Studies of Gluten Lipids. I. Distribution of Lipids in Gluten Fractions Separated by Solubility in 70% Ethanol

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ABSTRACT

Lipids extracted from flour and gluten by benzene-ethanol (1:1,v.:v.) had similar distributions as estimated by quantitative thin-layer chromatography. "Protein aggregates" (Cereal Chem. 42: 409; 1965), a poorly soluble fraction separated from 0.05*N* acetic-acid dispersions of gluten by centrifugation, had a lipid distribution comparable to that of both flour and gluten. Gliadin and glutenin, fractionated from gluten dispersions (after removal of aggregates) by the "classic" procedure involving 70% ethanol, had entirely different lipid distributions: gliadin lipids contained 75% polar material and 6% triglycerides; glutenin lipids had 24% and 58% respectively of these components. Flour lipids, by comparison, contained 46% polar material and 37% triglycerides. The possibility is considered that differences between gliadin and glutenin lipids may have been due to lipid relocation during the gluten fractionation procedure.

One approach toward an understanding of wheat flour gluten has been to isolate and study the classic glutenin and gliadin fractions. Because of the many investigations that have been conducted on glutenin and gliadin, and on gluten fractions in general—for recent reviews of wheat proteins see Sullivan (1) and Pence, Nimmo, and Hepburn (2)—it is interesting to note one research area that seems to have evaded close scrutiny—namely, the distribution of lipids associated with different gluten fractions. The importance of both the protein and lipid components to flour quality is often speculated upon, if not always conclusively demonstrated; if both components influence flour quality, then possible associations between particular lipids and particular proteins might also be a factor in flour quality and in breadmaking in general. Daniels and co-workers (3) have suggested recently that a study of lipid distribution and their involvement with various flour proteins could lead to a better understanding of dough mixing and baking.

Among the apparently few published papers dealing with lipids associated with gluten fractions was that of Olcott and Mecham (4); these workers reported in 1947 that the bulk of gluten lipids was associated with the glutenin fraction. Lee and Wan (5) subsequently confirmed these results. Earlier, Blish (6) observed that certain unidentified gluten fractions had lipid contents as high as 44%. More indirectly related to this general area of research was the work of Hess, quoted by Fisher (7), who reported that "Haft" protein contained three to four times as much lipid as "Zwickel" protein.

The objective of the work described in the present paper was to compare the distribution of lipids among flour, gluten, and certain gluten fractions. Glutenin and gliadin fractions utilized for this study were prepared by pH

adjustments of acetic-acid dispersions of gluten in 70% ethanol; this "classic" route was employed because it was desirable to characterize gluten fractions made by the procedure most often used (2). It should be recognized at the outset, however, that the use of ethanol to solubilize gliadin in the "classic" fractionation procedure raises the question as to whether gluten lipids are altered in any way by contact with the alcohol. This point will receive attention in this paper and in a succeeding paper.

MATERIALS AND METHODS

Gluten Fractionation

Flour (commercial bakers' patent) and water were made into simple doughs on a Hobart mixer, then the glutens were isolated by a hand-washing method as described previously (8). Portions (100 g.) of wet gluten were dispersed in 500 ml. 0.05*N* acetic acid by means of a Waring Blendor (low speed for 5 min.), then were heated to 98°C. to terminate enzyme activity (9). After quickly cooling to room temperature, the dispersions were centrifuged in a Model CS International centrifuge for 15 min. at 2,500 r.p.m. The soluble material was recovered by decantation, and the insolubles were washed with two portions of acetic acid solution; the washings were combined with the solubles. This insoluble fraction is referred to in this paper as "protein aggregates," or simply "aggregates," using the terminology of Mecham *et al.* (10), also adopted by Ponte *et al.* (8).

Portions of the above solubles were put into centrifuge jars and made up to 70% in ethanol; the pH of the suspensions was adjusted to 6.75 with *N* sodium hydroxide. Precipitation of the glutenin was allowed to proceed overnight in a refrigerator (4°C.). The glutenin was collected by centrifugation for 15 min. at 2,500 r.p.m., then was washed twice with 70% ethanol. The alcoholic solution was filtered through Whatman No. 1 paper and concentrated in a Buchler flash evaporator under vacuum at 35°C. The gliadin thus obtained, as well as the glutenin, protein aggregates, and portions of the starting gluten, was dispersed in 0.05*N* acetic acid and freeze-dried.

Extraction of Lipids

A portion (2 g.) of the freeze-dried gluten or gluten fractions was suspended in 200 ml. of a benzene-ethanol (50:50, v.:v.) mixture. The suspensions were agitated in a mechanical shaker for 15 min., then left overnight at room temperature. After centrifugation and decantation, the residues were re-extracted by two 10-min. periods of mechanical shaking with 100-ml. portions of the solvent system.

Concentration of the extracts was carried out in the flash evaporator under vacuum ($<$40°C.). When dry, the residues were taken up in petroleum ether (b.p. 30°–60°C.) and filtered through Whatman No. 1 paper into tared flasks; the flasks were attached to the flash evaporator and the solvent was removed. Last traces of the solvent were eliminated by directing a stream of nitrogen on the residues until no odor of solvent was evident. The lipids (petroleum-ether solubles of benzene-ethanol extracts) were made up to 10% solutions in redistilled chloroform and these were transferred to amber glass vessels for storage at 4°C. until use. To the solution was added

0.005% BHT (Eastman Tenox) to minimize oxidation, as suggested by Wren and Szczepanowska (11).

Wherever feasible throughout the above operations and during storage, the lipids were kept under an atmosphere of nitrogen.

Flour was extracted by mechanically shaking 20 g. of the material with 200 ml. of the benzene-ethanol for 15 min. After centrifugation, the supernatants were decanted and the residues were extracted once again. The extracts were then handled as described above.

Thin-Layer Chromatography

Slurries made with 40 g. Camag silica gel without binder (Muttenz, Switzerland) and 95 ml. double-distilled water were deposited on 20 × 20-cm. glass plates with a Desaga applicator. Qualitative and quantitative work was done with silica gel layers 0.5 mm. thick. The plates were allowed to air-dry overnight, then were washed with chloroform:methanol (1:1, v.:v.). Class separations were made on these plates without further preparation; polar separations were made on plates heated at 130°C. for at least 0.5 hr.

For qualitative comparisons of lipids, 10% solutions (10 μl.) were applied as spots with a syringe, at the origin located 2 cm. above the bottom of the plates; the silica gel layers were marked off into 2- to 3-cm. lanes. Development was carried out by the ascending technique for a total distance of 16 cm.

The developed plates were air-dried at room temperature; then the spots were visualized by exposure to iodine vapor, or by spraying with 0.1% Rhodamine 6 G (in ethanol), 0.1% 2,7-dichlorofluorescein (in ethanol), or a mixture of 70 parts saturated dichromate solution and 30 parts sulfuric acid, followed by heating on a hot plate. Modified Dragendorff's reagent (12) was used to help locate choline phosphatides and glycolipids, and 0.2% ninhydrin in butanol containing 1% pyridine (13) was utilized to locate amino-containing materials. All compounds, or classes of compounds, discussed in this paper were identified by reference to authentic samples.

Quantitative Thin-Layer Chromatography

A procedure was developed for quantification of lipid distribution, utilizing elements of the methods of Bragdon (14) and Amenta (15). Aliquots (50 μl.) of the 10% lipid solutions were deposited as narrow bands on the plates and developed with n-hexane:diethyl ether:acetic acid (65:35:2, v.:v.:v.). After drying, the plates were exposed to iodine vapor just until the spots could be barely visualized; the distributed lipids were outlined with a fine point in such a way that five fractions (to be subsequently identified) were demarcated. After the iodine had evaporated, the outlined areas of silica gel were scraped into test tubes with a razor blade. Blanks, comprising areas of silica gel identical with the various fractions, were obtained from each chromatogram and treated the same as the samples.

Three milliliters of an acid-dichromate solution (2.5 g. potassium dichromate made up to 1 liter with 36*N* sulfuric acid) was added to the test tubes containing the lipid fractions adsorbed on the silica gel scrapings.

The tubes were shaken and then placed in a boiling-water bath for 1 hr. with occasional shaking, then cooled in running water. Water (10 ml.) was added to the tubes; after mixing, the diluted solutions were filtered through Whatman No. 30 paper into colorimeter tubes, and read in a Spectronic 20 colorimeter at 580 mμ. The resulting absorbances were translated into mg. lipids by means of standard curves.

Preparative thin-layer chromatography (TLC) was employed to isolate materials for construction of standard curves. Solutions (20%) of flour lipids were streaked at the origin of 20 × 20-cm. plates coated with a 1.0-mm. layer of the silica gel; roughly 300 to 400 mg. of lipids was deposited. The plates were developed as described for qualitative TLC, then air-dried, and briefly exposed to iodine vapor. The lipids on these plates were carefully separated into the same five fractions as the samples; after evaporation of the iodine, the outlined areas were scraped into tubes and the lipids were extracted from the silica gel with diethyl ether. Good separations were achieved, as indicated by a qualitative examination of these materials on thin-layer chromatograms.

The separated five fractions of flour lipids were made into solutions, chromatographed, and analyzed exactly as described for the samples. Various concentrations of each fraction were run to obtain absorbances encompassing those obtained for the samples. Quantities of lipids in the samples were estimated by reference to regression equations, computed for each fraction. Identification of the five lipid fractions studied (with reference to principal components) and the regression equations obtained are as follows:

Fraction	*Principal Component*	*Regression Equation* (X = mg. lipid)
1	Polar lipids	Absorbance = 0.0380 X -0.0150
2	Diglycerides	Absorbance = 0.0463 X +0.0041
3	Fatty acids	Absorbance = 0.0535 X -0.0061
4	Triglycerides	Absorbance = 0.0203 X +0.0002
5	Nonpolar lipids	Absorbance = 0.165 X -0.0095

The principal objective in developing the procedure outlined above was to compare trends in distribution of lipids for the gluten fractions under study, rather than to develop a highly quantitative method. Nevertheless, the reasonably good reproducibilities achieved suggest that the procedure could be developed into a useful analytical tool (16).[1] As an example, some statistical data on lipids from the flour used in this study are given below.

Fraction	*Principal Component*	*Coeff. of Variation* %
1	Polar lipids	4
2	Diglycerides	3
3	Fatty acids	7
4	Triglycerides	4
5	Nonpolar lipids	19

The coefficient of variation is based on two separate lipid extractions of flour, each analyzed in duplicate; thus, four quantitative TLC analyses were

[1] When this study was nearing completion, a paper by Walsh, Banasik, and Gilles (16) appeared, describing a quantitative TLC procedure similar in part to the present one, but more rigorously detailed.

conducted. Poorest reproducibility was found in fraction 5, nonpolar lipids, probably because of the small amounts of material involved (1.4% of flour lipids).

Gas-Liquid Chromatography

Triglyceride areas were scraped from some of the thin-layer plates; after elution from the silica gel, the triglycerides were saponified and esterified (17), and analyzed by GLC as described previously (18).

RESULTS

Composition of Gluten Fractions

Data on the gluten fractions as well as on the starting flour and gluten studied in this investigation are provided in Table I.

TABLE I
ANALYSES OF FLOUR, GLUTEN, AND GLUTEN FRACTIONS

	PART OF GLUTEN [a]	PROTEIN (N × 5.7)	LIPID	PROTEIN:LIPID
	%	%	%	
Flour	....	13.9	2.1	6.6
Gluten	....	78.7	9.5	8.3
Aggregates	16.6	49.2	6.9	7.1
Glutenin	41.1	80.0	6.7	11.9
Gliadin	42.3	73.6	9.1	8.1

[a] Based on recovered material; recovery was roughly 92% of starting gluten. All data expressed on dry-solids basis.

On the basis of recovered material, the aggregates, prepared as described, comprised about 17% of the gluten; the glutenin and gliadin made up the remainder of the gluten in about equal proportions.

Protein was somewhat higher in the glutenin fraction than the gliadin fraction; both of these had substantially higher protein than the aggregates. The lower protein of the aggregates was probably due, at least in part, to residual starch that would be expected to come down with this fraction. (The aggregates quickly produced a blue color when exposed to iodine; the glutenin yielded color only after standing for some time, and the gliadin developed no starch color at all.)

Lipids were higher in the gliadin than in the other two gluten fractions, being present in about the same concentration as in the gluten. It should be noted that approximately 81% of the gluten lipids was recovered from the three gluten fractions compared to about 92% for protein. This indicates a binding of lipids during the fractionation procedure.

The high lipid content of the gliadin fraction disagrees with the data of Olcott and Mecham (4); this point will be discussed further.

Protein-lipid ratios, also shown in Table I, indicate that the flour had somewhat more lipid compared to protein than the gluten. Of the three gluten fractions, the aggregates appeared to have more lipid associated with protein than both the gliadin and the glutenin, particularly the latter.

Distribution of Lipids

Figure 1 shows the separation, by TLC, of lipids from the flour, gluten, and gluten fractions into broad classes.

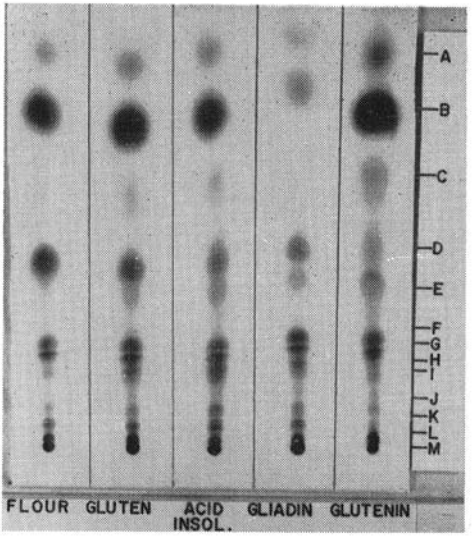

Fig. 1. Thin-layer chromatogram of lipids extracted from flour, gluten, aggregates (i.e., acetic-acid insolubles), gliadin, and glutenin. A, nonpolar material; B, triglycerides; D, free fatty acids; F, 1,3-diglycerides, G, 1,2-diglycerides; L, monoglycerides; M, polar material; all others unknown. Solvent: n-hexane:ethyl ether:acetic acid (65:35:2, by volume); 1.0 mg. lipids deposited. Visualized by exposure to iodine vapor.

The lipids from gluten and aggregates (i.e., acid insolubles) had distributions roughly similar to that of flour lipids. The lipids from gliadin and glutenin, however, had distributions markedly different. Much less nonpolar material and triglycerides was observed in the gliadin lipids compared to the flour lipids; the reverse was true for the glutenin lipids.

For purposes of quantification, the lipid classes were grouped into five fractions as shown in the table below. Polar lipids were phosphatides, glycolipids, and monoglycerides.

Fraction	*Components (see Fig. 1)*	*Major Constituent*
1	L, M	Polar lipids
2	F, G, H, I, J, K	Diglycerides
3	D, E	Free fatty acids
4	B, C	Triglycerides
5	A	Nonpolar lipids

Quantitative comparisons of the lipids are summarized in Table II.

TABLE II

LIPID DISTRIBUTION IN FLOUR, GLUTEN, AND GLUTEN FRACTIONS

	LIPIDS				
	Flour	Gluten	Aggregates	Gliadin	Glutenin
	%	%	%	%	%
1. Polar lipids	46	51	44	75	24
2. Diglycerides	6	5	12	6	6
3. Fatty acids	11	7	13	12	11
4. Triglycerides	37	36	30	6	58
5. Nonpolar lipids	1	1	2	1	2

Data on the distribution of flour lipids appear to be in line with the results of Nelson, Glass, and Geddes (19) on "endosperm" lipids (allowing for the slightly different nature of their substrate and system for grouping lipid classes) and also with the data of McKillican and Sims (20) and Fisher *et al.* (21), except that the flour lipids of the latter were richer in diglycerides. In the present study, the major classes of polar lipids (i.e., phosphatides, glycolipids, monoglycerides) and triglycerides accounted for about 46 and 37% respectively of the total extracted lipids of flour.

Although some differences were noted in the distribution of lipids from the gluten and aggregates compared to that of flour, these did not seem of major importance and will not receive further discussion.

The major changes in distribution appear to have occurred in the polar material and triglycerides of the gliadin and glutenin lipids. In the gliadin lipids, the polar material and triglycerides were present in 75 and 6% concentrations respectively; in the glutenin lipids these fractions were present in amounts of 24 and 58%.

Possible qualitative differences in the triglyceride fractions were explored by comparing the fatty acid distributions of the gluten and glutenin triglycerides; no appreciable differences were noted in a preliminary experiment by GLC.

Differences between the polar fractions of the various lipids are further illustrated in Fig. 2. A solvent system specifically designed to separate phosphatides was used.

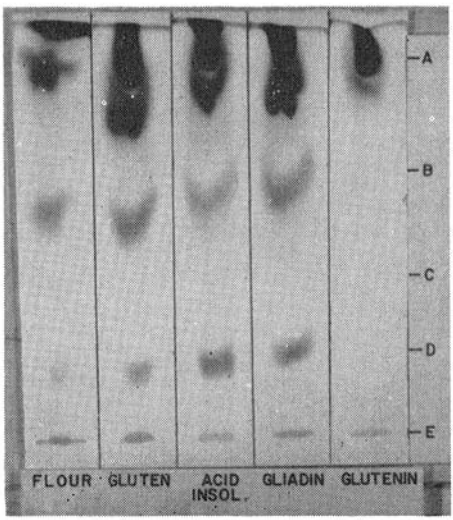

Fig. 2. Thin-layer chromatogram of lipids extracted from flour, gluten, aggregates (i.e., acid insolubles), gliadin, and glutenin. A, top spot is relatively neutral material, spot immediately below is glycolipids; B, phosphatidyl choline; C, unknown, visible when sprayed with ninhydrin; D, lysophosphatidyl choline; E, unknown. Solvent: chloroform:methanol:acetic acid:water (50:25:8:4, by volume); 2.0 mg. lipids deposited. Visualized by exposure to iodine vapor.

This chromatogram, developed by a relatively polar solvent system, shows that the glutenin lipids contained less of glycolipids compared to the lipids from flour, gluten, aggregates, and gliadin. At the concentrations employed (2.0 mg. lipids was deposited), virtually no phosphatidyl choline or lysophosphatidyl choline was evident in the glutenin lipids; similarly, unknown component C, visible when stained with ninhydrin, was noted in all the lipids except that from glutenin.

DISCUSSION

The data presented in this paper show that pronounced differences in distribution occur between the lipids of gliadin and glutenin, at least between lipids of fractions prepared as herein described. Gliadin was considerably richer in polar lipids and poorer in triglycerides compared to glutenin.

Although these differences were real, the question arises as to whether they accurately reflect lipid-protein associations in the original gluten. Specifically, it must be asked whether or not the fractionation procedure itself caused a relocation of lipids.

Ethanol, usually 95% or absolute, is a well-known lipid extractant for cereal products (22); 70% ethanol (hot) has also been used to remove lipids from dried gluten (23). Apparently ethanol is an effective agent for such extractions because of its ability to rupture "lipo-protein" bonds (e.g. 4, 24). Therefore, when gluten is exposed to 70% ethanol during the classic fractionation procedure, the possibility exists that lipid-protein associations could be broken; when the glutenin is subsequently precipitated out, the freed lipids could remain in solution to come down later with the gliadin. Lending some support to the possibility that lipids were transferred from glutenin to gliadin is the paper by Olcott and Mecham (4), who reported very little lipid in gliadin prepared from gluten by a procedure not involving alcohol. In the present study, somewhat more lipids were found in gliadin than in glutenin. To obtain more information in this area, further work is under way in this laboratory, including a study of lipid distribution in gluten fractions prepared by an alternate route.

Whether or not the classic gluten fractionation procedure causes a relocation of lipids, it is of importance to recognize the differences in lipid distribution between the resulting gliadin and glutenin; these differences in lipid distribution could conceivably influence properties of the gluten fractions in certain studies. Even in those instances where "defatted" flour is used to prepare gluten, it is unlikely that all lipid is removed, and therefore some differences in the distribution of residual lipids could be expected.

Acknowledgments

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Literature Cited

1. SULLIVAN, BETTY. Wheat protein research: fifty years of progress. Cereal Sci. Today 10: 338–344, 360, 361 (1965).

2. PENCE, J. W., NIMMO, C. C., and HEPBURN, F. N. Proteins. *In* Wheat: Chemistry and technology, ed. by I. Hlynka; Monograph Series, vol. III, chap. 6. American Association of Cereal Chemists: St. Paul, Minn. (1964).
3. DANIELS, N. W. R., RICHMOND, J. WENDY, EGGITT, P. W. RUSSELL, and COPPOCK, J. B. M. Studies on the lipids of flour. III. Lipid binding in breadmaking. J. Sci. Food Agr. 17: 20–29 (1966).
4. OLCOTT, H. S., and MECHAM, D. K. Characterization of wheat gluten. I. Protein-lipid complex formation during doughing of flours. Lipoprotein nature of the glutenin fraction. Cereal Chem. 24: 407–414 (1947).
5. LEE, C. C., and WAN, K-M. Studies with radioactive tracers. VII. Investigations with flour containing P^{32}. Cereal Chem. 40: 415–422 (1963).
6. BLISH, M. J. The wheat flour proteins. Cereal Chem. 13(5, Suppl.): 16–23 (1936).
7. FISHER, N. Wheat lipids. *In* Recent advances in food science, ed. by J. Hawthorn and J. M. Leitch; vol. I, pp. 226–245. Butterworths: London (1962).
8. PONTE, J. G., JR., DE STEFANIS, V. A., TITCOMB, S. T., and COTTON, R. H. Study of gluten properties as influenced by certain organic solvents. Cereal Chem. 44: 211–220 (1967).
9. JONES, R. W., BABCOCK, G. E., TAYLOR, N. W., and SENTI, F. R. Molecular weights of wheat gluten fractions. Arch. Biochem. Biophys. 94: 483–488 (1961).
10. MECHAM, D. K., COLE, ELAINE G., and PENCE, J. W. Dough-mixing properties of crude and purified glutens. Cereal Chem. 42: 409–420 (1965).
11. WREN, J. J., and SZCZEPANOWSKA, ANNA D. Chromatography of lipids in presence of an antioxidant, 4-methyl–2,6–di–tert.–butylphenol. J. Chromatog. 14: 405–410 (1964).
12. MANGOLD, H. K. Thin layer chromatography of lipids. J. Am. Oil Chemists' Soc. 38: 708–727 (1961).
13. LEPAGE, M. The separation and identification of plant phospholipids and glycolipids by two-dimensional thin layer chromatography. J. Chromatog. 13: 99–103 (1964).
14. BRAGDON, J. H. Colorimetric determination of blood lipids. J. Biol. Chem. 190: 513–517 (1951).
15. AMENTA, J. S. A rapid method for quantification of lipids separated by thin layer of chromatography. J. Lipid Res. 5: 270–272 (1964).
16. WALSH, D. E., BANASIK, O. J., and GILLES, K. A. Thin-layer chromatographic separation and colorimetric analysis of barley or malt lipid classes and their fatty acids. J. Chromatog. 17: 278–287 (1965).
17. METCALFE, L. D., SCHMITZ, A. A., and PELKA, J. R. Rapid preparation of fatty acid esters from lipids for gas liquid chromatography. Anal. Chem. 38: 514–515 (1966).
18. PONTE, J. G., JR., TITCOMB, S. T., CERNING, JUTTA, and COTTON, R. H. Dough-improving effect of some aliphatic hydrocarbons. II. Studies of dough lipids. Cereal Chem. 41: 431–442 (1964).
19. NELSON, J. H., GLASS, R. L., and GEDDES, W. F. Silicic acid chromatography of wheat lipids. Cereal Chem. 40: 337–343 (1963).
20. MCKILLICAN, MARY E., and SIMS, R. P. A. The endosperm lipids of three Canadian wheats. J. Am. Oil Chemists' Soc. 41: 340–344 (1964).
21. FISHER, N., BELL, BRENDA M., RAWLINGS, CHRISTINE E. B., and BENNET, RUTH. The lipids of wheat. III. Further studies of the lipids of flours from single wheat varieties of widely varying baking quality. J. Sci Food Agr. 17: 370–382 (1966).
22. MECHAM, D. K. Lipids. *In* Wheat: Chemistry and technology, ed. by I. Hlynka; Monograph Series, vol. III, chap. 8. American Association of Cereal Chemists: St. Paul, Minn. (1964).
23. DILL, D. B. The composition of crude gluten. Cereal Chem. 2: 1–11 (1925).
24. GLASS, R. L. The role of lipids in baking. Cereal Sci. Today 5: 60–62, 75 (1960).

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