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The role of vitamin A on the inhibitors of nonheme iron absorption: Preliminary results

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The interaction of vitamin A and inhibitors of iron absorption from a basal breakfast containing bread from either 100 g of precooked maize flour or 100 g of wheat flour + 50 g of cheese + 10 g of margarine was studied. These breads were labeled with either ^{55}Fe or ^{59}Fe. This basal breakfast was given alone on the first day of the study, and a beverage containing coffee or tea at different concentrations was administered with this breakfast on the following days. In the first three experiments performed, the bread was made from commercially available flours, fortified with iron as ferrous fumarate and vitamins. It can be noticed that whereas the iron absorption from the breakfast containing wheat bread was significantly reduced when given with different concentrations of coffee beverages, the bioavailability of iron from the breakfast containing precooked maize bread remained the same in spite of being administered with increasing concentrations of coffee beverages. The only ingredient present in precooked maize bread and not in wheat bread was vitamin A. In the other experiments, iron and vitamin A were added to the non-fortified precooked maize flour in our laboratory. In presence of vitamin A, nonheme iron absorption from the basal breakfast containing either coffee or tea was not statistically different from the breakfast without coffee, meaning that vitamin A can overcome the inhibition of coffee and tea on iron absorption and also prevents the inhibitory effect of phytates. The high performance liquid chromatography and spectrophotometric studies seem to indicate that during the digestive process, iron and vitamin A form a new prouct or complex, that keeps iron soluble even at pH6. All these data suggest that vitamin A binds iron liberated during digestive process and acts as a quelating agent, keeping iron soluble in the intestinal lumen and preventing the inhibition of polyphenols and phytates on nonheme iron absorption. (J. Nutr. Biochem. 8:61–67, 1997.)

Introduction

Nonheme iron absorption is inhibited by several components: phytates, polyphenols, calcium, manganese, zinc, and fibers. The principal inhibitors are the phytates contained in cereals and legumes[1] and polyphenols found in high concentration in tea and coffee. Among beverages, tea has a high amount of tannines, a polymer of galloyl compounds that show a strong inhibition on nonheme iron absorption either as iron salts or the iron contained in food.[2–4] Coffee, containing mainly cathecol compounds, also inhibits absorption of nonheme iron, but to a lesser extent.[5–6] The fibers play an inhibiting function in a meal;[7] however, the majority of meals containing high concentrations of fiber also contain a high amount of phytates.[8] There are also three minerals that possess inhibiting functions on nonheme iron absorption, they are: calcium when the intake is more than 500 mg in a meal;[9] manganese when the proportion in a meal is more than 300 times that of iron; and finally, zinc, when its content in a meal is 5 times higher than iron.[10]

After our laboratory studied the iron absorption from diets consumed by different socioeconomic strata from the Venezuelan population,[11,12] we continued the studies of in-

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teraction of various micronutrients on iron absorption especially since 1993. At that time, a national program of fortification of precooked maize flour and wheat flours with iron and vitamins began.[13] The present communication deals with iron absorption from a breakfast in which the main ingredient is either precooked maize bread or wheat bread and the effect of vitamin A on this process.

Methods and materials

Ninety four adult subjects, 76 women and 18 men from Valencia City, Impact Sector, voluntarily participated in this study. The Committee for the Protection of Human Subjects from the Venezuelan Institute for Scientific Research approved the studies. These subjects were in apparent good health and some of them presented moderate iron deficiency anemia that did not interfere with their daily work. To each person, in addition to iron absorption determinations, blood was taken to perform hemoglobin concentration PCV,[14] serum iron[15] unsaturated binding capacity,[16] and serum ferritin concentration.[17]

Preparation of meals

Two basal breakfasts were prepared; one with bread from 100 g of precooked maize flour + 10 g of margarine + 50 g of cheese and the other with bread from 100 g of white wheat flour + 10 g margarine + 50 g of cheese. The precooked maize flour and wheat flour had been fortified with iron and vitamins during the industrial process (*Table 1*).

This type of breakfast was used in the first part of the study. In the second part, the meal administered was the same, but bread was prepared from unfortified precooked maize flour + 5 mg of iron as ferrous fumarate.

The vitamin A used was palmite of retinol kindly supply by Roche Laboratories of Venezuela, which was soluble in cold water.

Absorption studies. Four meals were given to each subject. The basal breakfast tagged with ^{59}Fe was given the first day after an overnight fast. The second meal containing basal breakfast tagged with ^{55}Fe + coffee or tea beverage was given in the afternoon of the same first day. Blood was drawn 15 days later to determine the hematologic profile of the subject and to measure radioactivity in blood samples. The subjects were fed again in the morning and in the afternoon of day 15 with basal breakfast + beverages containing different concentrations of coffee or tea. Blood was drawn again on day 30.

Duplicated 10 mL blood samples, together with triplicate samples of the radioactive foods were prepared for radioactive counting using the technique of Dern and Hart.[18,19] Radioactivity was measured in a liquid scintillation counter. Iron absorption from the food was calculated from the radioactivity in the subject's blood using estimated blood volume based on sex, weight, and height.[20]

The administration of radioactive food in the morning after an overnight fast and the afternoon of the same day was based on experiments previously published.[12] Four-hr intervals between meals are sufficient for iron absorption studies.

Chemical analysis. The total iron concentration of foods was determined by digestion method,[21] phytates by the method of Haug and Lantzsch,[22] tannate by the method of Price and Butler,[23] and vitamin A by the method of Strohecker and Heming,[24] modified by Covenin.[25]

Dialyzable iron from maize flour. Dialyzable iron from food was determined using the method of Miller,[26] modified by Hickson and Fairwather (personal communication).

Ten grams of maize flour (enriched or not) were mixed with water to get a homogeneous slurry. The substances studied (tannic acid, catechin, and vitamin A) were added mixed with water. pH was adjusted to 2.0 with HCl 6M and 37 KBq of ^{59}Fe were added and mixed carefully.

After 2-hr pepsin digestion, titratable acidity was determined and a dialysis tube (MW cut off: 12,000 D) containing the amount of $NaHCO_3$ required to adjust the pH to 7.5, was inserted and after 30 min a bile-pancreatin solution was added and digested for another 2 hr. Duplicate samples of pepsin digest, dialysates, and retenates were counted in a gamma counter and results expressed as percent dialyzable iron.

Spectrophotometry and high performance liquid chromatography. For spectrophotometric studies, solutions of vitamin A, $FeCl_2$, $FeCl_3$, and ferrous fumarate were scanned from 220 to 900 nM by UV-VIS spectrophotometer using a Milton-Roy 3000 instrument, to detect the peak of maximum absorbance. Vitamin A and iron salts were disolved in distilled water or in HCl 0.1 N pH2. From these individual solutions, vitamin A-iron combinations were prepared. The spectrophotometric pattern was established for each compound and for combinations of these solutions and analyzed to detect changes in absorbance.

In HPLC experiments, 10 μL samples of 0.01M ferrous fumarate, $FeCl_2$, $FeCl_3$, vitamin A, as well as combinations of these solutions were injected in a HPLC system, Delta Prep 4000 water, equipped with a C-18 column using ethanol/water (20:80) as a mobile phase, at a flow rate of 0.8 mL/min, with monitoring at 220 nM.

Statistical analysis. The *t* test for unpaired and paired data was used. Geometric values and standard error were calculated for all absorption data and serum ferritin concentrations.

Table 1 Enrichment of food vehicles in Venezuela

	Precooked Maize Flour / Kg	White Wheat Flour / Kg
Vitamin A IU	9,500	-
Thiamin mg	3.1	1.5
Riboflavin mg	2.5	2.0
Niacin mg	51.0	20.0
Iron* mg	50.0	20.0

*Ferrous fumarate.

Results

Chemical results

The mean phytate content in the precooked maize flour was 257 mg/100 g and in the white wheat flour was 161 mg/100 g.

The mean of iron content in the fortified precooked maize flour was 6 mg/100 g, and in the white wheat flour was 3 mg/100 g. The ratio of iron to phytate content was 0.023 in the precooked maize flour and in the white wheat flour, the ratio was 0.0186. The ratio of iron to tannin content in coffee powder was 0.0024, and for tea, the ratio was 0.0006 in the experiment with precooked maize flour. The

ratio of iron to tannin in coffee contained in the meal with white wheat flour was 0.0012.

The tannin content in the coffee powder was 2,500 mg/100 g and in the tea, it was 10,224 mg/100 g.

The vitamin A content in the industrialized fortified precooked maize flour ranged from 720 to 1,020 IU/100 g, whereas in the non-enriched precooked maize flour, in which 1,000 IU/100 g were added, there was 806 IU/100 g in the dough, and 558 IU/100 g after baking procedures. When this baked bread was analyzed on the following day, the vitamin A content was 338 IU/100 g.

The mean vitamin A content of the white wheat bread enriched with 1,000 IU/100 g of this vitamin changed into 430 IU/100 g in the dough, 196 IU/100 g after baking procedures and 83 IU/100 g in the baked bread analyzed on the following day.

Iron absorption studies were performed 100 miles away from our laboratory, and the breakfast was administered 1 day after the bread was cooked.

Iron absorption studies

Table 2 shows three absorption studies from the basal breakfast prepared with fortified flour. In the first two, bread was prepared from industrialized enriched precooked maize flour. It can be noticed that there is no significant difference between the iron absorption from the basal breakfast given alone and the basal breakfast given with a beverage containing different concentrations of coffee. This finding contrasts with the other study in which the basal breakfast contained wheat bread. In this study, iron absorption is markedly reduced when the basal breakfast is given with the beverage containing several concentrations of coffee.

The only qualitative ingredient present in precooked maize flour and not in wheat flour is vitamin A. This difference motivated the following experiments. In *Tables 3, 4* and *5* the basal breakfast was prepared with 100 g of non-enriched precooked maize flour +5 mg of iron as ferrous fumarate.

In the experiments in *Table 3,* basal breakfast was given alone in test A, test B contained the basal breakfast prepared with 1,000 IU of vitamin A and administered with 8 g of coffee as a beverage. Finally, the basal breakfast was administered with 8 g of coffee as the beverage in test C. It was noticed that the iron absorption in test A was not significantly different from test B, indicating that vitamin A prevents inhibiting effect of coffee, but such effect was evident when comparing the iron absorption of test B (8.5%) with test C (2%).

Table 4 shows the results of the experiments in which the effect of Vitamin A on phytates contained in precooked maize bread and on 1 g of tea as a beverage was tested. Iron absorption from Tests A and B demonstrated that vitamin A prevented the inhibiting effect of phytate, by doubling the iron absorption in test B. Tea beverages significantly decreased iron absorption from 3.6% to 2.0% when the basal breakfast contained (test C) or not (test D) 1,000 IU of vitamin A. The mean iron absorption of test C should be taken as a base to compare with test D demonstrating the

Table 2 Iron absorption from a basal breakfast containing either precooked maize bread or wheat bread prepared from commercially available fortified flour and with several concentrations of coffee as a beverage

Subjects and sex	Hb g/dL	Serum transferrin saturation (%)	Serum ferritin concent. µg/L	Iron absorption (%) A Basal breakfast given alone	B Basal breakfast + American coffee (2g)	C Basal breakfast + espresso coffee (4g)	D Basal breakfast + cappuccino coffee (4g)	E Basal breakfast + espresso coffee (8g)
Basal breakfast with maize bread								
1) 4M 3F Mean	11.9	20	17	5.1	7.7	8.2	7.8	
SEM	0.2	0.5	1	1.4	1.4	1.4	1.3	
Statistics: A vs B- $P > 0.05$; A vs C- $P > 0.5$; A vs D- $P > 0.05$								
2) 1M 9F Mean	14.3	27	26	4.4	5.3	4.6		3.1
SEM	0.4	1	1	1.3	1.3	1.5		1.5
Statistics: A vs. B- $P > 0.05$; A vs C- $P > 0.05$; A vs E- $P > 0.05$								
Average: Mean 1 + 2	13.3	24	22	4.7	6.1	5.8		
SEM	0.4	2	1	1.3	1.5	1.5		
Basal breakfast with white wheat bread								
2M 8F Mean	12.9	29	28	6.8	1.2	0.4		0.7
SEM	0.8	1	2	1.2	1.4	1.4		1.2

Statistics: A vs B- $P < 0.05$; A vs C- $P < 0.05$; A vs E- $P < 0.05$.

Table 3 Iron absorption from a basal breakfast containing non-enriched precooked maize bread + ferrous fumarate, enriched with vitamin A in tests B and administered with coffee beverage in tests B and C

Subjects and sex	Hb g/dL	Serum transferrin saturation (%)	Serum ferritin concent. µg/L	Iron absorption (%) A Basal breakfast given alone	B Basal breakfast + 1000 IU vitamin A + espresso coffee (8 g)	C Basal breakfast + espresso coffee (8g)
1M 17F	12.5	25	13	5.8	8.5	2.0
	0.2	1	1	1.1	1.2	1.2

Statistics: A vs B- $P < 0.05$; C vs D- $P < 0.05$.

role of vitamin A in preventing the inhibiting effect of polyphenols. Further comments about this point will be addressed in the discussion section.

Table 5 summarizes the behavior of iron absorption from a basal breakfast containing bread prepared from commercially fortified wheat flour. In the first experiment vitamin A was incorporated into the flour whereas in the second experiment, 1,000 IU of vitamin A were dissolved in 20 mL of water, and swallowed slowly while eating the breakfast. In the first experiment, vitamin A is ineffective because iron absorption was reduced about half when the breakfast was administered with espresso coffee. In the second experiment, iron absorption from the basal breakfast showed a significant increase in 14 subjects from a mean of 6.9 to 8.3%. The small difference between tests A and B was significant because in only 1 subject out of 14, iron absorption was higher in test A than in test B.

Dialyzable iron from maize flour

After several attempts to employ the in vitro assay to demonstrate the effect of vitamin A preventing the action of inhibitors on iron absorption, we found that dializable iron from non-enriched precooked maize flour was 15%, whereas from iron- and vitamin A-enriched flour it was 15 to 18%, with vitamin concentrations ranging from 100 to 2,000 IU/10 g flour. Further increases in vitamin A concentrations did not change the amount of dialyzable iron.

HPLC and spectrophotometric studies

In an attempt to find out if there is a direct interaction between iron compounds and vitamin A in food during the digestive process, spectrophotometric and HPLC studies were performed. Changes in absorbance or elution profiles provide evidence of the formation of vitamin A-iron complexes. As shown in *Figure 1*, when 0.01 M solutions of vitamin A and $FeCl_3$, are combined and passed through a C-18 column in the HPLC system, there are important changes in elution profiles. The peak that elutes at 6.93 min, and corresponds to vitamin A (*panel B*), is reduced by almost 30% when compared to the mixture of vitamin A+ $FeCl_3$ (*panel C*). Likewise, there is a new peak at 14.75 min not seen when $FeCl_3$ (*panel A*) or vitamin A are injected

Table 4 Iron Absorption from a basal breakfast containing non-fortified precooked maize bread plus ferrous fumarate, enriched with vitamin A in tests B and C and administered with tea beverage in test C and D

Subjects and sex	Hb g/dL	Serum transferrin saturation (%)	Serum ferritin concent. µg/L	Iron absorption (%) A Basal breakfast given alone	B Basal breakfast + 1000 IU Vitamin A	C Basal breakfast + 1000 IU Vitamin A + Tea (1 g)	D Basal breakfast + Tea (1 g)
5 M 10F Mean	12.7	34	32	3.2	6.3	3.6	2.0
SEM	0.2	2	1	1.2	1.3	1.1	1.2

Statistics: A vs. B- $P < 0.05$; C vs D- $P < 0.05$.

Table 5 Iron absorption from a basal breakfast containing bread from wheat flour enriched with vitamin A in test B and C and administered with coffee beverage (8g)in tests C and D

Subjects and sex	Hb g/dL	Serum transferrin saturation (%)	Serum ferritin concent. µg/L	Iron absorption (%) A Basal breakfast given alone	B Basal breakfast + 1000 IU vitamin A	C Basal breakfast + 1000 IU vitamin A + espresso coffee (8g)	D Basal breakfast + espresso coffee (8g)
1) 2M 18F Mean	13.3	28	28	4.1		1.8	2.0
SEM	1.3	1	1	1.2		1.2	1.2
Statistics: A vs C- $P < .05$; A vs D- $P < -05$							
2) 2M 12F Mean	12.7	28	27	6.9	8.3		
SEM	1.3	2	1	1.3	1.2*		
Statistics: A vs B- $P < .05$							

*Vitamin A was administered as a beverage with the cooked bread.
The first line of each experiment corresponds to the geometrical mean value and the standard error for the second line.

separately. There are also changes in the elution patterns of the peaks eluting first in the chromatogram.

Regarding spectrophotometric measurements (*Figure 2*), there are changes in absorbance when we measure $FeCl_3$ and vitamin A as compared to combinations of these two solutions. For example, $FeCl_3$ has a maximum absorbance at 299 nM and vitamin A at 372 nM; when both solutions are combined the new maximum is at 311 nM. When the same experiments were done with ferrous fumarate, there was a maximum absorbance at 262 nM for ferrous fumarate, 372 nM for vitamin A and 371 for the combination. These results show the formation of a new product or complex, with a slightly different absorbance than the reactants.

Total iron and radioiron measurements of ferrous fumarate, vitamin A and mixtures of both solutions at pH 2 and 6, were performed to study the effect of vitamin A on iron solubility at pH values that reflect the pH of the gastric and intestinal lumen. Iron and vitamin A solutions were prepared in the same concentrations that were used to prepare the bread. When iron solutions at pH 2 were adjusted to pH 6, iron solubility decreased to only 25% of the iron soluble at pH 2. However, when solutions containing iron and vitamin A at pH 2 were adjusted to pH 6, 88% of the iron remained soluble. These experiments provide indirect evidence of iron-vitamin A interactions in the stomach, preventing iron precipitation at the basic pH of the intestinal lumen.

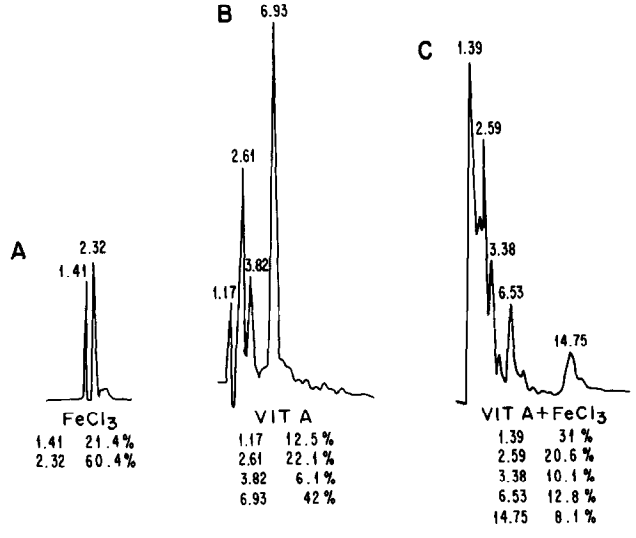

Figure 1. Elution profile of $FeCl_3$, vitamin A, and $FeCl_3$ + vitamin A solutions in HPLC. Left column of each panel represents time in minutes, and the second column represents the proportion of each peak.

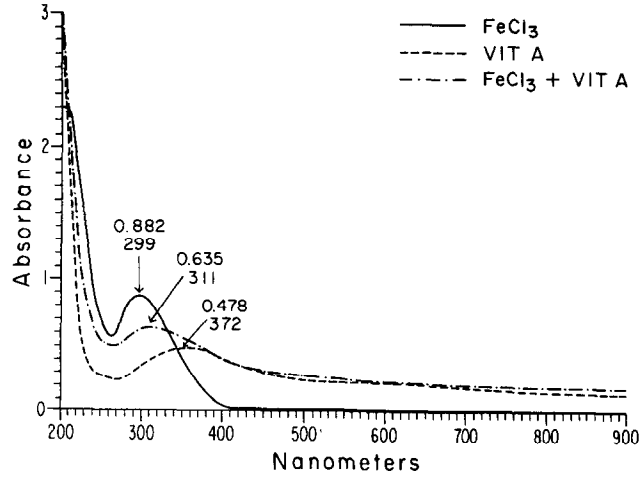

Figure 2. Spectrophotometric measurements of absorbance for $FeCl_3$, vitamin A, and $FeCl_3$ + vitamin A solutions.

Discussion

Vitamin A is an essential nutrient for vision, bone growing, cellular differentiation, reproduction, and integrity of the immune system.[27] It has been suggested that this nutrient is also essential in erythropoiesis. Deficiency of this vitamin results in anemia in humans and animals, which is only revered with vitamin A supplementation.[28,29,30,31,32]

Mejia et al.[33] reported the iron turnover in control and vitamin A-deficient rats using ^{59}Fe. No significant differences in iron absorption were observed in the two groups, but the distribution of radioactive iron in the body was different, the red cell incorporation in deficient rats was 40 to 50% less than the control and conversely, there was significant ^{59}Fe retention in the liver and spleen in vitamin A-deficient animals.

The results of the experiments shown in this article represent new information that deserves further explanation. It is demonstrated that vitamin A in precooked maize bread prevents binding of iron to hydroxyl radicals present in high proportion in phytate molecules. Due to this reaction, the iron absorption was increased about 100% when 1,000 IU of this vitamin reacted with 5 mg of iron as ferrous fumarate + 1 mg of food iron. Iron absorption from non-enriched precooked maize bread was 3.0% and 6.0% in normal and iron-deficient subjects, whereas the iron absorption from precooked maize bread enriched with 1,000 IU of vitamin A/100 g flour was 6.4% and 11.0% in normal and iron-deficient subjects, respectively.

The results also demonstrated that vitamin A prevents the inhibitory effect of polyphenols present in tea and coffee, which otherwise reduced by more than 50% the iron absorption from the breakfast given alone. The differences between mean iron absorption from precooked maize bread enriched with vitamin A and the mean iron absorption from precooked maize bread enriched with vitamin A and administered with tea (*Table 4*) deserve further comments. Such differences in iron absorption could be because during the digestive process, part of the iron contained in the maize bread reacted with vitamin A and the other part with the hydroxyl groups of the polyphenols.

In the case of wheat bread, we have limited information. The first experiment of *Table 5* demonstrated that vitamin A incorporated into the wheat flour did not prevent the inhibitory effect of polyphenols, perhaps due to adverse effect of the yeast and the prolonged high temperature of the baking procedure, which reduced the vitamin A concentration in about 80% of the initial enrichment value.

The second experiment in *Table 5* shows the advantage of giving the vitamin A separately, and slowly swallowing while eating the wheat bread. In this case, the iron absorption showed a significant increase. This preliminary information provided a basis for trying other ways to improve iron bioavailability from wheat bread. Results from in vitro digestion of maize flour showed that the method did not reproduce the effect found in vivo. This could be due to the lack of sensitivity of the method under the conditions used or that iron-vitamin A complexes do not dialyze. We are performing new studies changing membrane cut offs and other experimental conditions trying to reproduce the effect observed in humans.

This unexpected behavior of vitamin A in preventing inhibition of iron absorption by phytates and polyphenols is provocative for an explanation in an attempt to find the mechanism producing this reaction. The fact that vitamin A prevents the effect of these chemical compounds (phytates and polyphenols) that react with the iron through hydroxyl radicals, along with spectrophotometric results, HPLC studies, and the solubility of iron and vitamin A at different pH, suggests that under the conditions of the assays, vitamin A binds iron liberated during the digestive process and forms a complex that acts as a quelating agent preventing the inhibition of the polyphenols and phytates on nonheme iron absorption. This observation agrees with the studies that show that the chemical compounds containing double bonds are capable of reacting with iron.[34] This hypothesis also agrees with the results of Mejia,[30] who demonstrated interaction between vitamin A and iron metabolism. The dramatic reduction of the prevalence of iron deficiency shown in a recent Venezuelan survey is probably due to the effect of vitamin A preventing phytate inhibition, which is present in fortified precooked maize flour.[13]

The poor socioeconomic population living in Asia, Africa, and Latin America have low iron bioavailability from their diets due to the high consumption of cereals, legumes, and tubers.[1,35] The fortification with vitamin A not only prevents this vitamin deficiency, but also potentiates the iron fortification.

As mentioned in the title of this article, these results are preliminary and further research is needed in relation to the effect of vitamin A when the dose is increased, more detailed studies on the effect of this vitamin preventing phytate inhibition in some other cereals and legumes and further information on the in vitro test. Finally, the effect of carotenoids on the inhibition of phytates and polyphenols on iron absorption also needs to be assessed.

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