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CHANGES IN NONPROTEIN NITROGEN COMPOUNDS DURING DRY SAUSAGE RIPENING

INTRODUCTION

IT IS well known, that the concentration of water soluble nitrogen compounds in dry sausage increases during ripening and can reach values up to 25% of the total nitrogen (Maillet and Henry, 1960; Niinivaara et al., 1961; Mihalyi and Körmendy, 1967). The composition and concentration of several groups of these compounds, such as free amino-acids, peptides, nucleotides and nucleosides determine to a large extent the final aroma of dry sausage (Dahl, 1970).

The availability of automated analysis has recently intensified research into free amino acid production in dry sausage (Reuter and Langner, 1968; Langner, 1969) which is at least partly due to bacterial protease activity (Pohja and Niinivaara, 1966; Sajber et al., 1971). Also, Canto.ii et al. (1967) stated that the major nucleotide present initially in sau- is inosinic acid (IMP) formed by deamination of adenylic acid, soon after rigor mortis. During ripening, phosphomonoesterase and nucleosidase activity produce inosine nucleoside and hypoxanthine respectively from inosinic acid. Langner (1972) determined ammonia on 12 different brands of dry sausage, whereas Niinivaara et al. (1961), Körmendy and Gantner (1962) and Stanculescu et al. (1970) report values for total free α-amino acid nitrogen (α-NH_2-N) in dry sausages.

We are not aware, however, of any work describing the quantitative contribution of different compounds to the total nonprotein-nitrogen fraction (NPN fraction) at various stages of dry sausage ripening.

In this paper, we report changes in different groups of NPN compounds during dry sausage ripening as influenced by the presence of a "starter culture." The NPN compounds studied include ammonia, free amino acids, peptides, nucleotides, nucleosides and amines.

EXPERIMENTAL

Preparation of sausages

Two batches of sausages, referred to as expt 2 and 3 respectively, were used. The preparation, composition, change in dry matter content (D.M.), pH, concentration of carbohydrates and concentration of carbohydrate metabolism products of these sausages are reported in an accompanying paper (De Ketelaere et al., 1973).

Sampling procedure

The samples used for analysis were those obtained as described by De Ketelaere et al. (1974).

Analytical methods

Determination of total NPN and individual NPN fractions. Different NPN extraction methods (ethanol 80% v/v, trichloroacetic acid 10% w/v, $ZnSO_4$ 10% w/v treated with an equal volume of 0.5N NaOH, and $HClO_4$ 0.6N) were compared. As it was found that $HClO_4$ 0.6N extracted the highest amount of total N (Table 1) it was used in further experiments.

5g of sample were homogenized in 25 ml of 0.6N $HClO_4$ as described earlier (De Ketelaere et al., 1974). After filtration, neutralization

Table 1—Comparison of different NPN extraction methods (mg N/g sausage extracted)

Extraction agent used	EtOH 80%	TCA 10%	$HClO_4$ 0.6N	$ZnSO_4$ 10%
Nitrogen recovered				
Total N (NPN)	5.40	6.09	6.33	4.34
NH_3	0.55	0.67	0.69	0.62
Free α-NH_2-N	2.46	2.19	2.19	2.50

Table 2—Concentration of NPN compounds at various stages of ripening (mg N/100g) of dry matter

	Expt 2 Stage of ripening (days)						Expt 3 Stage of ripening (days)					
	0	3	9	15	22	36	0	3	9	15	22	36
NH_3	24	30	40	58	62	76	25	27	43	61	57	73
Free α-NH_2-N	141	188	204	234	243	255	155	200	225	230	255	302
Peptide bound α-NH_2-N	161	195	209	152	147	145	225	235	204	168	171	113
Nucleot.-N	34	33	15	13	12	12	37	21	17	16	13	14
Nucleos.-N	33	41	54	78	83	83	31	42	51	75	89	89
Total NPN												
Determined	537	775	790	789	803	820	544	706	805	802	806	889
Calculated[a]	494	615	660	664	677	704	600	670	683	683	727	730
% Recovery	92.2	79.3	83.5	84.1	84.3	82.0	110.2	94.9	84.8	85.1	90.1	82.1

Table 3—Concentration changes of NPN compounds at various stages of the ripening process (mg N/100g dry matter)

	Expt 2 Period (days)				Expt 3 Period (days)			
	0–3	3–15	15–36	0–36	0–3	3–15	15–36	0–36
NH_3	6	28	18	52	2	34	12	48
Free α-NH_2-N	47	46	21	114	45	30	72	147
Peptide bound α-NH_2-N	34	–43	–7	–16	10	–67	–55	–112
Nucleot.-N	–1	–20	–1	–22	–16	–5	–2	–23
Nucleos.-N	8	37	5	50	11	33	14	58

OH 30% w/v, filtration and dilution to volume, total NPN was determined by ro-Kjeldahl method [as described in the d methods of E.E.G. (Europese Eco- ie Gemeenschap)] (Anonymous, 1972). s of the extract were used for de- itions of NH_3 (1 ml) (Conway, 1962), ree α-NH_2-N (1 ml) using leucine as d (Rosen, 1957), total peptide bound N after acid hydrolysis (24 hr) and ion for free α-NH_2-N (Weidner and 1966), total nucleotides (1 ml) ex- as IMP and total nucleosides, expressed xanthine (Macy et al., 1970).

omated analysis of free amino acids and Free amino-acids and weak amines were ed from a separate sausage sample with cid (Stein and Moore, 1954) and quan- using a standard Technicon "Auto er" and norleucine as Internal Standard man et al., 1958). Part of the highly mines were extracted from a third sam- sausage (Hill et al., 1970) and separated standard Technicon "Auto Analyzer" as ed by Vandekerckhove and Henderickx

Table 4—Concentrations of free amino acids[a] at three stages of ripening (mg α-NH_2-N/100g dry matter)

	Expt 2 Stage of ripening (days)			Expt 3 Stage of ripening (days)		
Free amino acids	0	15	36	0	15	36
Asp	0.74	1.90	5.30	0	3.79	7.25
Thr	0.82	3.30	25.20	0	4.95	6.94
Ser	1.73	5.50	9.10	0	7.20	9.85
Glu	19.00	7.20	5.60	25.40	24.30	18.30
Pro	0	3.40	5.50	0	5.72	6.45
Gly	3.00	6.15	8.80	0	7.65	14.20
Ala	10.20	20.90	25.20	3.92	23.10	22.30
Val	1.44	6.35	8.85	0	7.41	11.95
Met	0.56	2.72	3.84	1.60	3.38	5.26
Ileu	1.60	3.74	5.45	2.24	4.50	8.10
Leu	1.06	11.50	13.30	8.30	13.20	17.60
Phe	0.93	4.50	5.25	2.98	5.00	7.15
Lys	2.07	4.67	6.35	2.30	3.94	6.46
His	0.73	1.46	0.01	2.77	2.20	0
Tyr	0.77	0	0	0	0	0.30
γ-N BA[b]	0	2.72	4.07	1.22	7.89	12.50
Orn[c]	1.16	0	0	1.98	0	0.83

[a] Shorthand notation
[b] γ-amino butyric acid (γ-amino-N calculated as α-amino-N)
[c] Ornithine

RESULTS and DISCUSSION

E 2 shows the concentration of the nt NPN compounds investigated, sed as mg N/100g of dry matter at s stages of the ripening process. It e seen that the major NPN fraction t is peptide bound α-amino-N at rt, whereas free α-amino-acids (free -N) dominate at the end of the g period. Addition of individual for each stage results in values than the total NPN determined. liscrepancy is obviously related to nces in color and color intensity of nhydrin reaction products between nt amino acids, the presence of -NH_2-N in the free amino acids ximately 25% of total amino acid d the expression of all nucleotides P and of all nucleosides as hypoxan- Indeed, besides nucleoside mono- hates, di- and triphosphates may be t, whereas nucleosides are present s hypoxanthine. However, addition components, after correction of -N values for the presence of 25% -NH_2-N, results in an average re- of 91.3 ± 4.2% (expt 3) and 83.6 % (expt 2) of determined NPN (Table 2). From data in Table 2, the concentration changes (as mg N/100g dry matter) for the different compounds at various stages of the ripening process were calculated and presented in Table 3.

These data show that during the first 3 days of ripening, the rate of free α-NH_2-N production is maximal and exceeds the rates of NH_3 production and peptide production from proteins. During this period intensive carbohydrate metabolism and bacterial growth also takes place (De Ketelaere et al., 1974). In the following periods the rate of ammonia production increases, but remains inferior to the rate of free α-NH_2-N production, whereas the concentration of peptide bound α-NH_2-N decreases. These results indicate, that free amino acids are produced at a faster rate than ammonia and peptides: % free α-NH_2-N in total NPN increases from ca 35% to 50% at the end of the ripening period. This is in contrast to results reported by Langner (1969) indicating an initial fast production rate for ammonia, whereas production of free amino acids only starts after an "initiation period." However, Niinivaara et al. (1961) and Körmendy and Gantner (1962) also observed the fastest rate of free amino acid production during the first 3 days of ripening. The final values obtained for NH_3 are within the range reported by Langner (1972) for 12 commercial sausages (16–103 mg NH_3/100g sausage) and are comparable to data reported by Stanculescu et al. (1970) (approx 60 mg/100g sausage) and by Körmendy and Gantner (1962) (approx 80 mg/100g sausage). Values for free α-NH_2-N are somewhat lower than those reported by Stanculescu et al. (1970): approx 600 mg/100g sausage.

Nucleotides decrease in concentration, whereas nucleosides and bases increase in concentration. The lack of stoichiometry between nucleotide disappearance and nucleoside formation, is probably related

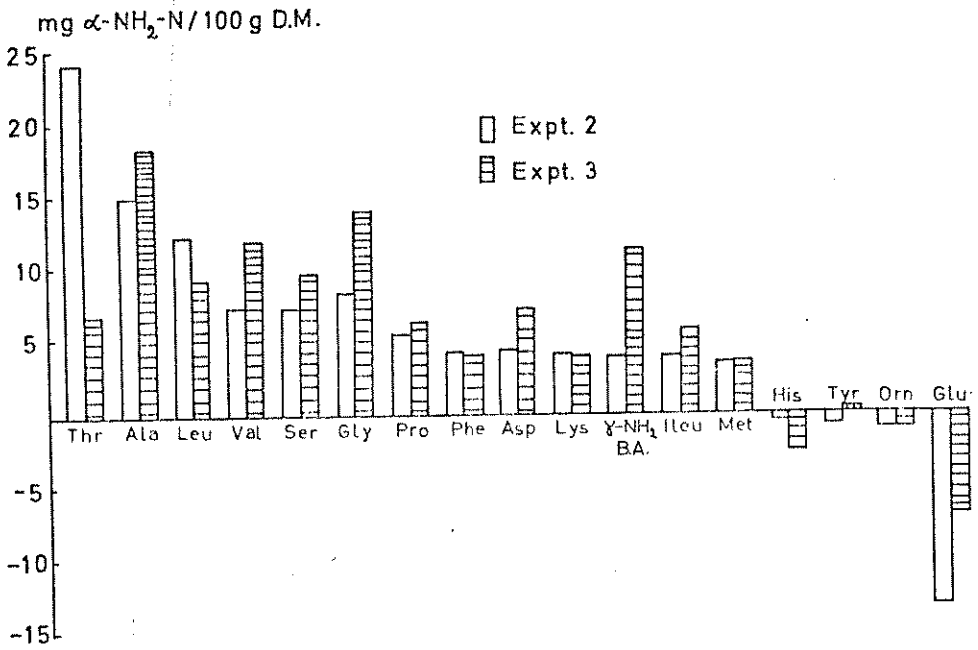

Fig. 1—Concentration changes of individual free amino acids between final and initial stage of ripening (mg α-NH_2-N/100g dry matter).

the expression of results as IMP and poxanthine, as explained earlier. The esence of a starter culture (expt 3) oduced no striking differences, except r a higher final concentration of free NH_2-N in expt 3, coupled to a lower ncentration of peptide bound α-NH_2-N ese findings may suggest a higher ypeptidase activity in expt 3 or may lated to a higher initial peptide ncentration in the same experiment able 2). In both experiments, the most nificant increase was observed for free NH_2-N (total free amino acids). In der to determine the individual amino ids responsible for the increase, amino id analyses were carried out on samples tained after 0, 15 and 36 days of pening. The results are presented in ble 4. They show that glutamic acid is e predominant amino acid in the initial mples, because of its presence as an ditive. The second predominant free ino acid initially present is alanine, nfirming data reported by Niinivaara et (1961), Stanculescu et al. (1970) and ngner (1969).

Concentration changes for individual ino acids were calculated between final d initial samples, and presented in gure 1. They show that the major ino acids responsible for the increase total free α-NH_2-N are alanine, leucine, line, serine, glycine and proline (increase larger than 5 mg γ-NH_2-N/100g y matter), followed by phenylalanine, spartic acid, lysine, α-amino butyrate, oleucine and methionine (increase smaller than 5 mg α-NH_2-N/100g dry matter). Threonine shows the largest increase in expt 2, but not in expt 3. For most amino acids, increases observed are larger in expt 3, confirming the data obtained for total free α-N. Final concentrations for free amino acids are within the range of values reported by Langner (1972). These results are partly in agreement with data presented by Reuter and Langner (1968), Niinivaara et al. (1961), Körmendy and Gantner (1962) and Stanculescu et al. (1970) as these authors also observed the most prominent concentration increase for alanine and leucine.

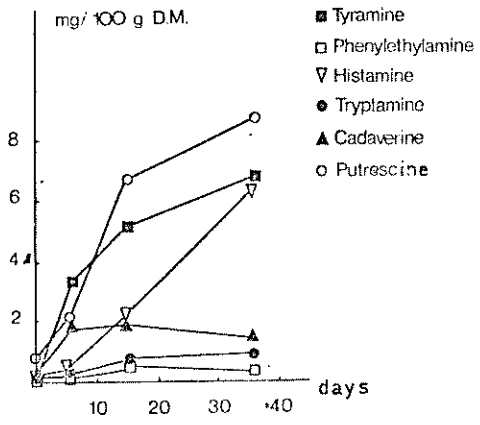

Fig. 2—Concentration of some amines at various stages of ripening in expt 3 (mg/100g dry matter.

In both experiments, a considerable part of the added glutamate disappears, and is at least partly decarboxylated to γ-amino-butyric acid, confirming results obtained by Langner (1972). Because of these results the use of glutamate as a flavor additive in dry sausage may be questioned (Langner, 1972). Other amino acids may be decarboxylated during dry sausage ripening, as indicated by the disappearance of histidine, tyrosine and ornithine. The decarboxylation products of these amino acids are histamine, tyramine and putrescine, respectively. Here, our results are in contrast to earlier findings of Langner (1969) and Reuter and Langner (1968), who also reported an increase for these three amino acids. However, tyrosine disappearance was reported by Niinivaara et al. (1961), as well as the formation of tyramine. Maillet and Henry (1960) reported the presence of histamine, whereas Langner (1972) even suggests production of cadaverine by decarboxylation of lysine.

Analysis of highly basic amines was carried out on samples obtained from expt 3. Although only very small amounts were detected, the concentration of histamine, tyramine and putrescine was increased at least tenfold, the rate of increase being maximal, during the first 3 days of ripening (Fig. 2).

The results are in line with the decrease in the concentration of histidine, tyrosine and ornithine observed in our experiments. Cadaverine, a decarboxylation product of lysine, was also detected in significant amounts.

Table 2—Recoveries of volatile fatty acids added to sausage

	Acetic acid	Propionic acid	Butyric acid
μmoles present	155.9	1.4	1.0
μmoles added	35.3	13.4	17.4
Total	191.2	14.8	18.4
Total μmoles recovered	196.6	17.8	18.3

method (Herbert et al., 1971) on samples containing lactose showed that differences were within the experimental error.

Volatile fatty acids (VFA) were isolated by steam-distillation: 5g of sample were mixed with 10 ml of H_2O and 3g of $MgSO_4 \cdot 7H_2O$ in a Virtis homogenizer. The mixture was transferred to a Markham Still and 5 ml of 85% H_3PO_4 (A.R. Merck, Darmstadt, Germany) added. The outlet of the condensor was immersed in 10 ml NaOH 0.1N containing phenolphtaleine and 200 ml of condensate was collected. The alkaline distillate was evaporated under reduced pressure in a rotary evaporator and the dry salts dissolved in 2.5 ml of 10% H_3PO_4. The VFA were separated as free acids by gas-liquid chromatography using 5 μl of solution and an F&M 700 apparatus (Hewlett-Packard, Brussels) equipped with a flame ionization detector, as described in earlier work from this laboratory (Van Nevel et al., 1969). Quantitation was carried out by comparison of sample peak heights with peak heights of standard mixtures, also subjected to steam distillation and injected at regular intervals between samples. Table 2 shows recoveries obtained for known amounts of VFA added to a sausage sample. Carbonyl compounds were determined as saturated aldehydes (mean M.W. 91) using the benzidine reagent as described earlier (Demeyer et al., 1974).

Quantitative determination of bacteria

In expt 1, before grinding the sausage, a slice was removed with a knife. The sample was weighed, homogenized (1 min) and diluted tenfold in a Waring Blendor, (14,000 rpm) using a solution containing 0.1% peptone, 0.85% NaCl and 0.04% agar. Inoculation, incubation and counting of bacteria was carried out using the ringed-plates technique described by Van der Heyde (1963, 1964). Lactobacilli were incubated anaerobically on Rogosa SL agar and Micrococci aerobically on S 110 agar (Difco).

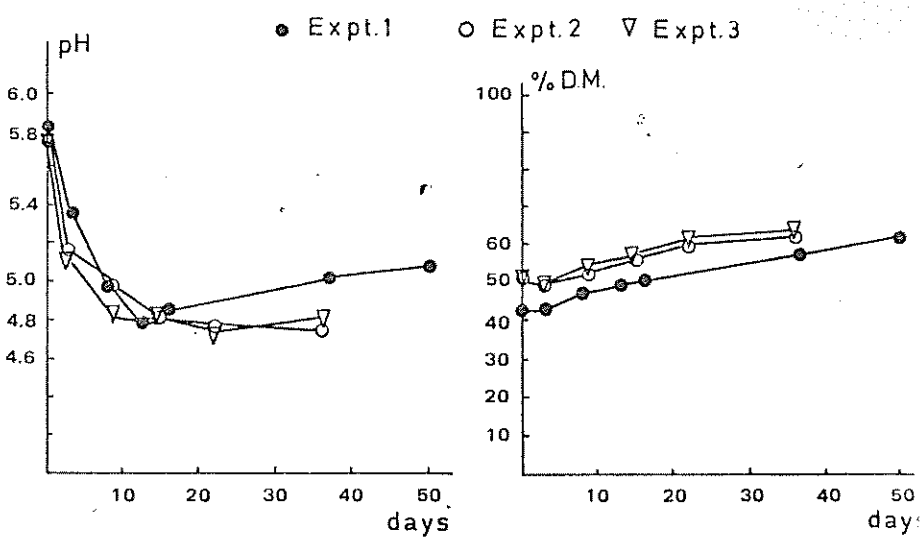

Fig. 1—Changes in pH and dry matter (D.M.) during dry sausage ripening.

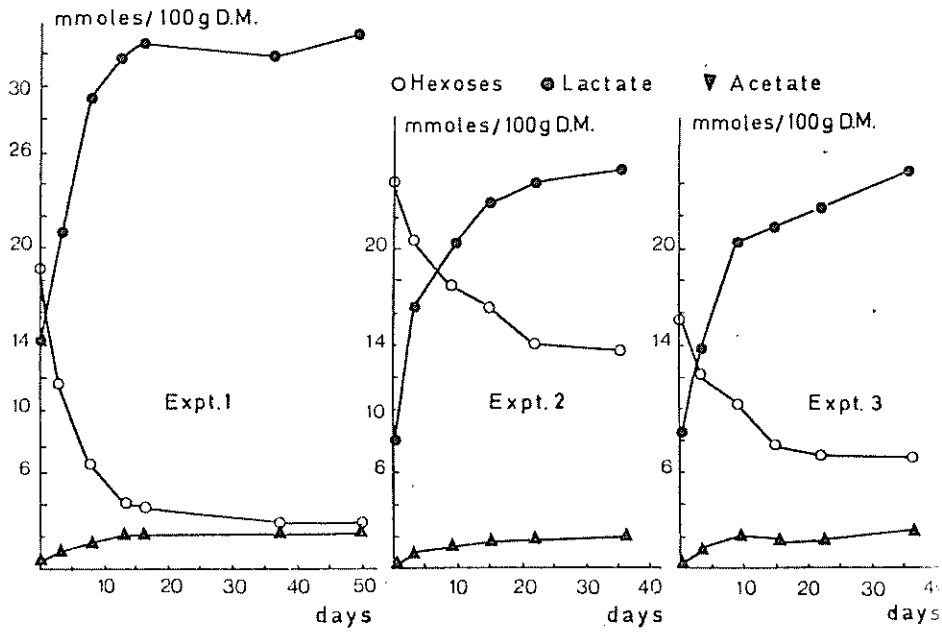

Fig. 2—Changes in concentration of hexoses, lactate and acetate during dry sausage ripening.

RESULTS & DISCUSSION

FIGURE 1 SHOWS that in all experiments, dry matter content increased to approximately 60% during the ripening process. Values of pH dropped from an initial value of about 5.8 to approximately 4.8 during the first 15 days of ripening, and changed little afterwards, except for expt 1 where an increase was observed. The drop in pH coincides with an accumulation of lactic acid and the disappearance of carbohydrates (Fig. 2), both of these processes being nearly completed after 15 days of ripening. Together with lactic acid, smaller amounts of acetic acid are formed (Fig. 2) and very small, but significant amounts of propionic and butyric acids (10–20 μmoles/100g dry matter). No α-keto acids could be detected by the method used, whereas total carbonyl concentration never exceeded 0.5 mmole/100g dry matter. The percentage of total crude protein, soluble as "myofibrillar protein," decreased from approximately 45% to 25% during the first 15 days of ripening, whereas "sarcoplasmic protein" decreased from approximately 18% to 5% after 35 days of ripening. The presence of a starter culture in expt 3 did not produce significant chan for any of the characteristics measur (Fig. 1, 2 and 3).

From the amounts of carbohydrat expressed as mmoles of hexose, and t amounts of lactate and acetate produc fermentation balances can be calculat according to the reactions:

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$$

and $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH +$
$2CO_2 + 8H$

It is clear from these reactions, that f

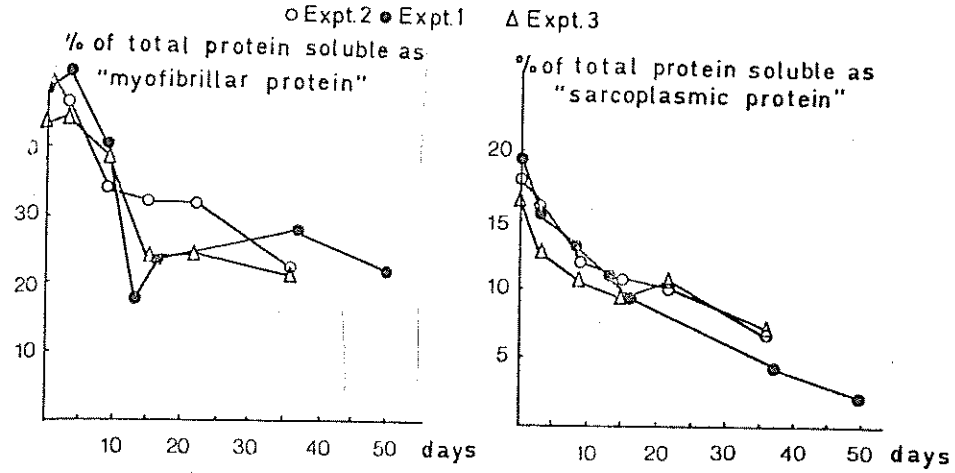

Fig. 3—Changes in protein solubility during dry sausage ripening.

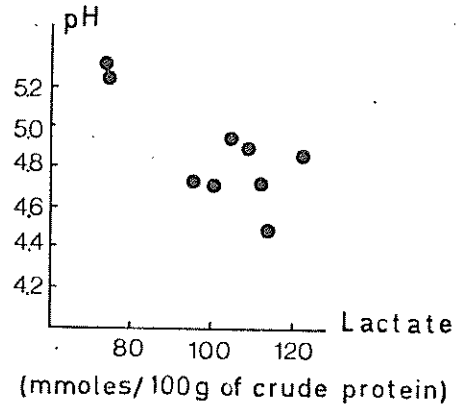

Fig. 5—Relationship between pH and lactate concentration (data from Table 4).

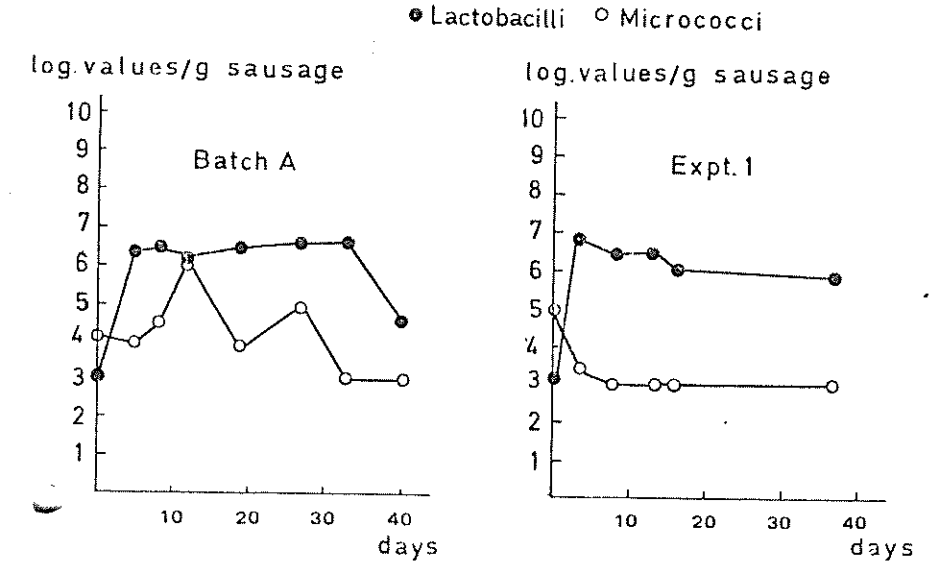

Fig. 4—Counts of lactobacilli (•) and micrococci (○) at different stages of ripening.

ach mole of hexose disappearing, two oles of lactate and/or acetate should be rmed. The theoretical amounts of these ids, calculated from hexose metabo-ed, are compared to the amounts tually found for the different periods the ripening process, as well as for the hole period, in Table 3.

It can be seen that for the whole peri-l, in expt 2 and 3, the amounts of lac-e and acetate found correspond to the ounts calculated, indicating that all ose metabolized was anaerobically verted to lactate and acetate, the ner being the major end-product. For period 15–36 days (Table 3), the nts of lactate + acetate found differ icantly from the amounts calcu-This may indicate lactate produc-rom substrates other than carbohy-drates (expt 3) or further metabolism of lactate formed (expt 2). However, these differences are within experimental error when the whole period is considered. In expt 1, lactate and acetate found can only account for about 2/3 of all hexose metabolized, indicating that other end-products were formed. The small amounts of propionate, butyrate and carbonyl compounds formed cannot explain this discrepancy. However, regeneration of reduced cofactors in anaerobic carbohydrate fermentation may produce other reduced compounds such as ethanol and other low molecular weight alcohols, not determined in these experiments. In view of the magnitude of the discrepancy, and the low concentration of ethanol reported elsewhere (Pezacki and Szostak, 1962), a more likely explanation may be related to the initial presence of more oxygen in the sausages of expt 1, as compared to expt 2 and 3. Indeed, whereas sausages were vacuum filled in the latter experiments, they were not in the former. A higher oxygen concentration may induce a complete oxidation of part of the carbohydrate, with production of CO_2 and H_2O. Such oxidative dissimilation of carbohydrates has been suggested for the last stages of ripening by Pezacki and Fiszer (1966). However, as is clear from Table 3, the discrepancy between end-products found and substrate metabolized, is most prominent for the first 3 days of ripening. In all experiments, fermentation balance discrepancies were observed for the last period of ripening (Table 3), but the amounts involved are of minor importance, compared to the first two periods.

Although very early in the ripening period lactobacilli become the predominant flora of dry sausages, ripening under the conditions described, the number of micrococci initially present is comparable to the number of lactobacilli (Reuter et al., 1968). Micrococci may contribute to complete oxidation of carbohydrate during the first days of the ripening period. In expt 1, micrococci and lactobacilli were enumerated and comparable numbers were only observed for the first sample (Fig. 4). Numbers of micrococci tended to be higher, however, in samples obtained from batch A, described in the preceding paper (Demeyer et al., 1974), ripened under similar conditions as batch B (expt 1) and for which preliminary results on carbohydrate metabolism indicated even more prominent fermentation balance discrepancies (Fig. 4). Although the stoichiometry clearly indicates a different pattern of carbohydrate metabolism in expt 1, compared to expt 2 and 3,

Table 3—Fermentation balances, calculated at various stages of dry sausage ripening

Period (days)	Hexose fermented[a]			Lactate + Acetate formed[a]		
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
0–3	7.17	3.83	3.56	7.82(14.34)[b]	9.07(7.66)	6.01(7.12)
3–15		4.30	4.56		7.17(8.60)	8.33(9.12)
3–16	7.68			13.12(15.36)		
15–36		2.38	0.75		2.42(4.76)	3.88(1.50)
16–50	0.84			0.61(1.68)		
0–36		10.51	8.87		18.66(21.02)	18.22(17.74)
0–50	15.69			21.55(31.38)		

[a] All results expressed as mmoles/100g of dry matter
[b] Numbers in parentheses are theoretical values calculated from glucose fermented.

Table 4—Composition of dry sausage

	Brand									
	1	2	3	4	5	6	7	8	9	Mean ± S.E.
Dry matter (D.M.) (%)	58.6	62.2	73.1	64.0	70.2	65.6	65.0	66.4	61.6	65.1 ± 1.5
Protein (% D.M.)	29.7	27.5	25.4	26.7	31.0	28.2	30.0	27.9	27.0	28.1 ± 0.6
Fat (% D.M.)	60.0	—	66.8	61.2	61.1	60.6	60.3	56.5	62.1	61.0 ± 1.0
pH	4.86	4.70	5.23	4.72	5.31	4.94	4.90	4.72	4.48	4.87 ± 0.09
% of protein as										
Myofibrillar	35.4	18.8	23.4	20.2	31.5	26.1	17.1	17.0	27.8	24.1 ± 2.2
Sarcoplasmic	8.9	7.1	8.6	7.1	9.5	6.2	7.5	9.4	8.1	8.0 ± 0.4
Organic Acids										
Lactate[a]	36.4	27.7	18.8	25.4	22.7	29.3	32.7	31.2	30.8	28.3 ± 1.8
Acetate[a]	4.2	1.7	1.8	2.1	3.1	3.4	1.4	1.8	2.4	2.4 ± 0.3
Butyrate[b]	21.0	22.7	19.8	9.5	13.4	8.2	4.2	13.1	19.1	14.5 ± 2.1
Propionate[b]	17.2	6.2	7.8	4.6	45.3	5.9	6.8	8.4	3.9	11.7 ± 4.4
Carbonyl compounds[b]	222	360	253	246	213	416	796	345	162	334 ± 64
Hexoses[a]	7.8	10.6	20.0	7.5	1.9	1.6	6.9	21.3	5.7	9.3 ± 2.4

[a] mmoles/100g of D.M.
[b] μmoles/100g of D.M.

the absolute amounts of lactic and acetic acid formed in all experiments are similar (Table 3).

Also, the final concentrations of these acids, as well as other characteristics measured are similar to the mean values calculated for nine samples obtained commercially (Table 4). Individual values of pH for these samples were found to be inversely related to the concentration of lactic acid, expressed per 100g of crude protein, as suggested by Andersen and Ten Cate (1965) (Fig. 5).

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