Selected Chemical and Microbiological Changes in Refrigerated Pork Stored in Carbon Dioxide and Oxygen Enriched Atmospheres

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Bacterial populations and lactic acid isomers, short-chain fatty acids, acetoin/diacetyl, and diamine concentrations were measured in refrigerated pork stored at 2 °C in CO_2/air (20/80) (v/v) and CO_2/O_2 (20/80) (v/v) atmospheres. *Brochothrix thermosphacta* was the dominant bacterium in both atmospheres, reaching levels of about 10^8 CFU cm^{-2} after 2 weeks of storage. Lactobacilli and Enterobacteriaceae increased consistently although final levels were 3–4 logarithmic units lower than the aerobic count at 24 °C. No consistent patterns were obtained with L-(+)-lactic acid and acetoin/diacetyl. D-(−)-Lactic acid concentration increased with lactobacilli counts. Most short-chain fatty acids (acetic, propionic, isobutyric, and isovaleric) also increased with aerobic count at 24 °C. Cadaverine and putrescine levels increased along with the Enterobacteriaceae. Relationships between bacterial counts in different culture media and the byproducts formed during storage have been established.

INTRODUCTION

The slime-producing psychrotrophic Gram-negative bacteria that are usually responsible for spoilage of refrigerated meat (Dainty et al., 1983) are effectively inhibited by 20% CO_2 (Christopher et al., 1979). This flora is replaced, to a large extent, by CO_2-resistant organisms, e.g., lactic acid bacteria and *Brochotrix thermosphacta* (Newton et al., 1977; Asensio et al., 1988). When refrigerated meat is stored in CO_2-enriched atmospheres, the myoglobin oxidation becomes the limiting factor of the shelf life of meat (Ordóñez and Ledward, 1977; Asensio et al., 1988). To overcome this, the use of carbon dioxide and oxygen enriched atmospheres has been proposed (McDougall and Taylor, 1975) to delay metmyoglobin formation (McDougall and Taylor, 1975; Ordóñez and Ledward, 1977; Asensio et al., 1988).

Many workers [e.g., McDougall and Taylor (1975), Ordóñez and Ledward (1977), and Asensio et al. (1988)] have studied the factors limiting the shelf life (microflora, metmyoglobin formation, and lipid oxidation) of refrigerated meat stored in carbon dioxide and carbon dioxide and oxygen enriched atmospheres. Likewise, the metabolism of *B. thermosphacta*, the dominant organism in CO_2-enriched atmospheres (Asensio et al., 1988), has been studied in both aerobic (Dainty and Hibbard, 1980, 1983; Dainty and Hofman, 1983) and anaerobic (Hitchner et al., 1979; Dainty et al., 1979) conditions. However, no information is available about the chemical changes occurring during the storage of refrigerated meat in the modified atmospheres above mentioned. The present paper deals with these aspects.

MATERIALS AND METHODS

Samples. Naturally contaminated pork (*Longissimus dorsi* 24 h post-mortem; pH 5.4–5.6) was cut into ca. 1 cm thick slices and individually packed in laminated film bags (20 × 22 cm) of low gas permeability (Cryovac BB1; diffusion coefficient of 25 mL/m^2/24 h/atm to O_2 and 100 mL/m^2/24 h/atm to CO_2). Gas mixtures consisted of 20% CO_2 plus 80% air (v/v) and 20% CO_2 plus 80% O_2 (v/v) and were supplied by Sociedad Española de Oxigeno. After most of their air content had been evacuated by gentle pressure, plastic bags were flushed for 10 s at a flow rate of about 6000 mL/min; the bags were then filled with ca. 1000 mL of gas mixture and heat-sealed with a Moulinex 629 thermsealer. Samples were stored at 2 ± 1 °C. In each experiment, two bags were drawn periodically for sampling.

Microbial Analysis. At each sampling time, the odor of the headspace gas of the bags was assessed after 15 min of equilibration at room temperature, by an untrained panel of six members. They were asked if the odor was acceptable or if any off-odor was present.

Microbial analyses were done on duplicate samples by swabbing a 2 × 2 cm area of the meat surface with sterile cotton swabs. Aerobic psychrotrophs were determined with plate count agar (PCA, Oxoid) incubated at 24 °C for 36 h. To characterize the dominant organisms, 20% of the colonies from PCA plates were randomly chosen (Ordóñez, 1979) and subcultured into brain heart infusion (BHI, Oxoid). Characterization of microorganisms was carried out according to the methodology previously described (Dainty et al., 1979). Enterobacteriaceae members were determined on double-layer violet–red–bile–glucose agar (VRBG, Oxoid) plates incubated at 30 °C for 24 h (Edwards et al., 1983). Lactobacilli were counted on double-layer pH 5.6 MRS agar (Oxoid) incubated at 24 °C for 3 days.

Chemical Analysis. A portion of the meat slices (30 g) was ground with 90 mL of distilled water in an Omni-mixer Sorvall, Model 17.106. For short-chain fatty acids determination, an aliquot of the former mixture was used; 0.1 mL of a 1.2% solution of hexanoic acid (BDH) was added as internal standard. The homogenate was centrifuged (10000*g* for 10 min) and the resulting pellet reextracted with an additional volume of water and recentrifuged. The two supernatants were combined, and the volume was recorded. Proteins were precipitated by adding 5 N KOH (final pH of 12.0) and $ZnSO_4$ (final concentration 0.1% w/v), followed by heating in a boiling-water bath. Denatured proteins were removed by filtration, and the filtrate was taken to dryness on a rotatory evaporator. The residue was dissolved in a small volume of distilled water and acidified with 5 N HCl to pH 2.0 for extraction into ethyl ether. Immediately after extraction, ethyl ether extracts were analyzed on a 910 Perkin-Elmer chromatograph equipped with a column (2 m × 4 mm) filled with Carbowax 20 M plus 1% H_3PO_4 on 80/100-mesh Chromosorb W. The apparatus was operated under isothermal conditions (120 °C) using nitrogen as carrier gas (20 mL/min). Fatty acid identification was made by comparing the retention

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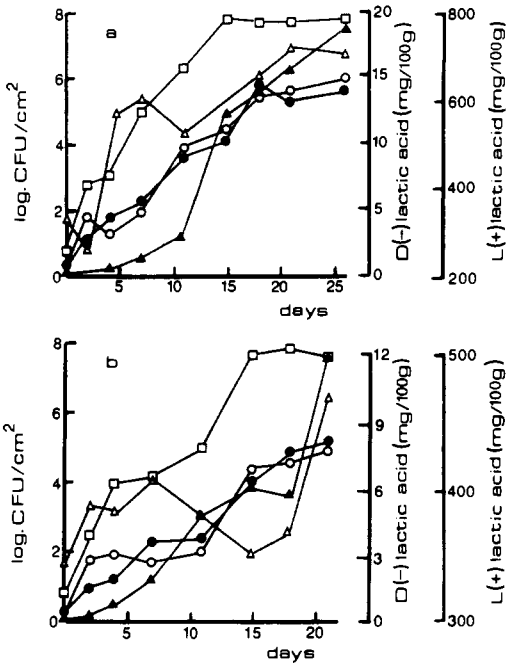

Figure 1. Changes in aerobic psychrotrophs (□), Enterobacteriaceae (●), and lactobacilli (○) counts and D-(−) (▲) and L-(+)-lactic acids (△) contents on refrigerated pork (2 °C) stored in CO_2/air (20/80) (v/v) (a) and CO_2/O_2 (20/80) (v/v) (b) atmospheres.

times with those of authentic standards (BDH) and quantified by peak measurement against a normalized internal standard.

For diamine determination, another aliquot of ground meat was treated, for protein precipitation, with $HClO_4$ (final concentration 7% w/v), and after centrifugation (10000*g* for 10 min) at 5 °C, the supernatant fluid was neutralized with $KHCO_3$ and filtered through No. 54 Whatman paper. Diamines were extracted according to the scheme of Spinelli et al. (1974) and analyzed by HPLC in a Beckman Model 332 apparatus equipped with Model 110 A pumps, a Spectra/flo fluorometer and a column (25 cm × 4.6 mm) filled with ODS-2 on Spherisorb. The chromatography was carried out according to a modification of the method described by Wiedmeir et al. (1982). Resolution of the peaks was accomplished by using a gradient elution, with the mobile phase in pump A consisting of pH 4.2, 0.01 M sodium acetate buffer/tetrahydrofuran (95/5) (v/v) and that in pump B of acetonitrile/tetrahydrofuran (90/10) (v/v). The mobile phase was begun at a flow rate of 1.5 mL/min at 40% B and increased to 90% B over 25 min and then held at this ratio for 2 min. At the end of the isocratic period the percentage of B was lowered to 40%. The column was then reequilibrated at this percentage of B for an additional 8 min. Diamine quantification was made by area measurements normalized against 1,6-hexanediamine (Sigma) used as internal standard.

The meat extract used for the diamine determination was also utilized for the assay of D-(−)- and L-(+)-lactic acid and acetoin/diacetyl. Lactic acid isomers were determined enzymatically by the lactic acid dehydrogenase method, with kits from Boehringer Mannheim, according to the instructions provided. Acetoin/diacetyl was assayed according to the method described by Westerfield (1945). Since this method is based on the oxidation of acetoin to diacetyl in alkaline medium, the reading of absorbance (530 nm) of samples after 5–10 min of reaction was assumed to be due to the diacetyl content and after 90 min to the acetoin/diacetyl concentration.

Statistical Analysis. The relationship among microbial and chemical data was analyzed by utilizing the Cricket Graph program (Cricket software) with a Macintosh ED computer.

RESULTS AND DISCUSSION

Microbial Flora. Figure 1 shows the changes in the different microbial groups during storage of refrigerated pork in CO_2/air (20/80) (v/v) and CO_2/O_2 (20/80) (v/v) atmospheres. After 15 days of storage, the growth of the different microbial groups was similar under both gas mixtures. Bacterial numbers on pork reached 10^8 CFU cm^{-2} by day 15 and remained at this value for 10 additional days. Counts on MRS and VRBG were quite similar (10^4–10^5 CFU cm^{-2}) and thus represented, at the end of the experiment, from 0.01 to 0.1% of the aerobic count. This indicates that neither *Lactobacillus* spp nor Enterobacteriaceae was the dominant flora. Characterization of the dominant flora after 22–25 days of storage showed that the dominant organism (85–92%) was *B. thermosphacta*. *Lactobacillus* spp and Enterobacteriaceae were present in much smaller numbers at a ratio of approximately 1:1. Similar results have been found for meat stored in CO_2-enriched atmospheres (Asensio et al., 1988). These changes are due to the well-known inhibition of Gram-negative organisms by CO_2. The similar microbial changes observed in BB-1 bags filled with each CO_2-enriched gas mixture indicate that oxygen concentrations, at least between 16 (in CO_2/air) and 80% (in CO_2/O_2), have no influence on the dominant organisms. However, the microbial pattern observed in CO_2-enriched atmospheres is different from that found in vaccum-packed meat. In the latter, lactic acid bacteria are the dominant organisms (Dainty et al., 1979; Simard et al., 1985) and *B. thermosphacta* and Enterobacteriaceae have been also detected in small proportions (Simard et al., 1985; Asensio et al., 1988).

In both atmospheres, the members of the panel described the odors after 15 days of storage as dairy/cheesy/sour/acid, not clearly unpleasant.

D-(−)- and L-(+)-Lactic Acids. Figure 1 also shows the levels of D-(−)- and L-(+)-lactic acids accumulated during storage. No consistent changes were observed in the L-(+)-lactic acid levels (350–450 mg/100 g) of samples in either CO_2 or CO_2/O_2 enriched atmospheres throughout the first 10–15 days of storage. However, there was a small but progressive increase in L-(+)-lactic acid concentrations during the final days of storage, reaching levels of 500–700 mg/100 g at the end of the storage period. The mean levels of L-(+)-lactic acid observed during the first phase of storage are in the range of those reported for the typical composition of meat (Nottingam et al., 1981). Thus, it seems appropriate to infer that the L-(+)-lactic acid comes from its major source in muscle, i.e., anaerobic glycolysis, while the increase observed in the final period may be due to the metabolism of glucose by *B. thermosphacta*, which generates only the L-(+) isomer (Davidson et al., 1968).

It was possible to detect small amounts of D-(−)-lactic acid after storage for about 10 days. Its concentration then increased progressively during storage, reaching final levels of 12–18 mg/100 g. These values are very low when compared to those reached in vacuum-packed meat, where values about 10-fold higher have been reported (de Pablo et al., 1989). Obviously, the D-(−) isomer does not come from either *B. thermosphacta* or anaerobic glycolysis because in both instances only L-(+)-lactic acid is produced. Therefore, it has to come from the LAB metabolism that may generate D-(−)-, L-(+)-, or DL-lactic acids (Shaw and Harding, 1984). The low levels found are consistent with the relatively small counts of LAB observed (no more than 10^6 CFU cm^{-2}).

Short-Chain Fatty Acids. Table I shows the levels of short-chain fatty acids at various times throughout the storage period. Results in both CO_2 and CO_2/O_2 enriched atmospheres were similar. Acetic acid showed a clear rising trend from the beginning. A similar trend was observed for propionic, isobutyric, and isovaleric acids but only from about the 10th day of storage. No consistent patterns

Table I. Concentrations (μg/g) of Short-Chain Fatty Acids on Meat during Refrigerated Storage in CO_2/Air (20/80) (v/v) (Upper) and CO_2/O_2 (20/80) (v/v) (Lower) Atmospheres

time, days	acetic	propionic	isobutyric	*n*-butyric	isovaleric
0	5.2	Tr[a]	Tr	1.6	ND[b]
2	7.5	Tr	Tr	2.0	ND
4	8.3	Tr	Tr	1.5	ND
7	11.0	Tr	Tr	1.5	ND
11	37.0	Tr	Tr	1.5	0.7
15	NA[c]	1.6	4.6	2.0	1.6
21	49.0	Tr	4.0	Tr	6.0
26	63.0	0.6	6.0	Tr	8.0
0	5.2	Tr	Tr	1.6	ND
2	8.0	Tr	Tr	0.5	ND
4	9.1	Tr	Tr	0.4	ND
7	13.6	Tr	Tr	1.1	ND
11	20.6	0.3	0.4	0.2	0.2
15	27.2	1.1	0.9	1.1	2.3
21	44.3	2.2	5.4	Tr	4.4

[a] Tr, trace amounts. [b] ND, not detected. [c] NA, not analyzed.

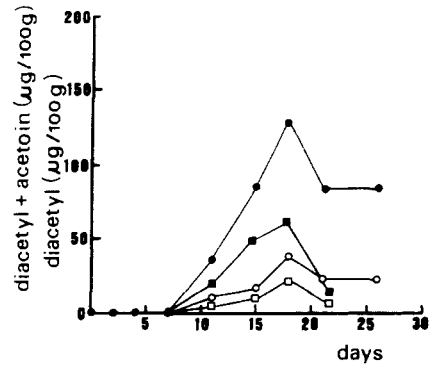

Figure 2. Changes in diacetyl (open symbols) and diacetyl plus acetoin (solid symbols) contents on refrigerated pork (2 °C) stored in CO_2/air (20/80) (v/v) (circles) and CO_2/O_2 (20/80) (v/v) (squares).

emerged for *n*-butyric acid, and *n*-valeric acid was not detected. The metabolism of *B. thermosphacta* may be either anaerobic or aerobic. Under anaerobic conditions, glucose is metabolized by *B. thermosphacta* yielding, as main metabolites (85–90% of utilized glucose), L-(+)-lactic acid and ethanol in proportions close to 3:1 (Hitchner et al., 1979) but no acetoin (Hitchner et al., 1979; Dainty et al., 1979) and no (Hitchner et al., 1979), or small amounts of (Dainty et al., 1979), short-chain fatty acids are produced. The *n*-butyric acid is probably not produced by the microorganisms present (Dainty et al., 1979). On the other hand, under aerobic conditions, *B. thermosphacta* produces acetoin and acetic, isobutyric, and isovaleric acids (Dainty and Hibbard, 1980, 1983; Dainty and Hofman, 1983), 3-methylbutanol (Dainty and Hofman, 1983), and 2-methylbutyric acid (Dainty and Hibbard, 1983). In addition, several environmental factors might greatly influence its aerobic metabolism, such as oxygen and glucose concentration and pH (Dainty and Hibbard, 1980). No report on the effect of CO_2 or CO_2 and O_2 enriched atmospheres on the short-chain fatty acids formation during storage is available. However, the data presented in Table I suggest that *B. thermosphacta* present on refrigerated meats stored in modified atmospheres showed aerobic metabolism.

Acetoin/Diacetyl. The formation of acetoin/diacetyl is shown in Figure 2. These compounds, although absent at the beginning of the experiment, increased from day 7 to day 18 of storage and then decreased afterward. As it has been suggested above, the metabolism of *B. thermosphacta* in refrigerated meat stored in CO_2 or CO_2 and O_2 enriched atmospheres might be mainly aerobic. Thus, this bacterium could produce diacetyl/acetoin from glucose (Dainty et al., 1983), a reaction which is, in turn, favored by the low pH of meat (Dainty and Hibbard, 1980, 1983). The decrease observed in the diacetyl/acetoin levels during the final days of storage may be due to the microbial activity once the glucose has been depleted. *B. thermosphacta*, *Lactobacillus*, and Enterobacteriaceae contain the enzyme diacetyl reductase and reversible butyleneglycol dehydrogenase, which would reduce diacetyl to acetoin and the latter to butyleneglycol (Branen and Keenan, 1970). In addition, the butyleneglycol may be converted to other substances, such as acetic acid (Dainty and Hofman, 1983). Both butyleneglycol and the substances resulting from the enzymatic reduction of butyleneglycol give a negative response in the Westerfield test used for diacetyl/acetoin detection.

Diamines. Table II shows the amine concentrations of refrigerated pork meat stored in CO_2/air (20/80) (v/v) and CO_2/O_2 (20/80) (v/v) atmospheres. Putrescine and cadaverine showed a constant increase during storage, with cadaverine reaching higher values than putrescine. Spermine and spermidine stayed at similar levels during the whole period of storage. A small increase in tyramine levels was observed during the last half of storage. Several workers have reported the formation of diamines on meats stored aerobically (Nakamura et al., 1979; Edwards et al., 1985) and in vacuum packages (Edwards et al., 1985), but there do not appear to be any studies on the effect of modified atmospheres on diamine formation. In contrast to the formation of short-chain fatty acids and D-(−)-lactic acid, formation of diamines in meat stored under these atmospheres was quite similar to that in vacuum-packed meat (Edwards et al., 1985). In meat held under aerobic conditions, increasing levels of putrescine and cadaverine were always detected (Nakamura et al., 1979; Edwards et al., 1983); however, the levels of putrescine were always higher than those of cadaverine (Edwards et al., 1983). These amines are produced by the metabolic activities of *Pseudomonas* and Enterobacteriaceae, respectively (Slemr, 1981). In vacuum-packed meat the situation is different. Even though increasing amounts of both amines were observed (Edwards et al., 1985), their levels were always lower than under aerobic conditions, with cadaverine being dominant (Edwards et al., 1985). Lactic acid bacteria are not diamine producers (Dainty et al., 1986), and Enterobacteriaceae are regularly present on vacuum-packed meat (Dainty et al., 1979). Thus, the latter bacterial types are the ones likely responsible for the amine formation. Similarly to lactic acid bacteria, *B. thermosphacta* does not produce amines (Edwards et al., 1985). Therefore, although the microbial profile of vacuum-packed meat is different from that of meat stored in CO_2/air (20/80) (v/v) or CO_2/O_2 (20/80) (v/v) atmospheres, no difference exists in amine production. The formation of these latter compounds is due to the Enterobacteriaceae, most probably *Hafnia alvei* and *Serratia liquefaciens*, which are very common in meats (Lee et al., 1985). Cadaverine is probably formed by lysine decarboxylation by these bacteria without the cooperation of other organisms, while putrescine generation requires the presence of bacteria which use arginine for ornithine production, the latter being decarboxylated by Enterobacteriaceae (Dainty et al., 1986).

The relationship between bacterial count and the concentration of the various compounds produced on pork during storage in modified atmospheres was investigated by plotting the $\log_{10}$ bacterial count against the $\log_{10}$

Table II. Concentrations (μg/g) of Amines on Meat during Refrigerated Storage in CO_2/Air (20/80) (v/v) (Upper) and CO_2/O_2 (20/80) (v/v) (Lower) Atmospheres

time, days	tryptamine	putrescine	cadaverine	tyramine	spermidine	spermine
0	ND[a]	0.1	0.1	0.6	0.9	15.3
2	ND	0.3	0.2	ND	1.0	14.3
4	1.8	0.2	3.2	ND	0.6	8.3
7	ND	0.2	0.6	ND	0.5	6.9
11	1.3	3.9	4.3	0.3	2.8	3.8
15	3.4	3.5	6.6	0.2	0.6	7.1
18	ND	19.4	29.6	2.6	1.9	12.2
21	1.5	17.9	33.2	1.1	2.5	28.1
26	ND	28.6	60.0	1.3	0.9	10.0
0	ND	0.2	0.1	ND	0.9	15.3
2	ND	0.4	0.5	ND	1.0	16.6
4	ND	0.5	1.7	ND	3.5	45.2
7	ND	0.3	6.1	Tr[c]	1.2	18.5
11	ND	NA[b]	6.0	Tr	0.6	22.8
15	2.5	0.2	10.8	0.4	0.9	18.8
18	1.8	1.7	12.6	0.4	0.9	18.7
21	ND	6.6	39.6	0.7	3.2	26.5

[a] ND, not detected. [b] NA, not analyzed. [c] Tr, trace amounts.

Table III. Relationships between Bacterial Counts in Different Culture Media and Several Substances Formed on Meat during Storage in CO_2/Air (20/80) (v/v) and CO_2/O_2 (20/80) (v/v) Atmospheres[a]

relationship	equation	R^2	n
log acetic acid vs log PCA	$y = 0.4895 + 0.1485x$	0.90	13
log L-(+)-lactic acid vs log PCA	$y = 1.4400 + 0.0724x$	0.37	16
log D-(−)-lactic acid vs log PCA	$y = -0.5075 + 0.3259x$	0.86	16
log putrescine vs log PCA	$y = -1.2356 + 0.2429x$	0.58	15
log cadaverine vs log PCA	$y = -1.0748 + 0.3093x$	0.67	16
log acetic acid vs log VRBG	$y = 0.6863 + 0.1988x$	0.94	13
log putrescine vs log VRBG	$y = -1.0864 + 0.3799x$	0.82	15
log cadaverine vs log VRBG	$y = -0.6625 + 0.4158x$	0.89	16
log acetic acid vs log MRS	$y = 0.6643 + 0.1912x$	0.90	13
log D-(−)-lactic acid vs log MRS	$y = -0.0720 + 0.4111x$	0.81	16
log L-(+)-lactic acid vs log MRS	$y = 1.4738 + 0.1105x$	0.49	16

[a] Results from meat stored in both atmospheres were combined. See text. y = $\log_{10}$ of substance concentration (μg/g); x = log of bacterial counts (CFU cm^{-2}) in the corresponding culture medium. PCA, total viable counts; VRBG, Enterobacteriaceae counts; MRS, lactic acid bacteria counts.

concentration of the compounds produced during storage (the CO_2/air and CO_2/O_2 data were combined). The results are shown in Table III. There was a correlation ($R^2 \geq 0.90$) between the total aerobic count, the lactobacilli count, and the Enterobacteriaceae count and the production of acetic acid. Also, there was a correlation between the level of Enterobacteriaceae and the production of cadaverine and putrescine ($R^2 \geq 0.82$). However, there was no correlation between these amines and the aerobic count ($R^2 \leq 0.67$). Obviously, these latter results are related to the ability of Enterobacteriaceae (Edwards et al., 1985) and the inability of *B. thermosphacta* (Edwards et al., 1985) to produce these diamines. Similarly, a correlation ($R^2 = 0.81$) was obtained between lactobacilli counts and D-(−)-lactic acid concentration. Since *B. thermosphacta*, the dominant organism, produces only L-(+)-lactic acid, the high correlation ($R^2 = 0.86$) obtained for D-(−)-lactic acid versus aerobic counts might well be due to the parallelism observed in the counts on PCA and MRS media (Figure 1), although the levels of the latter were about 3 log units lower. As expected, a very low correlation ($R^2 \leq 0.49$) was found between L-(+)-lactic acid and the counts of the different bacterial groups. The data shown in Table III indicate that determination of acetic acid and cadaverine concentrations in meats stored in modified atmospheres may show promise as a rapid method for assessing shelf life.

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