* plasma contained both $\alpha$- and $\beta$-lipoproteins and the amounts did not differ grossly from those in controls.

## DISCUSSION

The present studies indicate that the thymus, the tissue most heavily involved in the foam cell reticulosis of mice, shows an absolute increase in the content of sphingomyelins, cholesterol, and total phospholipids in this disease. We have no explanation for the discrepancy between our chemical findings and those reported by Lyon, Hulse, and Rowe. The differences revolve mainly around the identification of lysolecithin and sphingomyelin, for which they employed one-dimensional paper chromatography. We also repeated their extraction procedure, which employed hot ethanol–ether (1), but the phospholipid compositions obtained in both *fmfm* and control thymuses were similar to those obtained with chloroform–methanol extraction. The data leave little doubt that the metabolism of sphingomyelin rather than lysolecithin is in some way affected in this genetic disorder.

The similarities between the sphingomyelin–sterol lipidosis in mice with similar disorders observed in man are of considerable interest. Current information suggests that Niemann–Pick disease, as it was first described (13), represents only one of several similar lipid storage diseases (14). In each of these, foam cells appear in many tissues and organs, most abundantly in those rich in reticuloendothelial cells. In man the thymus, Peyer's patches, lymph nodes, and uterus, as well as the thyroid and parathyroids, are involved as they are in the *fmfm* mouse.[1] On the other hand, the liver, spleen, brain, and eyes,[1] little if at all affected in the *fmfm* mouse, are nearly always heavily involved in classical Niemann–Pick disease and frequently in other forms of human "sphingomyelin lipidoses."

In affected humans all of the involved organs have an increased content of both sphingomyelin and cholesterol. The latter is largely nonesterified, as it is in the *fmfm* thymus (Fig. 2). In the mouse thymuses we examined, the excess of cholesterol was roughly three times the excess of sphingomyelin on a molar basis. This is a greater relative preponderance of cholesterol than is found in the spleen in most cases of Niemann–Pick disease, but similar stoichiometry has been seen in some patients (14). The percentage of tissue phospholipids represented by sphingomyelin in Niemann–Pick disease varies considerably, and may be as much as 60% (14).

Patients with sphingomyelin lipidoses are subject to further clinical and possibly biochemical classification (15, 16). The rapidly fatal infantile form (Crocker's "Type A" or classical Niemann–Pick disease), and a form seen in older children who have severe visceral changes without evidence that the central nervous system is affected ("Type B"), have deficient sphingomyelinase activity in liver or spleen (2, 17). Older patients who may have much less visceral involvement and only late nervous system manifestations ("Type C") and possibly a Nova Scotia variant ("Type D") either may have no sphingomyelinase deficiency or a lesser degree than the other types (17).

If *fm* reticulosis in mice is analogous with one of these, it is one of the forms not associated with clear-cut

[1] We are grateful to Dr. Harold DeBlanc and Dr. Vernon Wong for histochemical examination of mouse tissues.

sphingomyelinase deficiency. It is not known why the disease is uniformly lethal in mice, or whether the involvement of different tissues both in the mouse and in man may be due to basic differences in the diseases or to a more general difference between species in tissue responses to lipid storage.

More can be learned from the *fm* mouse in regard to the above questions and to possible abnormalities in other biochemical pathways, including the synthesis of sphingomyelin and cholesterol. There is also the possibility that further study of the *fm* mouse may provide methods for altering the expression of similar lipidoses in man. As one of the relatively rare animal models of genetically determined diseases of lipid metabolism, the strain deserves continuation and further study. Sufficient animals are now available at the National Institutes of Health to renew the original generous offer of the Harwell Laboratory to make the stock available to those interested in further studies of this disease.[2]

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[2] Those interested should contact Dr. Carl T. Hansen.

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