Table V. Results of Tests for Significance of Differences between Slopes for Different Analytical Procedures[a]

Pigment	Mean square diff between slopes (1)	Pooled within error mean squares (2)	$F = (1)/(2)$[b]
	Diethyl Ether		
$ZnPy_a$	1.6288	0.0920	17.70NS
$ZnPy_b$	0.9337	0.0194	48.13*
C_a	0.0039	0.0035	1.11NS
C_b	0.0432	0.0006	72.00**
Py_at	0.0187	0.0353	0.53NS
Py_bt	0.0718	0.0302	2.38NS
Py_a	0.1724	0.1689	1.02NS
Py_b	0.1545	0.0414	3.73NS
	80% Acetone		
$ZnPy_a$	0.6312	0.0841	7.51*
$ZnPy_b$	0.1180	0.0816	1.45NS
C_a	0.0546	0.0408	1.34NS
C_b	0.0021	0.0813	0.11NS
Py_at	0	0.0234	0 NS
Py_bt	0.0949	0.0262	3.62NS

[a] Pigments in diethyl ether and 80% acetone. [b] NS indicates nonsignificance; * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level.

To evaluate further the instrumental methods of analysis, comparisons were made of the regression coefficients for each pigment based on estimation by spectrophotometry and fluorometry. The results of these comparisons are shown in Table V. With the exception of the difference in slopes for $ZnPy_b$ and C_b in diethyl ether and $ZnPy_a$ in 80% acetone, all differences were nonsignificant. Therefore, the conclusion was drawn that the proposed fluorometric procedure (F) was essentially as reliable as the spectrophotometric method (S) and was suitable for estimation of zinc complexes in diethyl ether and in 80% acetone solutions in a range of concentrations 1/100 that required for spectrophotometric procedures. The large F value for C_b in diethyl ether as shown in Table V is highly significant because of the very small value for "pooled within error mean square" rather than because of a large value for "mean square difference between slopes".

No attempt has been made to estimate pheophorbides, chlorophyllides, and the zinc pheophorbides in mixtures. If proper precautions were taken, the authors would expect that these components of mixtures containing nonphytylated pigment could be estimated with reasonable accuracy by the procedure previously published (White et al., 1963). Pheophorbides are highly subject to loss by adsorption on the laboratory glassware and precautions against such loss should be followed, as previously set forth by White et al. (1972). Also, as indicated earlier, pheophorbides in mixtures subjected to analysis for pheophytins will appear as pheophytins unless the pigment mixture is extracted for pheophorbide removal before the pheophytin estimation step.

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Experimental Formation of Zinc and Copper Complexes of Chlorophyll Derivatives in Vegetable Tissue by Thermal Processing

Ivan D. Jones,* Raymond C. White, Eleanor Gibbs, Lillian S. Butler, and Larry A. Nelson

Zinc and copper complexes of the pigments pheophytins a and b and/or pheophorbides a and b were experimentally formed in green vegetable tissue by prescribed thermal treatments after the addition of zinc or copper salts. Estimates of the pigment components present in 80% acetone extracts of the vegetable tissue before and after treatment are presented. Procedures for such estimates were based on spectrophotometry if copper complexes were absent or on a combined spectrophotometric–fluorometric method if copper complexes were present. The concentration levels for zinc and copper salts at which appreciable complexing occurred were about 10 and 1 μmol per μmol of total pigment, respectively.

The intentional formation of zinc or copper complexes in vegetable tissue to which zinc or copper salts were added has been observed. Fishbach and Newburger (1943) and Fishbach (1943) reported the presence of a zinc-containing pigment in canned okra to which a weak solution of zinc chloride had been added during the can-filling operation prior to thermal treatment. The purpose of the addition of zinc chloride was to reduce or prevent the loss of the typical green color of fresh okra during canning. Schanderl et al. (1965) observed regreening of some stored, thermally processed lots of commercially preserved vegetables. They investigated the experimental formation of zinc and copper

Department of Food Science (I.D.J., E.G., L.S.B.), Department of Chemistry (R.C.W.), and Department of Statistics (L.A.N.), North Carolina State University, Raleigh, North Carolina 27607.

complexes by refluxing pea puree to which zinc or copper was added at levels of 5–100 ppm. They observed that formation of zinc and copper complexes was complete after refluxing 60 and 20 min, respectively.

Thermal treatment of varying intensity is given green plant tissue in the preparation of vegetables for table use and during processing for preservation by canning. Such thermal treatment is responsible for a change in the color of the tissue from the characteristic blue-green of fresh material to a drab olive-brown color typical of most canned green vegetables. Chemically, the color change generally has been considered to be due to the conversion of chlorophylls a and b to pheophytins a and b. Such changes have been observed to occur readily when fresh green tissue, at pH values only slightly less than 7.0, was heated at temperatures near that of boiling water.

Weast and Mackinney (1940) noted marked chlorophyllase activity in some plant tissue subjected to hot water treatments. Maximum activity was reported to be at 75 °C. End products of the action of chlorophyllase on the chlorophylls and pheophytins are chlorophyllides and pheophorbides, respectively. Jones et al. (1963) indicated that in the blanching of some green plant tissue chlorophyllides and pheophorbides were formed from the chlorophylls. Such changes occurred to a considerable or a slight extent, depending upon whether blanching was at 180 °F (82 °C) or 212 °F (100 °C), respectively.

White et al. (1963, 1972) cited a great spectrophotometric and fluorometric similarity between the chlorophylls a and b and the chlorophyllides a and b, respectively, and likewise a great similarity of the pheophytins a and b and the pheophorbides a and b, respectively. Methods were described for the estimation of the chlorophylls, chlorophyllides, pheophytins, and pheophorbides in mixtures. The above-mentioned authors emphasized that unless the extraction step for separation of the chlorophyllides from the chlorophylls and of the pheophorbides from the pheophytins was followed the chlorophyllides and the pheophorbides would be estimated as chlorophylls and pheophytins, respectively.

Jones et al. (1968) in a study of the absorption spectra of the pheophytins and pheophorbides of zinc and copper reported that the spectral curves of zinc pheophytins a and b were identical with those of zinc pheophorbides a and b, respectively, and that the spectral curves of copper pheophytins a and b were identical with those of copper pheophorbides a and b, respectively. Jones et al. (1972b) described thin-layer chromatographic methods for the qualitative identification of the copper and zinc pheophytins and pheophorbides in the presence of chlorophylls, chlorophyllides, pheophytins, and pheophorbides.

The spectral similarity between chlorophylls and the zinc pheophytins and by implication between the chlorophyllides and zinc pheophorbides has been discussed (Jones et al., 1977). Such similarity precludes the direct estimation of the zinc complexes in the presence of the chlorophylls (or chlorophyllides) either by spectrophotometry or fluorometry. Methods were proposed in that paper, however, for the estimation of each component present in either diethyl ether or 80% acetone solutions of the zinc complexes in mixtures with chlorophylls, chlorophyllides, pheophytins, and pheophorbides. Furthermore, White et al. (1977) have proposed a method for the estimation of the copper complexes in diethyl ether solution mixtures of chlorophylls, chlorophyllides, pheophytins, and pheophorbides. This is a report of a study of the experimental formation and subsequent estimation of zinc and copper complexes of chlorophyll derivatives in green plant tissue, to which zinc and/or copper salts had been added, under conditions resembling thermal processing for food preservation.

Table I. Record of Experimental Treatments Given Vegetable Tissue Samples[a]

Heating periods, min	Sample and salt added, μmol	pH values 6.8	8.5	5.1	4.5	3.8
		No. of replications				
0	Control	5	2			
30	Control	9				
30	Copper					
	0.5	1				
	1.0	4				
	1.5	4				
	2.0	3				
	2.5	2	1		1	1
	3.0	2				
30	Cu + Zn					
	0.5 + 175	1				
	2.5 + 75	1				
60	Control	2	2			
60	Copper					
	1.0	2				
	1.5	2				
	2.5	2				
60	Zinc					
	2.5		2			
	5.0		2			
	10.0	1	1	1		
	20.0	1	1	1		
	30.0	1		1		
	40.0	3	1	2	2	2
	50.0		1			
	100.0		1			
	150.0		1			
	200.0		1			

[a] Quantities of $ZnCl_2$ and/or $CuSO_4$ added in 4 ml of distilled water to replicated samples (1.42 ± 0.04 g) of spinach slurry, pH values of samples, and heating periods at 100 °C.

EXPERIMENTAL SECTION

Frozen spinach, chosen as a pigment source, was comminuted in a Virtis blender with sufficient water to permit sampling with a plunger-type repetitive pipetter. The slurry pH was adjusted by adding concentrated acetic acid or 12 N NaOH by micropipet. Following deaeration, aliquots of the slurries were pipetted into 8-ml glass tubes fitted with screw caps. Aliquots were taken from each experimental lot immediately following preparation and were held at −18 °C until needed for study. Aliquot size was determined gravimetrically but further reference thereto was in volumetric terms. Aliquots were 1.42 ± 0.04 g which was considered to be 1.4 ml.

Complexing was accomplished by heating the sealed tubes in boiling water for 30 or 60 min following addition of suitable quantities of copper ($CuSO_4$) and/or zinc salt ($ZnCl_2$) solution. To 1.4 ml of slurry was added 4 ml of the metal salt solutions at suitable concentration. The total volume of the reaction medium was therefore 5.4 ml. Following heating for a selected time, the tubes were cooled in running water. Table I provides a record of treatments given samples.

A control study to evaluate the proposed procedure of sample treatment and of pigment extraction on pigment estimation was conducted. For the control samples an aliquot, 4 ml, of distilled water was substituted for the salt solution. Some samples were not heated; others were heated in boiling water for 30 or 60 min. The pH of the controls was 6.8.

The pigment was extracted by the addition of five 3-ml

Table II. Absorptivities (Micromoles^{-1} Centimeters^2) of Pheophytins and Copper(II) Pheophytins in 80% Acetone

Pigment[a]	Max Py_a, 665.8 nm	Max Py_b, 653.4 nm	Max $CuPy_a$, 652.2 nm	Max $CuPy_b$, 631.8 nm
Py_a	46.4	18.5	16.1	4.88
Py_b	9.36	31.0	30.6	3.95
$CuPy_a$			62.2	17.5
$CuPy_b$			8.15	43.3

[a] Symbols Py_a, Py_b, $CuPy_a$, and $CuPy_b$ represent pheophytins a and b and copper(II) pheophytins a and b, respectively.

portions of acetone, each addition followed by agitation and decantation. After each addition of acetone the tube was sealed, mechanically shaken, and centrifuged. The supernatant was decanted through a small funnel into a 25-ml volumetric flask and the solution was made to volume. The extracts were permitted to stand in the refrigerator for about 10 h to promote clarification and were then centrifuged.

Estimation of the pigments was based upon the analyses detailed in Chart I. These analyses were according to the procedures previously discussed by Jones et al. (1977) and a modification of the combined spectrophotometric–fluorometric method of White et al. (1977) for copper complexes to permit their determination in 80% acetone solution. For this modification it was necessary to establish the λ_{max} and absorptivities of $CuPy_a$ and $CuPy_b$ in 80% acetone.

RESULTS AND DISCUSSION

Shown in Table II are the λ_{max} and absorptivity values for $CuPy_a$ and $CuPy_b$ in 80% acetone. Listed also are the corresponding values for Py_a and Py_b to indicate the marked spectral similarity between $CuPy_a$ and Py_b. This similarity was found to be so great as to preclude the estimation of the copper complexes and the pheophytins in mixtures in 80% acetone by spectrophotometry. However, the copper complexes were found to be nonfluorescent and to not interfere with fluorometric determinations of fluorescent pigments. Therefore, the combined spectrophotometric method of White et al. (1977), when modified for use with 80% acetone, provided a means for estimation of copper complexes and pheophytins in mixtures.

The chlorophylls, zinc pheophytins and, in the absence of copper complexes, the total pheophytins were calculated by the equations published by Jones et al. (1977). If copper complexes were known to be present or were suspected of being present, Py_at and Py_bt were estimated fluorometrically based on the fluorescence of a diluted aliquot of solution 3 (Chart I), according to the method previously described (White et al., 1972). The Py_at and Py_bt values of a diluted aliquot of solution 3 (Chart I) obtained by fluorometry were adjusted for concentration comparability of solutions estimated spectrophotometrically and fluorometrically. The adjusted fluorometric values of Py_at and Py_bt and the absorbances of solution 3 at the maxima of copper pheophytins a and b were inserted into the following equations (1 and 2) to calculate the concentrations of the copper pheophytins.

$$[CuPy_a] = 17.0A^{652.2} - 3.20A^{631.8} - 0.258[Py_at] - 0.507[Py_bt] \quad (1)$$

$$[CuPy_b] = -6.87A^{652.2} + 24.4A^{631.8} - 0.0084[Py_at] + 0.114[Py_bt] \quad (2)$$

The concentrations of the free pheophytins a and b were calculated by equations given previously (Jones et al., 1977).

Chart I. Scheme for Estimation of Pigments in 80% Acetone

Instrumental Analyses

Solutions 1, 2, and 3 formed by extract treatments listed below were read on the spectrophotometer. If the extract was known to contain or suspected of containing copper complexes, solution 3 was read on a spectrophotometer and a diluted aliquot was read on a fluorometer with filter systems designated 440 and 405[a,b] according to the procedure of White et al. (1972).

Extract

May contain C_a, C_b, Py_a, Py_b, $ZnPy_a$, $ZnPy_b$, $CuPy_a$, $CuPy_b$.

Treatments

Solution 1: Dilute 10 ml of extract to 25 ml with 80% acetone. Read at 664.0 and 646.5 nm.

Solution 2: To 10 ml of extract add 1.0 ml 0.5 M oxalic acid in acetone; let stand 90 min. Dilute to 25 ml with 80% acetone. Read at 664.0, 646.5, 659.0, and 642.0 nm. Pigments possibly present—Py_a, Py_b originally present and that formed from C_a, C_b; also $ZnPy_a$, $ZnPy_b$, $CuPy_a$, and $CuPy_b$.

Solution 3: To 10 ml of extract add 0.32 ml of 12 N HCl; let stand 30 min; add 0.23 ml of ethanolamine; dilute to 25 ml with 80% acetone. Read at 659.0 and 642.0 nm. If copper is known to be absent read at 665.8 and 653.4 nm. For extracts known to contain or suspected of containing copper read as 652.2 and 631.8 nm. Furthermore, to estimate copper complexes read a diluted aliquot of solution 3 on fluorometer with filter systems 440 and 405. Pigments present—Py_at (original Py_a + Py_a from C_a + Py_a from $ZnPy_a$); Py_bt (original Py_b + Py_b from C_b + Py_b from $ZnPy_b$); and also $CuPy_a$ and $CuPy_b$ if originally present.

[a] Filter systems used are: 440—at primary, interference, nominally 440; at secondary, interference, nominally 650 + #2-60; aperature 3×; 405—at primary, interference, nominally 405; at secondary, interference, nominally 680 + #2-60; aperature 10×. [b] #2-60 designates Corning Series, glass, sharp cut, transmission less than 0.5% at 599 nm, greater than 37% at 619 nm.

Table III. Pigment Component Means as Influenced by Thermal Treatment[a]

No. of obsvtns	Time, min	Pigment components, μmol × 10					
		Py_at	Py_bt	C_a	C_b	Py_a	Py_b
5	0	8.74	3.01	8.01	2.35	0.71	0.66
9	30	8.76	2.78	2.20	1.25	6.56	1.54
2	60	8.89	2.58	0.32	0.62	8.57	1.97
LSD 0.05		NS	0 vs. 30 = 0.057	0.799	0.252	0.979	0.319
			30 vs. 60 = 0.081	1.119	0.354	1.372	0.449

[a] Pigment components, estimated spectrophotometrically, in 1.42-g spinach puree samples at pH 6.8 following heating in boiling water for periods indicated.

Table IV. Zinc Chlorophyll Pigment Formation in Green Plant Tissue during Thermal Processing in the Presence of Zinc Salt[a]

Zinc complex	Reaction medium pH	a (intercept)	b values		N[d]	R^2	Complex[e] formed, %
			C_1[b]	C_2[c]			
$ZnPy_a$	6.8	−1.6	2.05	−0.0184	7	0.981	51.1
$ZnPy_b$	6.8	−5.1	0.251	0.00412	7	0.866	11.5
$ZnPy_a$	5.1	−6.8	2.75	−0.0337	5	0.968	49.3
$ZnPy_b$	5.1	7.2	−0.0611	0.0108	5	0.684	22.0
$ZnPy_a$	8.5	1.6	3.51	−0.0445	10	0.991	70.4
$ZnPy_b$	8.5	−1.3	1.13	−0.0105	10	0.886	27.1
$ZnPy_a$	4.5				2		30.1
$ZnPy_b$	4.5				2		5.8
$ZnPy_a$	3.8				2		6.5
$ZnPy_b$	3.8				2		0

[a] Statistics for regression of zinc complex formed on concentration of added zinc chloride at different reaction media pH values. Thermal treatment was for 60 min at 100 °C. [b] C_1 = linear regression coefficient. [c] C_2 = quadratic regression coefficient. [d] N = number of observations. [e] Zinc complexes formed in the presence of 40 μmol of $ZnCl_2$ per μmol of total pigment in sample. The complexes were estimated as $ZnPy_a$ or $ZnPy_b$ and were reported in terms of percentage of Py_at or Py_bt present, respectively.

In Table III are shown statistics of the analysis of the data from the control study. These data provide an indication of the effect of the thermal treatments on the stability of the chlorophylls and the pheophytins and of the consistency of the method proposed. The treatments induced a highly significant change of the chlorophylls to the pheophytins. The magnitudes of the conversion of C_a to Py_a were 72 and 96% for the 30- and 60-min periods, respectively; for C_b to Py_b they were 47 and 74% for the 30- and 60-min periods, respectively. Such conversion is a well-recognized change (Mackinney and Weast, 1940; Tan and Francis, 1962).

The thermal treatments caused no loss of the total a component, as indicated by the nonsignificant differences in the Py_at values in Table III. Py_bt was less stable then Py_at. A linear decrease in Py_bt was apparent as the thermal treatment was prolonged. The observed decrease during both heating periods was highly significant. Mackinney and Weast (1940) reported that degradation more drastic than conversion of chlorophyll b to pheophytin b occurred during the thermal processing of canned peas. Such thermal treatment was somewhat more severe than that in this study.

In Table IV are presented the regression statistics of zinc complexes formation. In the analysis of the zinc studies data comparisons were made of the quantities of zinc complexes formed by reaction of the pigment samples with different quantities of $ZnCl_2$ at given pH values as detailed in Table I. The quantities of zinc salt added were expressed as ratios of micromoles of zinc salt added per micromole of total pigment ($a + b$) in the aliquot analyzed. The zinc complexes were expressed as ratios, $[ZnPy_a]/[Py_at]$ and $[ZnPy_b]/[Py_bt]$. These ratios when multiplied by 100 represent the percentage of total pheophytin a and total pheophytin b complexed.

From the data in Table IV it will be seen that formation of zinc pheophytin b was comparatively much lower than for zinc pheophytin a and that, in general, the more acid the reaction medium the less complex formed. This latter comparison relates to the quantity of complex observed at 40 μmol of $ZnCl_2$ per μmol of total pigment.

A graphic presentation of some of the results of the zinc complexing study is made in Figure 1. Given are curves showing the percentage of the a and b components which were complexed upon addition of specified quantities of $ZnCl_2$ to the samples. These curves indicate that the percentage of complexing was somewhat greater at pH 8.5 than at 6.8 and 5.1 under similar conditions of zinc salt concentration. Additional observations at pH 8.5 were made at $ZnCl_2$ concentrations up to four times the maximum shown in Figure 1 (200:1). The percentage of zinc pheophytin a complexed under such conditions was essentially that observed at a $ZnCl_2$/total pigment ratio of about 40:1. For the b component the quantity of the complex formed was 30% greater at the 200:1 ratio than at the 40:1 ratio. From the data of Table IV it appeared that little or no formation of zinc complexes occurred under conditions of pH 3.8.

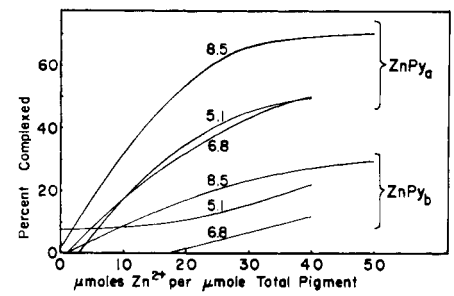

Figure 1. Relationship between zinc complex formation and concentration of zinc salt in reaction medium at various pH values.

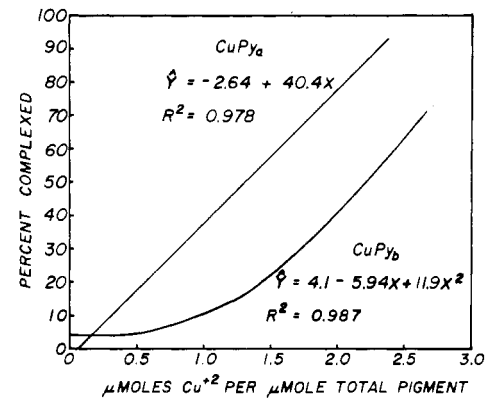

Figure 2. Relationship between copper complex formation and concentration of copper salt in reaction medium at pH 6.8.

Observations of the relationship between copper complex formation and relative salt concentration are summarized in Figure 2. Shown in the figure are curves plotted from data pertaining to copper complexes formed at pH 6.8. The relationship is expressed in terms of the percentage of total a and total b complexed at various levels of added $CuSO_4$ (micromoles) per micromole of total pigment ($a + b$) present. Data were obtained but are not shown which indicated that the percentage of copper(II)

Table V. Zinc and Copper Complexes Formation; Formation of Zinc and Copper Pheophytins as Influenced by Concentration of Zinc and Copper Salts in Reaction Medium[a]

Metal salt concn as μmol per μmol of total chlorophyll (a + b)		Zinc and copper complexes formed			
		As % total chlorophyll a present		As % total chlorophyll b present	
$ZnCl_2$	$CuSO_4$	$ZnPy_a$	$CuPy_a$	$ZnPy_b$	$CuPy_b$
175	0.5	53	21	29	12
75	2.5	6	92	0	80
150	0.0	70	0	42	0
50	0.0	70	0	26	0

[a] Reaction medium pH value, 6.8.

pheophytin a formed was not appreciably increased by extending the thermal treatment from 30 to 60 min but the amount of copper pheophytin b formed was increased by about 25% by such extended heating. At pH values of 3.8, 4.5, and 8.5 the percentages of copper complexes formed were essentially the same as at pH 6.8. Such were the observations with respect to the formation of both the copper a and copper b complexes.

From a preliminary study of the complexing of chlorophylls in the presence of both copper and zinc salts it was observed that the formation of the copper complexes occurred much more readily than that of the zinc complexes. Accordingly, even relatively small amounts of copper salt present greatly reduced the amounts of zinc complexes formed. This relationship is indicated in the data presented in Table V.

Because of the spectral similarity of the zinc and copper complexes to the chlorophylls, the formation of these metal complexes had the visual effect of retention of the characteristic blue-green color of the vegetable tissue during and following the thermal treatment applied. The subjective color evaluation of samples during treatment was not extensive; therefore, this subject will not be discussed further.

The concentration levels for the zinc and copper salts at which appreciable complexing occurred were about 10 and 1 μmol/μmol of total pigment, respectively. These values represent about 120 and 12 ppm for zinc and copper salts, respectively, in the reaction medium.

In this investigation there have been no direct observations relative to whether the complexes were pheophytins or pheophorbides. As previously reported by Jones et al. (1963) and Clydesdale and Francis (1968) it would be expected that chlorophyllides or free pheophorbides would be formed to some extent due to the heat treatment given. In the presence of either chlorophyllides or free pheophorbides, zinc and copper(II) pheophorbides would presumably be formed as readily with suitable concentrations of zinc and copper salts as would zinc and copper(II) pheophytins in mixtures containing chlorophylls and pheophytins. The pheophorbides, including those of zinc and copper, could be identified (Jones et al., 1972a,b). Estimation would be from diethyl ether solutions as indicated by White et al. (1972, 1977) and Jones et al. (1977), observing the special precautions cited in these references to avoid losses through adsorption on glassware.

Attention is called to the possibility of the estimation of copper complexes by spectrophotometry only. This estimation may be accomplished by transferring the pigments of solution 3 from 80% acetone to diethyl ether and then following the method of White et al. (1977). Futhermore, the original extract may be transferred to diethyl ether and all pigments estimated spectrophotometrically by the methods of Jones et al. (1977) and White et al. (1977).

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