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THE FATTY ACID COMPOSITION OF COD LIVER OIL

by

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INTRODUCTION

The chemical composition of cod liver oil has been quite extensively studied, but due to the complexity of the problem detailed information has emerged only recently. Novel analytical principles have been applied resulting in greatly extended knowledge. The present paper gives the result from investigations at our laboratory on the fatty acid composition of cod liver oil. Since the development of gaschromatographic methods a fairly large number of investigations on this problem has been reported. Based on these investigations it is now possible to establish reliable values for the fatty acid composition of this important fish oil. The status of the investigations further justifies a review of the historical aspects together with a critical evaluation of the results from the more recent studies.

HEYERDAHL (1895) has compiled a chronological synopsis of the early chemical researches on cod liver oil. He found that the first chemical examination apparently was made by WURZER in 1822. Of more interest in the present connection is that SPAARMANN (1828) in a study on cod liver oil claimed that 97 per cent of the oil consisted of the fatty acids: margaric acid (17%), oleic acid (74.5%) and delphinic acid (valeric acid) (5.5%). This composition was very similar to that given for most fats investigated at his time. These findings were generally adopted in text-books. Most studies, however, were concerned with other components in order to establish a basis for the pharmacognostic properties and therapeutic principles of cod liver oil. De JONGH (1843) gave a major treatise on this subject.

The first rational studies on the chemistry of cod liver oil were carried out by HEYERDAHL (1895). He understood that the fatty acid composition was more complex than usually assumed, and proved by studies on bromine absorption that fatty acids with more than one double bond were present. He tried to isolate some of these acids and named two of them, jecoleic acid and therapic acid. Unfortunately these acids could not be identified by more advanced techniques. HEYERDAHL (1895) also pointed out that the rancidity of cod liver oil was a result of oxidation of polyenoic acids.

BULL (1899) continued the investigations, and quickly grasped some fundamental principles which had to be taken into consideration. He

proved the presence of several unsaturated acids, and introduced methods of handling which prevented oxidation during the analysis. He found that saltprecipitations and other crystallization methods gave erroneous results because of the complex mixture. He tried fractionation by steam distillation of the free fatty acids, but abandoned this principle for a method based on fractional distillation in vacuum of the methylesters. He designed a column for this work, and his principle and methods dominated completely the field of fat analysis up the 1950's (BULL, 1906). He proved the presence of saturated acids of chain lengths C_{14}, C_{16} and C_{18}, and of the monoenoic acids C_{16}, C_{18}, C_{20} and C_{22} in cod liver oil. The name gadoleic acid, as proposed by BULL (1906), is still applied to the C_{20}-acid, and the C_{16}-acid was named „BULL's acid" for many years. Unfortunately this gifted chemist had to postpone his planned work on polyenoic acids for more trivial duties.

In the following years, up to 1930, no important investigations regarding the fatty acid composition of cod liver oil were reported, as the interest almost totally centred around the discovery of the fat soluble vitamins. Japanese investigators, however, continued the studies of fatty acids in other marine oils, and isolated and named a series of polyenoic acids. The complexity of the problem led in many cases to wrong results and conclusions. In the 1930's the school of T. P. HILDITCH (1940) revived the analytical studies in the fat field. They applied group separation followed by fractional distillation of the methyl esters. They further introduced systematic and thorough calculations of the many fractions obtained, and gave important contributions to the identification and characterization of the different fatty acids. They also investigated samples of cod liver oil, and one of these analyses is discussed below (LOVERN, 1942).

The introduction of the spectrophotometer resulted in the development of spectrophotometric methods for the analysis of fatty acids based on the alkaline isomerization of the double bonds to conjugated positions, resulting in characteristic U.V.-spectra. The method was simple, and therefore appealing, but it was soon found that the results were based on a set of empirical equations which needed proper standards. Such standards did unfortunately not exist for the polyenoic acids in cod liver oil. Any errors from the highly unsaturated groups were multiplied through the steps of calculation. The principle was, however, widely accepted in the 1950's. As an example of the results obtained by this method a study by NOTEVARP et al. (1954) has been discussed below.

In 1957 gaschromatographic methods were introduced for the analysis of fatty acids. These methods led to a rapid progress in fat research, and have extended our knowledge in this field. Gaschromatography is

today the preferred method of analysis. For biochemical and medical reasons the interest in marine oils has increased in later years. Nine results of gaschromatographic analyses of cod liver oil are compared in detail with the present findings, and the average composition of fatty acids in cod liver oil is given.

METHODS

Preparation of methyl esters.

Five g of the oil was saponified for 30 min. with 40 ml methanol (aldehyde free) + 5 ml 60% (W/W) potassium hydroxide in the presence of pyrogallol and ascorbic acid for protection against oxidation. The methanol was quickly removed under vacuum, thus a cooling of the mixture took place. The saponification mixture was transferred to a separatory funnel with 100 ml ethyl ether (peroxide free) and 100 ml distilled water. The unsaponifiable matter was extracted into the ethyl ether, and the extract washed with 30 ml distilled water to which was added 1 ml KOH-solution. The water phase was added to the soap solution, and the ether phase discarded. 50 ml of 15% (v/v) hydrochloric acid was added to the soap solution. The free fatty acids were extracted three times with 50 ml ethyl ether, and the combined extracts washed four times with 50 ml distilled water. The ethyl ether was evaporated in vacuum at approx. 40° C. 20 ml of 12% borontrifluoride (FLUKA) in methanol was added quickly. The solution was boiled for five minutes, transferred to a separatory funnel with 25 ml distilled water, and extracted three times with 25 ml ethyl ether. The combined extracts of methyl esters were washed four times with 25 ml distilled water, and the ethyl ether evaporated in vacuum at low temperature. The methyl esters were dissolved in a few ml of hexane (B.p. 67–72° C), and oxidation products removed by passing this solution through a column (ϕ 12 mm) of soft alumina (40 g Al_2O_3 Brockmann + 10% water). Elution was carried out with 50 ml hexane which was removed in vacuum at low temperature. The esters were dissolved in methylhexanoate (about ten times their weight), and stored in the freezer.

Hydrogenation.

To an aliquot of the hexanoate solution, usually 3 ml, was added a pinch of palladium on activated carbon (FLUKA) as a catalyst. The mixture was shaken in a small flask for 3 to 4 hrs. under approx. 20 p.s.i. of hydrogen, and filtered.

Thin layer chromatography.

Glas plates (20 × 10 cm) were prepared with 15 g "Kieselguhr G" (Merck) suspended in 15 ml distilled water. The plates were dried at 120° C for 3–4 hours. Sets of nine plates were used in the present study. They were impregnated with paraffin (b.p. 180–200° C). A 10% paraffin solution in pentane (b.p. 45–50° C) was allowed to ascend to within 2.5 cm from the top, the pentane was evaporated in the air. 7–8 spots of 5 µl of the methyl esters solution were applied to the nonimpregnated parts of the plates. The elution was carried out with a mixture of acetonitrile (Merck): isopropanol: water (80 : 15 : 5) saturated with paraffin prior to use. After about 45 min. elution, the plates were dried under nitrogen at a slightly elevated temperature, and developed by spraying with a solution of 0.001 per cent of sodium fluoresceinate in water. The spots were plotted in UV-light, carefully removed by scraping them off the plates and transferred to centrifuge tubes containing 20 ml hexane and 10 ml distilled water. The tubes were shaken, centrifuged and frozen. The hexane phases were collected, concentrated in vacuum and transferred to small tubes with conical bottoms. The rest of the hexane was removed by a current of nitrogen, and the methyl esters stored dissolved in the accompanying paraffin.

Calculation of results.

Each peak was quantitated by calculation of the area using the formula: height x width at half height. The trienes, tetraenes and pentaenes, with the exception of $C_{18:4}$ and $C_{20:5}$, were recorded as two peaks. This was assumed to be caused by the presence of positional isomers.

The chromatograms from the fully hydrogenated fraction were used for the calculation of chain length percentages and the percentages of odd-numbered and branched fatty acids. The sum was calculated to 99.0%, thus giving 1.0% as the sum of uncalculated peaks.

From each of the six fractions obtained by thin layer chromatography, a major fatty acid peak was chosen as representing the fraction. In the present investigation these fatty acids were: $C_{22:1}$ for fraction 1, $C_{20:1}$ for fraction 2, $C_{18:1}$ for fraction 3, $C_{14:0}$ for fraction 4, $C_{18:3}$ for fraction 5, and $C_{22:6}$ for fraction 6.

These six peaks were quantitated on the chromatograms of the original methyl esters to obtain relative values between the thin layer fractions. The peaks from the chromatograms of each of the thin layer fractions could then be calculated on the basis of their representative fatty acid. All areas were summed up according to the relations found in the chromatograms of the original methyl esters.

Lastly the acids from each chain length were summed up and corrected according to values found in the chromatograms of the fully hydrogenated fraction.

All methods are discussed in details by LAMBERTSEN (to be published).

RESULTS AND DISCUSSION

For the sake of convenient discussion the results of the investigation have been tabulated in direct comparison with literature data.

The methods applied before the introduction of gaschromatography gave results which lacked in detail. In Table 1 are compared the findings of LOVERN (1942) and of NOTEVARP et al. (1954) with present results calculated into parallell groupings. It is apparent that these older methods gave results of the same order, but neither gave values for single acids. The method applied by LOVERN (1942) showed generally lower unsaturation compared to the present finding. Most probably this arose from oxydation loss during the prolonged distillation time. This drawback of the method was greatly improved by modern vacuum-distillation techniques ("Spinning band" columns etc.) but in the meantime gaschromatography took over as the preferred method. The alkali-isomerization method applied on cod liver oil (NOTEVARP et al. 1954) gave good values for average unsaturation.

In Table 2 are reported analyses of the fatty acids found in cod liver based on three samples: The first was prepared by total saponification of the liver. The second was prepared by centrifugation at 25° C of a liver suspension in which the cells were broken down by preliminary freezing. The third sample is a typical production sample from a cod liver oil processing plant. The three samples were analyzed by gaschromatography according to the described method but *without* preliminary thin layer chromatography. The analyses were carried out in 1961, before the introduction of the combined thin-layer/gas liquid chromatography Thus only twenty fatty acids are tabulated compared to fortyone in Table 4, column 10. The fourth column in Table 2 is again a modified presentation of the most recent results for the sake of comparison. The laboratory prepared samples (column I & II) show higher values for the contents of saturated fatty acids, 25.8 and 23.0 per cent as compared with 17.5 and 14.0 per cent for the industrial samples. Most probably this difference can be ascribed to loss of more saturated glycerides during the cold-clarification process which involves filtering at a temperature slightly below 0° C. The difference between 18 : 1 in column III and IV will be discussed below. In general the results in Table 2 are of a

Table 1. *Comparison of the results from fractional vacuum distillation method and from alkali isomerization method with the present analysis by GLC.*

	Lovern (1942)	Vit. lab. (Table 4–10)		Notevarp et al. (1959)	Vit. lab. (Table 4–10)
14 : 0 ...	4%	3.5%	Saturated	18.0	16.3%
16 : 0 ...	11%	8.5%	monoenes	50.0	47.8%
18 : 0 ...	1%	2.0%	dienes	4.0	3.5%
14 : u ...	trace	0.4%	trienes	2.5	2.5%
16 : u ...	11% (2.0H)	9.2% (2.3H)	tetraenes	6.9	4.4%
18 : u ...	27% (2.5H)	26.6% (2.9H)	pentaenes	8.4	12.2%
20 : u ...	27% (5.0H)	23.8% (5.8H)	hexaenes	10.2	12.2%
22 : u ...	19% (7.1H)	21.4% (8.6H)	Others	—	1.1%
Others		4.6%		100.0	100.0%
	100%	100.0%			

Table 2. *The fatty acid composition of cod liver, a coldcentrifuged liver oil, and industrial cod liver oils (Percentage).*

Designation	I Saponified liver	II Frozen liver centrifuged at 25° C	III Production sample	IV Commercial sample
14 : 0	6.1	5.1	3.3	3.5
15 : br	0.6	0.7	0.5	0.5
15 : 0 + 1	0.4	0.4	0.3	0.3
16 : 0	16.7	15.0	11.9	8.5
16 : 1	8.3	8.6	12.3	8.3
16 : 2	0.4	0.7	0.3	0.6
17 : br	0.9	0.8	0.8	0.9
17 : 0 + 1	1.0	1.0	0.8	0.6
18 : 0	3.0	2.9	2.3	2.0
18 : 1	19.7	22.0	27.3	20.6
18 : 2	2.0	2.2	2.0	2.2
18 : 3 + 4	3.2	3.0	2.3	3.8
20 : 1	9.9	9.2	9.3	11.8
20 : 4	0.9	0.8	0.6	0.9
20 : 5	7.6	8.7	8.7	10.1
21 : 5	0.4	0.5	0.4	0.6
22 : 1	5.3	4.4	4.5	6.3
22 : 2 + 3 + 4	0.4	0.4	1.0	1.4
22 : 5	0.8	1.0	0.8	1.5
22 : 6	11.6	11.5	9.7	12.2
Others	0.8	1.1	0.9	4.7
	100.0%	100.0%	100.0%	100.0%

Table 3. *Gas-chromatographic investigations on cod liver oil fatty acids referred to in Table 4.*

Laboratory number	Authors	Country	Type of oil	Remarks
1	Ackman & Burger (1964)	Canada	Laboratory extracted liver	26.5% fat 155 I.V.
2	Century et al. (1963)	U.S.A.	U.S.P. Medicinal C.L.O.	
3	DeWitt (1963)	U.K.	Trawler-produced	Averages of 7 analysis
4	Gruger jr. et al. (1964)	U.S.A.	Laboratory extracted	52.6% fat
5	Hallgren & Stenhagen (1960)	Sweden	No information	
6	Kaufman & Khoe (1964)	Germany	Commercial sample C.L.O.	145.2 I.V.
7	Kingsbury et al. (1962)	U.K.	Commercial sample C.L.O.	
8	Klenk & Eberhagen (1962)	Germany	Commercial sample C.L.O.	159 I.V.
9	Reed (1964)	U.K.	B.P. Medicinal C.L.O.	
10	Present study	Norway	Ph. Nordica Medicinal C.L.O.	168.7 I.V.

precision comparable to studies 2–8 summarized in Table 3. This table summarizes informations regarding the determinations of fatty acids in cod liver oil from the North Atlantic cod (*Gadus morrhua*). The detailed results of these studies are compared in Table 4. The fatty acid composition has been determined by gaschromatography of the methyl esters, but the applied procedures differ in details and instrumentation. The authors are from 6 countries and the laboratories have research background varying from product control to medical research.

A few adjustments were found necessary to compile the literature data in a comparable form. In our opinion the acid $C_{22:1}$ from laboratory 2 was identified erroneously as $C_{20:4}$. These acids normally run parallel in the gaschromatograph. Similarly laboratory 6 has identified $C_{18:4}$ wrongly as $C_{20:0}$. Laboratory 5 did not originally report a value for the $C_{20:1}$ acid. The sum of fatty acids, however, came to 87.5% ,as compared to 100.0% for other samples in the same investigation, and we have assumed that the value 12.5% was lost in the print.

About 15 per cent of the fatty acids of the cod liver oil are saturated. Of these only small amounts of C_{12} and lower acids are known to be present, and only a trace of C_{20} could be ascertained. The main acids are myristic, palmitic and stearic acids as in most natural fats. Certain minor saturated constituents are always present, thus acids with odd num-

Table 4. *The fatty acid composition of cod liver oil.*

Designation	Laboratory (See Table 3)*									
	1	2	3	4	5	6	7	8	9	10
14 : 0	3.5	3.6	2.8	2.8	3.8	2.7	2.3	3.2	4.2	3.5
14 : 1	0.2		0.2			0.5	0.1		0.6	0.3
14 : 2			0.2							0.1
15 : br			0.6		0.5	0.4	0.6		0.4	0.5
15 : 0 + 1 .	0.7		0.3	0.6	0.4	0.4	0.6	0.3	0.3	0.3
16 : br										0.1
16 : 0	10.4	10.1	11.6	10.7	10.2	9.7	11.5	12.4	12.7	8.5
16 : 1	12.2	11.1	8.6	6.9	7.2	8.4	7.8	(4.7)	9.8	8.3
16 : 2	0.9		0.8	1.0	1.3		1.2	0.1	1.1	0.6
16 : 3	0.2				0.7			0.7		0.3
16 : 4	0.1									—
17 : brI			0.7							0.5
17 : brII ...			0.6		0.8	0.7	0.9	1.0	0.6	0.4
17 : 0 + 1 .	0.3		0.3	1.2	1.1	1.0	0.9	1.0	0.9	0.6
18 : br										0.2
18 : 0	1.2	1.2	2.7	3.7	2.2	2.4	3.6	3.0	2.9	2.0
18 : 1	19.6	26.3	25.2	23.9	26.9	20.7	25.6	23.6	26.6	20.6
18 : 2	(0.8)	1.9	2.5	1.5	2.1	1.7	2.7	1.3	1.8	2.2
18 : 3ω6....	0.1		0.7	0.9	0.6	0.9	1.4	1.0	0.8	0.3
18 : 3ω3....	0.1									1.0
18 : 4	(0.7)	—	2.2	2.6	2.1	2.1	2.7	1.3	2.8	2.5
19 : 0 + 1 .	0.1			0.6		0.9		0.3		0.2
19 : 4										0.1
20 : 0	0.1									0.1
20 : 1	14.6	12.6	13.1	8.8	12.5	9.9	11.7	9.4	9.0	11.8
20 : 2	0.2	2.2		0.5	0.3		0.5	0.3		0.3
20 : 3ω6....	0.1		0.9	0.1		0.8			1.1	0.1
20 : 3ω3....	0.1									0.2
20 : 4ω6....	1.2			1.0	0.3	0.5	1.4	0.7	1.0	0.4
20 : 4ω3....	0.5				0.4	0.5		0.3		0.9
20 : 5	(5.0)	(12.9)	9.3	8.0	8.2	10.3	8.2	8.0	10.0	10.1
21 : 5	0.4							2.2?		0.6
22 : 1	(13.3)	4.8	6.3	5.3	7.5	5.1	4.9	5.4	5.0	6.3
22 : 2				1.1		1.2	1.0			0.3
22 : 3ω6....			0.4						0.6	0.2
22 : 3ω3....						3.2				0.4
22 : 4ω6....										0.2
22 : 4ω3....	0.2			0.3			0.5			0.3
22 : 5ω6....	0.1		1.0	1.3	0.8	1.3	1.3	1.3	1.1	0.2
22 : 5ω3....	1.9					1.8				1.3
22 : 6	10.5	11.7	8.7	14.3	10.1	12.3	7.4	(19.0)	6.7	12.2
Other	0.7	1.6	0.3	2.9	—	0.6	1.2	÷0.5	—	1.1

*1. The authors further report the presence of 1.2% fatty acids C_{23}, C_{24} etc., and 0.8% miscellaneous branched chain acids.

2. See text.

4. The authors report 0.5% C_{24} and "odd" or "branched" acids. $C_{16:2}$ also comprises $C_{17:1}$, and $C_{19:0}$ comprises $C_{16:4}$.

5. See text.

6. The authors report the presence of C_8–C_{13} omitted in this table. See also text.

7. The authors report 0.8% C_{10} and 0.4% C_{12}.

8. $C_{21:5}$ has been reported as "C_{20}?", but tabulated after $C_{22:2}$. Corrected here.

bered chains and branched chains have been found, mainly in groups C_{15} and C_{17}. Some of these minor acids may be present as monoenoic acids, but they could not be identified in the present system of analysis. It may be pointed out that acids of the C_{17}-group are quite prominent in all fish oils (LAMBERTSEN, to be published). In cod liver oil this group may amount to 1.5–2.0% of the total.

The major group of acids in cod liver oil are the monoenoic acids of chain lengths 16, 18, 20 and 22, as already proved by BULL (1906). They total about 50 per cent of all fatty acids, and oleic acid represents half of this amount. A comparison of the oleic acid contents in Table 2 and 4 indicate two groups of values, respectively about 20% and 26%. This difference cannot be explained from the material available.

The major polyenoic acids are those of highest possible unsaturation. $C_{18:4}$, $C_{20:5}$ and $C_{22:6}$ comprise 20–25% of the total fatty acids. while the acids $C_{14:2}$, $C_{16:3}$ and $C_{16:4}$, are of less importance. In our system $C_{16:4}$ could not be identified, but its existence has been proved by KLENK & SKINBACH (1959) and others. All other polyenoic acids are present in quite small quantities, from 0.1 to 1%, with the exception of $C_{18:2}$, $C_{18:3}$ and $C_{22:5}$, which were found in amounts between 1 to 2%. From Table 4 may be seen that some trienes, tetraenes and pentaenes are each given in two isomeric forms, $\omega 3$ and $\omega 6$. The thin layer chromatography made identification and separation of these forms possible. ACKMAN & BURGHER (1964) have suggested the same identification by their system with two different polar GLC-columns. They also reported the presence of the two acids $C_{19:4}$ and $C_{21:5}$, and these could be verified by thin layer chromatography in the present study. In straight gaschromatographic methods these acids will be identified as $C_{20:2}$ and $C_{22:3}$. In Table 4 only the laboratories 1 and 10 have used methods which may justify the identification of the minor polyenoic acids.

If all $\omega 6$-acids are considered biologically essential as compared to the structurally related linoleic and arachidonic acids, the content of these acids in cod liver oil is 1.4%. THOMASSON (1953) found in biological tests a value of c. 3.9%, as compared to linoleic acid.

Under the group "other acids" in Table 4, the values from the present study has been calculated to 1.1% by difference. We assume this to be a reasonable figure considering the limits of the applied method. Probably half of these acids are C_{24} and higher acids, as indicated by studies with hydrogenated fats (LAMBERTSEN et al. 1965).

In Table 5 are summarized averages for the eleven major fatty acids in cod liver oil, based on the studies compiled in Table 3 & 4. These acids comprise 90% of the total fatty acids, and should give the main features of cod liver oil. We found it necessary to exclude four of the

Table 5. *Mean values and coefficient of variation for the main fatty acids in cod liver oil based on results from gas-chromatographic studies. (See Table 3).*

Designation	Mean value	Coefficient of variation	Number (n)
14 : 0	3.24%	±18.0%	10
16 : 0	10.78%	±11.9%	10
16 : 1	8.92%	±19.9%	9
18 : 0	2.49%	±35.0%	10
18 : 1	23.90%	±11.4%	10
18 : 2	1.97%	±23.1%	9
18 : 4	2.29%	±21.1%	8
20 : 1	11.34%	±17.3%	10
20 : 5	9.01%	±11.3%	8
22 : 1	5.62%	±16.0%	9
22 : 6	10.43%	±23.9%	9
	89.99%	±19.0%	

$$\text{Coefficient of variation} = \frac{s}{\bar{x}} \cdot 100\%, \quad \text{where } s = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{(n-1)}}$$

values from laboratory 1 from the calculations. The excluded values are quoted in brackets in Table 4. The reason for the discrepancy is probably that the sample analyzed at that laboratory represented the oil from the liver of a single cod. The fat content of this liver was given as 26.5% compared to about 50% in most cod livers. The values from laboratory 8 were obtained by calculations in connection with preparatory work, and this may explain the discrepancy of the values for $C_{16:1}$ and $C_{22:6}$, which also were omitted.

The average values in Table 5 should give a quite reliable picture of the fatty acid composition of cod liver oil. The coefficients of variation average ± 19.0% of the mean values. This variation is reasonable considering the complexity of the analysis and the varying analytical background of the investigators. The high coefficients of variation for $C_{18:0}$ and some of the other acids most probably reflects overlapping peaks in the gaschromatograms, resulting in less precise calculation of areas. A biological explanation of these variations is less probable.

SUMMARY

The fatty acid composition has been determined using group separation by thin layer partition chromatography in conjunction with gas liquid chromatography.

Values are given for 41 acids as percentages of the sum varying between 0.1% and 20.6%.

These results have been compared with the results from nine similar studies reported during the last five years. Mean values have been calculated for the eleven major fatty acids, and coefficients of variation are given.

A fairly uniform fatty acid composition for cod liver oil emerged from these data. The dominating acid is oleic acid $C_{18:1}$ (25%), followed by the five acids $C_{16:0}$ (11%), $C_{16:1}$ (9%), $C_{20:1}$ (11%), $C_{20:5}$ (9%) and $C_{22:6}$ (10%).

A comparison of laboratory extracted oils and commercial cod liver oil has also been reported.

A short historical review is given, and the results of two older different methods of analysis are compared with the present results.

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