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Fate of Free Amino Acids and Nucleotides in Spoiling Beef

JAMES M. JAY AND KALLIOPI S. KONTOU[1]
Department of Biology, Wayne State University, Detroit, Michigan 48202

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Fresh beef allowed to undergo microbial spoilage at 7 C showed decreases in quantity and types of amino acids as well as decreases in nucleotides when the initial number of bacteria per gram was high. When the initial number of bacteria was low, decreases were either not detectable or of low orders. It seems, therefore, that low-molecular-weight compounds of the above type support the growth of beef-spoilage bacteria rather than primary beef proteins. These low-molecular-weight compounds provide the probable sources of ammonia, hydrogen sulfide, and other such compounds associated with beef allowed to spoil at low temperatures.

Previous reports from this laboratory presented evidence for a general lack of breakdown of major beef proteins by the usual low-temperature spoilage flora when fresh beef was held until frank spoilage occurred (4). Some investigators have shown a lack of proteolysis under conditions of aseptic postmortem incubation of beef muscles, and others have shown the occurrence of this phenomenon but only to a small degree (1). In view of the fact that the evidence for some type of protein breakdown by either beef spoilage flora or postmortem autolysis seems not to be clear, it was of interest to try to determine the nature of the substrates upon which meat spoilage bacteria subsist while growing in and upon beef. In a previous report, it was shown that the low-molecular-weight proteins of the sarcoplasm constitute one source of readily utilizable compounds (4). Since fresh beef is known to contain around 0.42% low-molecular-weight compounds such as free amino acids and nucleotides, the aim of this study was to determine the fate of these substances in beef held from freshness to spoilage at low temperatures. The probable origin of volatile foul-smelling compounds often associated with such beef spoilage is also discussed.

MATERIALS AND METHODS

Meats employed in this study consisted largely of single beef muscles obtained 24 hr after slaughter and held at 5 to 7 C. The meat was trimmed of visible fat, ground in the laboratory, and placed in 100-ml beakers covered with several layers of aluminum foil. Enough samples were prepared to allow for the use of duplicates on test days, and the quantity of meat per beaker was sufficient to make all necessary determinations. For the determination of free amino acids, 10 g of beef was boiled for 10 min in 50 ml of *p*H 5.0 buffer. The supernatant fluid was collected after centrifugation and the supernatant fluids from two additional washings, each consisting of 10 ml of distilled water, were added to the original. The supernatant fluids, consisting of free amino acids, were desalted by essentially the procedure described by Furuholmen et al. (2) with the use of a Dowex 50-X8 cation-exchange resin in hydrogen form. After desalting, the amino acid mixture was reduced to a volume of approximately 5 ml by boiling in a water bath. Each sample was then made up to 10 ml by use of HCl at a concentration sufficient to give a final concentration of 1 N in the amino acid sample. The one-dimensional paper chromatography procedure of Furuholmen et al. (2) was employed. This procedure involves the use of Whatman no. 3 MM paper and three solvent passes with butanone-water-propionic acid-*t*-butanol (75:30:25:20, v/v) as solvent. The amino acid control consisted of 18 known amino acids made up in 1 N HCl to give a final concentration of 0.02 M. Samples were applied to the paper by means of a calibrated heavy-guage wire loop, with flaming of the loop between samples. For the application of many samples, preliminary work showed this procedure to be more practical than using microliter pipettes, and differences in accuracy between the two were insignificant. Two loops of each preparation (including the control) were applied at the point of origin, with complete drying of the first spot being allowed before addtion of the second. The papers were air-dried and developed by spraying with 0.2% ninhydrin in acetone.

For the extraction of nucleotides from beef, the method of Lento et al. (8) was employed. Samples containing extracted nucleotides were read at 2,600 A by use of a Beckman DB-G spectrophotometer. The blank was prepared in the same way as the extractions for nucleotides, but the meat sample was omitted.

[1] Present address: University of Michigan Medical School, Ann Arbor 48104.

TABLE 1. *ERV, log bacterial numbers, percentage of protein, and pH from unirradiated control SM muscle held 15 days*

Days held	ERV	Log no./g	Protein (%)	pH
0	27	3.40	14.7	6.30
5	23	8.43	17.5	6.68
8	4	9.81	—	7.20
12	1	10.38	18.5	7.24
15	0	10.00	18.5	7.60
Mean	11.00	8.40	17.30	7.00

To determine the effect of pure cultures, ground beef was placed in 100-ml beakers, covered with aluminum foil, and subjected to 1,000,000 rad of cobalt-60 radiation from a 10,000 c source (University of Michigan, Ann Arbor). Preliminary work showed that this dosage was sufficient to destroy all low-temperature growers without changing the capacity of the meat to undergo normal spoilage when inoculated with a mixed flora. Bacterial numbers, extract-release volume (ERV), and *p*H were determined as previously described (3).

RESULTS

In an attempt to establish whether or not beef spoilage was accompanied by an accumulation of amino acids from protein breakdown, the percentage of protein in fresh and spoiled semimembranous (SM) muscle was determined by the Kjeldahl method on trichloroacetic acid insoluble matter. As shown in Table 1, no decrease in percentage of protein occurred by this procedure when the meat was held for 15 days. There appeared to be a slight increase in protein upon spoilage, and this might possibly represent a conversion of nonprotein nitrogen to protein nitrogen by the spoilage flora. These data suggest that amino acids which exist in spoiling beef may be taken to represent free amino acids for the most part, and not those from protein breakdown. The degree of spoilage of this meat is reflected by both bacterial numbers per gram and ERV. The use of the latter phenomenon as a measure of beef microbial quality has already been discussed (5).

Figure 1 presents comparative one-dimensional chromatograms from beef SM muscle held 15 days at 7 C, along with a standard of 18 amino acids. This meat showed organoleptic signs of spoilage on the 5th day, at which time the log bacterial number per gram was 8.43. An inspection of the comparative chromatograms for the five test periods over the total 15-day incubation time reveals no consistent decrease in amino acids, except for cystine which almost disappeared by the 15th day. The 12- and 15-day samples contained higher quantities of amino acids than samples on earlier days. From these chromatograms alone, it appeared that spoiled beef contained approximately the same quantity of amino acids as fresh beef. A similar pattern was found with other samples of SM muscle, as well as with samples of mixed muscles. In all such cases, the initial bacterial number per gram was relatively low, being 3.40 in the case of SM muscle presented in Fig. 1. However, when the initial bacterial load was found to be relatively high, a greater decrease of amino acids occurred with the onset of spoilage and thereafter. This pattern appeared to hold whether pure cultures or mixed cultures were employed.

Figure 2 presents comparative chromatograms from irradiated beef inoculated with a fluorescent *Pseudomonas* sp. and held for 15 days also at 7 C. All amino acids decreased sharply upon holding. The log bacterial number per gram increased sharply, from 6.74 to 9.28 in 5 days and to 10.24 in 8 days. There was a decrease in numbers at 12 and 15 days. The only detectable amino acid on the 15th day was lysine.

That the decrease was not the result of the irradiation treatment can be seen from Fig. 3. Samples of irradiated but uninoculated SM muscle were held under similar conditions with no apparent decrease in amino acids. Of interest

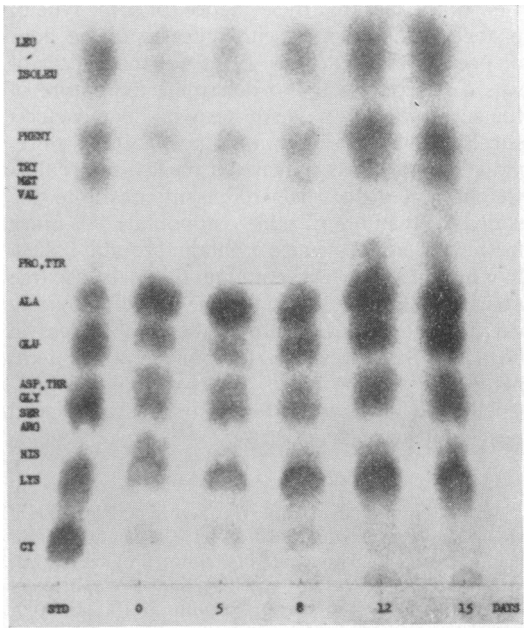

FIG. 1. *Comparative amino acid chromatograms from SM beef muscle allowed to spoil with its own flora at 7 C over a 15-day period along with a standard of 18 amino acids.*

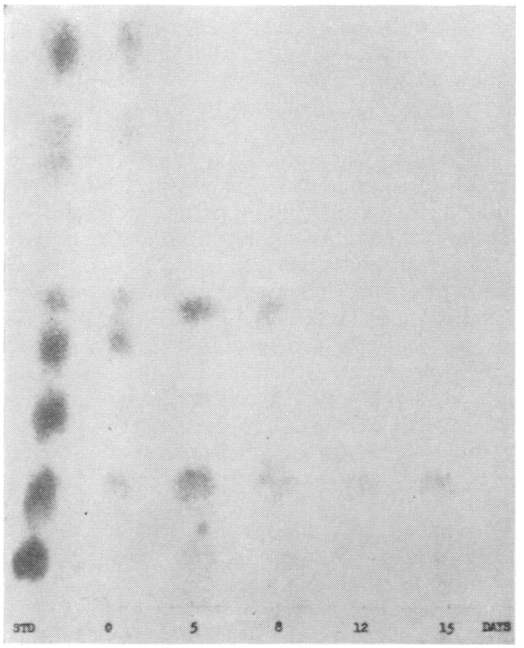

FIG. 2. *Comparative chromatograms of amino acids from irradiated SM muscle inoculated with a fluorescent Pseudomonas and held at 7 C for 15 days.*

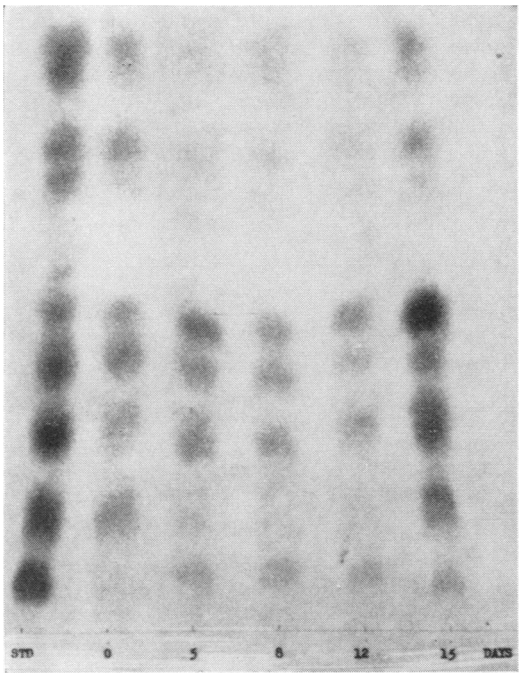

FIG. 3. *Comparative chromatograms of amino acids from irradiated but uninoculated SM muscle held at 7 C for 15 days.*

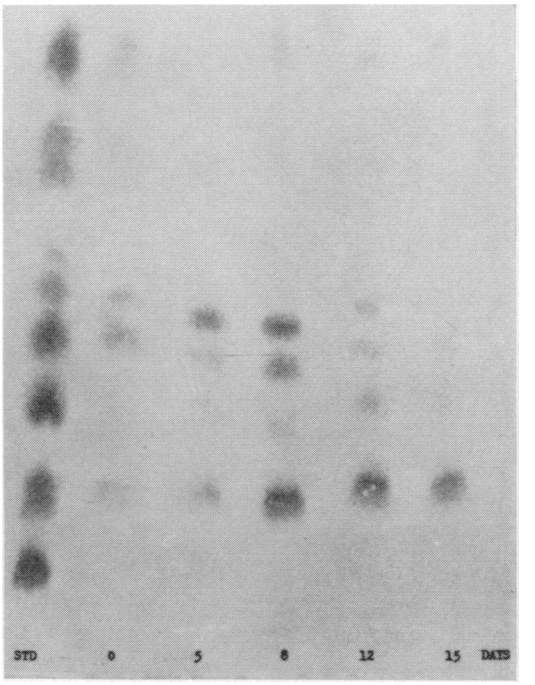

FIG. 4. *Comparative chromatograms of amino acids from irradiated SM muscle inoculated with approximately equal numbers of fluorescent Pseudomonas and Achromobacter sp. and held at 7 C for 15 days.*

here is the fact that the 15-day sample again showed higher concentrations than earlier samples. Since these samples remained essentially free from bacteria (no colonies on 1:100 plate dilutions), this finding might be interpreted to mean that some aseptic autolytic process was in effect which increased the amino acids upon holding despite the absence of detectable protein breakdown as previously mentioned.

Figure 4 presents chromatograms from irradiated SM muscle inoculated with approximately equal numbers of a fluorescent *Pseudomonas* sp. and an *Achromobacter* sp. Again, a decrease in amino acids occurred with increasing bacterial growth and spoilage. In this case, the initial count of log 7.30/g increased to 11.38 in 8 days before falling off to 9.99 in 15 days. Amino acids detectable on the 15th day were lysine, serine, glutamic acid, alanine, leucine, and isoleucine.

The decrease in amino acids as beef underwent microbial spoilage is substantiated by data of another type. Figure 5 presents data on ninhydrin-reactive substances from ERV extracts of ground beef held for 9 days at 7 C along with ERV and total numbers of cocci and gram-negative bacteria. As the meat spoiled, a significant decrease in

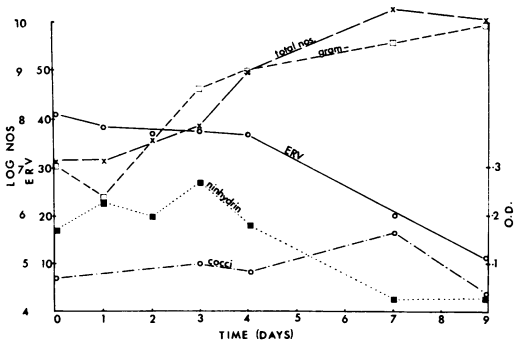

FIG. 5. *Relationship between ninhydrin-reactive substances (amino acids), bacterial numbers, and ERV on ground beef held for 9 days at 7 C.*

ninhydrin-reactive compounds occurred. It can be seen also from Fig. 5 that spoilage was the property of gram-negative bacteria (determined by growth on MacConkey agar) and not gram-positive cocci (determined by growth on mannitol-salt-agar).

The fate of nucleotides during the bacterial spoilage of SM muscle under five different treatments is presented in Table 2. In the unirradiated control samples which spoiled from their own natural flora, the initial optical density level of 0.62 showed a mean decrease of around 9% over the 15-day holding period, whereas the bacterial number increased from log 3.40 to 10.38 in 12, 10.00/g in 15 days. The correlation coefficient (r) between bacterial numbers and nucleotide decrease was of no statistical significance. Cobalt-60 irradiation had the effect of reducing the optical density nucleotide level from 0.62 to 0.56. It can also be seen from Table 2 that the irradiated but uninoculated samples showed essentially the same levels over the 15-day period with an overall mean decrease of around 10%.

To obtain the total flora from a sample of retail-store ground beef, 1 g was placed in a sterile dilution blank with 99 ml of tap water and was shaken briskly. After allowing meat particles to settle, 1-ml quantities were removed with needle and syringe and inoculated into meat samples by repeated jabs. When irradiated SM muscle was inoculated with such total beef flora, nucleotide levels decreased around 15% over a 15-day period, and the flora increased from log 6.18 to 10.46 at 12 days and 10.04/g over 15 days (r not significant). When an equal mixture of a fluorescent *Pseudomonas* sp. and an *Achromobacter* sp. (both recovered from spoiled beef) were inoculated into irradiated SM, nucleotides showed a mean decrease of over 43% and, the log bacterial number per gram increased from 7.30 to 10.61 in 12 days and then became 9.99 in 15 days ($P < 0.05$). Similar results were obtained when a fluorescent pseudomonad and *P. fragi* (ATCC 4973) were inoculated separately under similar conditions. The former organism caused about a 45% decrease in nucleotides ($P < 0.05$) and the latter reduced the initial level by around 40% ($P < 0.02$). Again, the greatest decreases in nucleotides were associated with the higher initial bacterial numbers, as was the case with amino acids previously mentioned.

The correlation between nucleotide level, ERV, and *p*H as beef spoiled is presented in Fig. 6

TABLE 2. *Log bacterial numbers and nucleic acid levels from five samples of beef held from freshness to spoilage at 7 C*

Days held	Control		Irradiated control		Irradiated + total flora[b]		*Pseudomonas-Achromobacter*		Fluorescent-pseudomonad		*Pseudomonas fragi*	
	Log no.	N.A.[a]	Log no.	N.A.	Log no.	N.A.	Log no.	N.A.	Log no.	N.A.	Log no.	N.A.
0	3.40	0.62	<2	0.56	6.18	0.56	7.30	0.56	6.74	0.56	7.28	0.56
5	8.43	0.38	<2	0.56	9.62	0.45	9.34	0.31	9.28	0.41	9.42	0.40
8	9.81	0.51	<2	0.46	10.02	0.35	11.38	0.22	10.24	0.41	10.19	0.19
12	10.38	0.45	<2	0.52	10.46	0.48	10.61	0.23	10.19	0.18	10.04	0.25
15	10.00	0.57	<2	0.55	10.04	0.42	9.99	0.18	9.95	0.18	9.81	0.22
Mean	8.40	0.51	<2	0.53	9.26	0.45	9.72	0.30	9.28	0.29	9.35	0.32
SD	2.59	0.08	0	0.04	1.57	0.07	1.39	0.13	1.32	0.16	1.07	0.14
r[c]	−0.523				−0.716		−0.908*		−0.932*		−0.942**	

[a] Nucleic acid.
[b] Irradiated samples were inoculated with the total flora from unirradiated beef.
[c] * Significant at the 5% level. ** Significant at the 2% level.

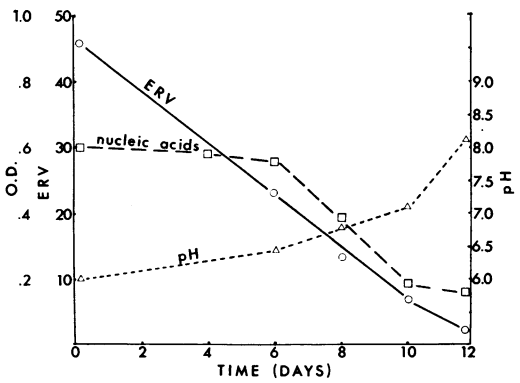

FIG. 6. *Correlation between nucleotides, ERV, and pH on LD muscle held at 7 C for 12 days.*

where longissimus dorsi (LD) muscle was employed and held at 7 C for 12 days. Between ERV and nucleotides, the data were statistically significant at the 2% level; between *p*H and nucleotides, they were significant at the 5% level.

To determine the relative effectiveness of beef isolates in utilizing or degrading beef nucleotides at 7 C, nucleotide preparations (without ether and trichloroacetic acid) were filter-sterilized by use of 0.2-μ Metricel membranes (Gelman Instrument Co., Ann Arbor, Mich.) and were placed into sterile tubes. Tubes were inoculated with 34 strains of beef isolates previously described and characterized (6) and were incubated for 11 days. The tubes were read at 2,600 A after filter sterilizing, and the percentage of decrease in nucleotides by the organisms from the initial level was calculated. Of the 34 strains inoculated, only 26 grew in the nucleotide preparation. The results are presented in Table 3, where the percentage of decrease or utilization ranged from 72 to 9, with an average of around 40 over the 11-day period. The most effective strain was a *Pseudomonas* sp., which was the most commonly occurring one in fresh and spoiled beef (6). It is of interest that *P. fragi* reduced nucleotide by only 28%, whereas the same organism caused a 40% decrease of these substances when inoculated in whole beef. The five most effective utilizers of nucleotides were *Pseudomonas* strains, and the five least effective strains consisted of only one *Pseudomonas* along with two strains of *Flavobacterium* and one each of *Achromobacter* and *Proteus*.

TABLE 3. *Decrease of nucleotides by 26 strains of bacteria of beef origin (6) and two control strains incubated at 7 C for 11 days*

Genus	No. of strains	Avg decrease
		%
Pseudomonas	18	44
Aeromonas	3	48
Achromobacter	2	28
Flavobacterium	2	17
Proteus	1	9
P. fragi (ATCC 4973) and *P. taetrolens* (ATCC 4683)	2	28

DISCUSSION

With respect to the apparent lack of amino acid decrease upon spoilage with an initially low microbial number, the possibility exists that the early flora subsisted upon a limited supply of other low-molecular-weight compounds which exerted a sparing effect upon amino acid utilization. The faster utilization of amino acids by a larger flora would then reflect faster depletion of the other limited but more utilizable substrates. Fresh beef is laden with numerous low-molecular-weight compounds such as carnosine, lactic acid, inosine, vitamins, and many low-molecular-weight enzymes (Table 4). Unfortunately, data on the relative rate of attack of such compounds and amino acids by beef spoilage bacteria seem not to be available. It is known that amino acids are quite readily utilized by bacteria, but it does not necessarily follow that the same is true when one considers the meat environment. On the basis of

TABLE 4. *Chemical composition of typical adult mammalian muscle after rigor mortis but before degradative changes post-mortem*[a]

Component	Percentage
Water	75.5
Protein	18.0
Myofibrillar	10
Sarcoplasmic	6
Others	2
Soluble nonprotein substances	3.5
Nitrogenous (creatine, inosine monophosphate, nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate amino acids, carnosine, anserine)	1.57
Carbohydrate (lactic acid, glucose-6-phosphate, glycogen, glucose)	1.18
Inorganic (soluble phosphorus, K^+, Na^+, Mg^{++}, Ca^{++}, Zn^{++})	0.63
Fat	3.0
Others (trace metals, vitamins, etc.)	0.1

[a] After Lawrie (7).

molecular size alone, one would expect amino acids to be utilized by bacteria preferential to nucleotides, and in this sense the slower attack on the latter compounds by an initially small flora as compared to a higher attack by a larger initial flora would seem to be consistent. The finding that uninoculated irradiated beef showed an increase in amino acids upon ageing suggests that additional compounds of this type were liberated, which, in the case of an initially small number of bacteria, would mean less decrease in the total quantity of amino acids during spoilage. In a study employing LD muscle held at 2 C for 30 days, Davey and Gilbert (1) found a 2.3% degradation of meat proteins, which was detectable as nonprotein nitrogen. The exact nature of the nonprotein nitrogen compounds was not reported, however.

In a previous report from this laboratory (4), beef sarcoplasmic proteins were shown to decrease upon beef spoilage, and the data in the present report appear to indicate that free amino acids and nucleotides are also utilized by the spoilage flora before complex proteins are attacked during low-temperature beef spoilage. Since evidence has not been presented to show that the organisms "chop off" amino acids, amino, and $-SH$ groups from proteins, it seems reasonable to conclude that ammonia, hydrogen sulfide, indole, and other foul-odor compounds usually associated with spoiling meats owe their origin to the free amino acids and other low-molecular-weight compounds rather than to the larger proteins. In regard to the overall question as to specifically how beef spoils, it is also conceivable that the complex beef proteins are not attacked until the more readily utilizable substances have been depleted. This view is consistent with the known facts surrounding the degradation of macromolecules under other conditions, such as in the bovine rumen, soils, etc. It is obvious, however, that more data are necessary before the exact mechanism by which beef spoils at low temperatures is known.

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