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Stoichiometry of Carbohydrate Fermentation during Dry Sausage Ripening.

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Summary.
During ripening of dry sausages, disappearance of carbohydrates and production of lactate, volatile fatty acids, pyruvate and carbonyls was followed. When expressed as mmoles/100 g of D.M., carbohydrate disappearance (ca 10 mmoles/100 g D.M.) could be accounted for by production of lactate (ca 17) and acetate (ca 2) in two similar experiments. No differences were observed due to the presence of a starter culture in one experiment. In a third experiment, carbohydrate disappearance (ca 16) could only partly be accounted for by lactate (ca 19) and acetate (ca 2) production. The low amounts of butyrate, propionate and carbonyls, present in all experiments cannot explain the latter discrepancy. Oxydative dissimilation of carbohydrates by Micrococci during the early stages of ripening in the third experiment is offered as possible explanation.

Résumé.
La disparition de glucides totaux et la production de lactate, d'acides gras volatils, de pyruvate et de composés cabonyls ont été suivies pendant la maturation de suacisson sec. En exprimant les résultats en mmoles/100 g M.S., la diminution en glucides (ca 10 mmoles/100 g M.S.) était conforme à la production de lactate

(ca 17) et d'acetate (ca 2) pour deux expériences, faites en donditions identiques. La présence d'une culture d'ensemencement dans une expérience n'avait pas d'influence sur les résultats. Dans une troisiéme expérience, seulement une partie des glucides disparus (ca 16) pouvait être expliquée par la production de lactate (ca 19) et d'acetate (ca 2). Les quantités minimes de butyrate, de propionate et de dérivés carbonyls, trouvées dans toutes les expériences, ne peuvent pas expliquer cette différence. Une dissimilation oxydative de glucides par les Microcoques durant les premiers jours de la maturation est suggerée pour expliquer les résultats obtenus dans la troisième expérience.

Zusammenfassung.

Die Abbau von Kohlenhydraten, und die Produktion von Milchsäure, flüchtigen Fettsäuren, Brenztraubensäure und Carbonyle wurde bestimmt während der Reifung von Rohwurst. Kohlenhydratabbau ausgedrückt als mmoles/100 g T.S. (ca 10 mmoles/100 g T.S.) stimmt stöchiometrisch mit der gefundenen Milchsäure Produktion (ca 17) und Essigsäureproduktion (ca 2) weitgehend überein in zwei Experimente. Die Anwesenheit einer "Startekultur" in einem dieser Experimente hat kein Einfluss auf die Resultate. In ein drittes Experiment, könnte nur ein Teil des Kohlenhydratabbaus (ca 16) verantwirtet werden durch Milchsäureproduktion (ca 19) und Essigsäureproduktion (ca 2). Die kleine Menge Buttersäure, Propionsäure und Carbonyle, gefunden in allen Experimenten, können diese Differenz nicht erklären. Teilweise oxydative Dissimilation der Kohlenhydrate durch Micrococci während der ersten Tage der Reifung in das dritte Experiment kan diese Resultate erklären.

Introduction.

According to Ter. Cate (1960), carbohydrates in dry sausages, are fermented during the ripening period mainly following the stoichiometry of homolactic fermentation :

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

However, besides lactic acid, other products, such as ethanol (Pezacki and Szostak, 1962), volatile fatty acids (Halvarson, 1973) and pyruvic acid (Pezacki and Szostak, 1962) were also reported as end products of carbohydrate metabolism during dry sausage ripening. Furthermore Pezacki and Jaroszewski (1963) and Pezacki and Fiszer (1966) suggest that besides fermentation, oxidative dissimilation of carbohydrates can occur during the last stages of ripening.

In view of the complexity and discrepancy of the available data, the experiments discribed in this paper were carried out to evaluate the stoichiometric relationship between the disappearance of carbohydrates and the production of organic acids during dry sausage ripening.

Materials and Methods.

Preparation of sausages:

Three experiments were carried out, involving three batches of sausages. In experiment 1, batch B described in the preceding paper (Demeyer et al, 1973) and prepared by a local butcher was used. In experiments 2 and 3, two batches were prepared simultaneously in a local factory. The compositon of the sausage mixtures is given in table 1 and differed by the addition in experiment 3 of a starter culture (Duploferment) whereas 1% less of a "Sugar mixture" was added. The latter mixture contained, 53.8 % of total carbohydrates, 25.5. % crude protein, 6.3 % ash and 6.0 % H_2O.

Table 1. Composition of sausage mixture
(Experiment 2 and 3)

Cooled, deboned and chopped beef	75 kg
Cooled, deboned and chopped pork	25 kg
Cooled and chopped lard	40 kg
Cooled and chopped pork-rind	8 kg
Salt (NaCl)	4.3 kg
Coloring salts ($NaNO_2$ + KNO_3)[1]	50 g
Sugar mixture[2]	6 kg
Smoke concentrate	150 g
Monosodium glutamate	150 g
Pepper	450 g
Starter culture[3]	75 g

[1] added with NaCl : 36 g of KNO_3 + 14 g of $NaNO_2$

[2] 4.5 kg in expt. 3

[3] Only in expt. 3, equivalent to 10^{10} cells/kg and 68 g of glucose.

The sausage mixture is filled into semi-synthetic casings using a vacuum filling machine. The sausages (approx. 3.5 kg each, diameter 105 mm) are then transferred to a conditioned chamber for 6 days. During this period, temperature was gradually lowered from 22° C to 18° C and R.H. from 95 % to 85 %, while cold smoke was applied 3 h. daily. They are then transferred to a drying chamber, where they are kept for a further 30 days at 16° C and 85 % R.H., until ready for consumption.

Analyses were als carried out on nine different brands of dry sausages (numbered 1 to 9) as obtained from various shops.

Sampling procedure:

At different stages of the ripening process, a sausage was transported to the laboratory (expt. 1) or a sample of approx. 500 g was removed, making a transverse cut (expt. 2 and 3). The cut surface of the remaining sausage was sealed off by immersion in liquid gelatin, after each sampling. Samples were treated as described previously (Demeyer et al, 1973).

Analytical Methods:

Samples were analyzed for Dry Matter (D.M.), Crude Protein (Kjeldahl method) and Crude Fat (ether extract) by conventional methods. Using a Radiometer 22 apparatus (Radiometer, Copenhagen) with expanded scale, pH was measured by careful insertion of pointed electrodes in the sample (casing removed).

For the determination of total carbohydrates, lactic acid and pyruvic acid, a sample was extracted with 0.6 N $HClO_4$. Aliquots of the extract were used for the determination of lactic acid (Conway, 1957), determination of pyruvic acid (Umbreit et al, 1959)

and determination of total carbohydrates using the anthrone reagent as described by Herbert et al (1971). Volatile fatty acids (vfa) were isolated by steam-distillation; evaporated under reduced pressure and the dry salts dissolved in 2.5 ml of 10 % H_3PO_4. The vfa were separated by Gas liquid chromatography as described earlier (Van Nevel et al, 1969). Carbonyl compounds were determined as saturated aldehydes (mean M.W. 91) using the benzidine reagent as described earlier (Demeyer et al, 1973).

Quantitative determination of Bacteria :

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

Results and Discussion.

Fig. 1 shows that in all experiments, D.M. content increased to approximately 60 % during the ripening process. Values for 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. 4 where an increase was observed. The drop in pH coincides with an accumulation of lactic acid and the disappearance of carbohydrates (fig.2), both these processes being nearly completed after 15 days of ripening.

Fig. 1 Changes in pH and Dry Matter (D.M.) during dry sausage ripening.

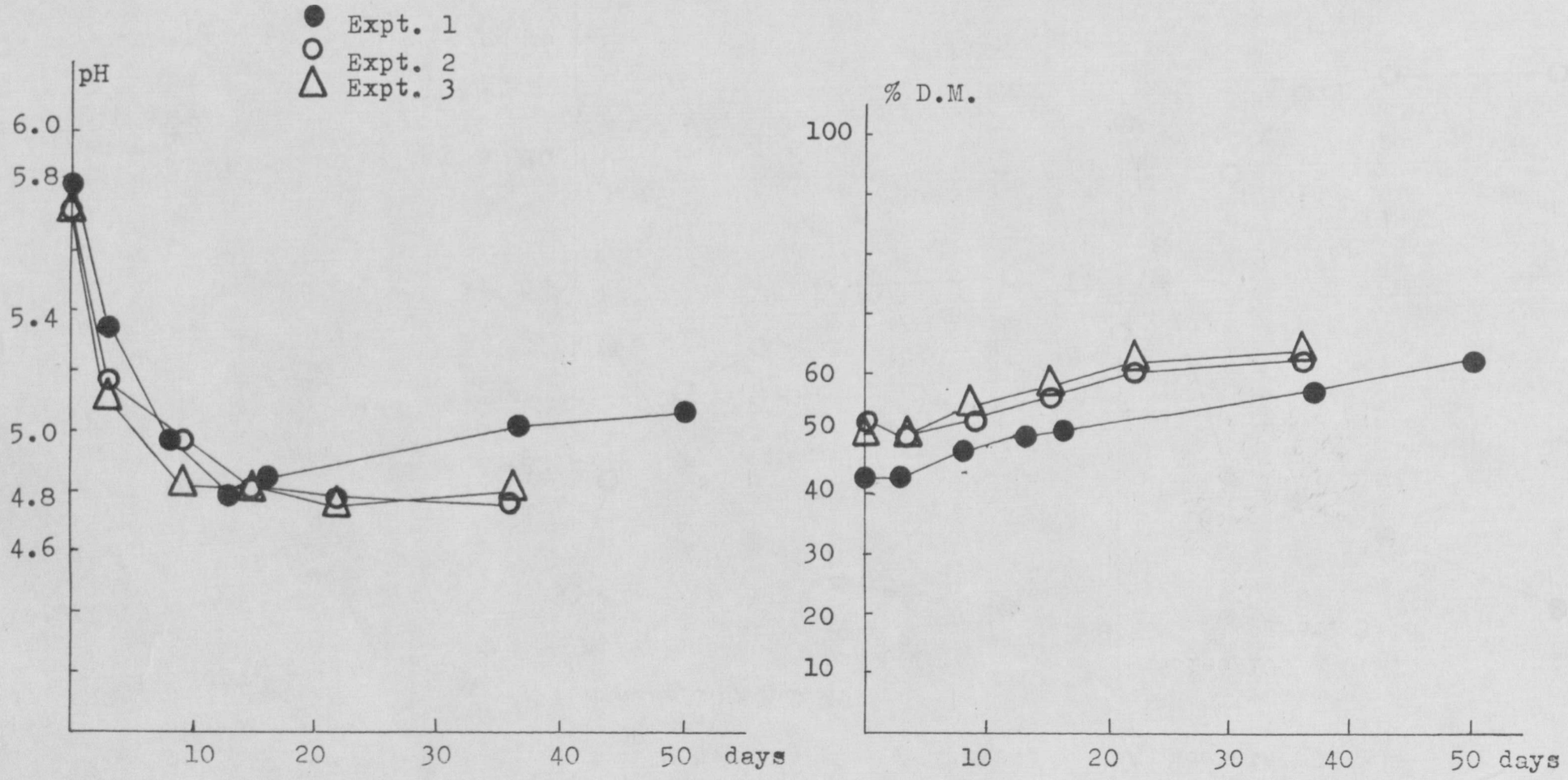
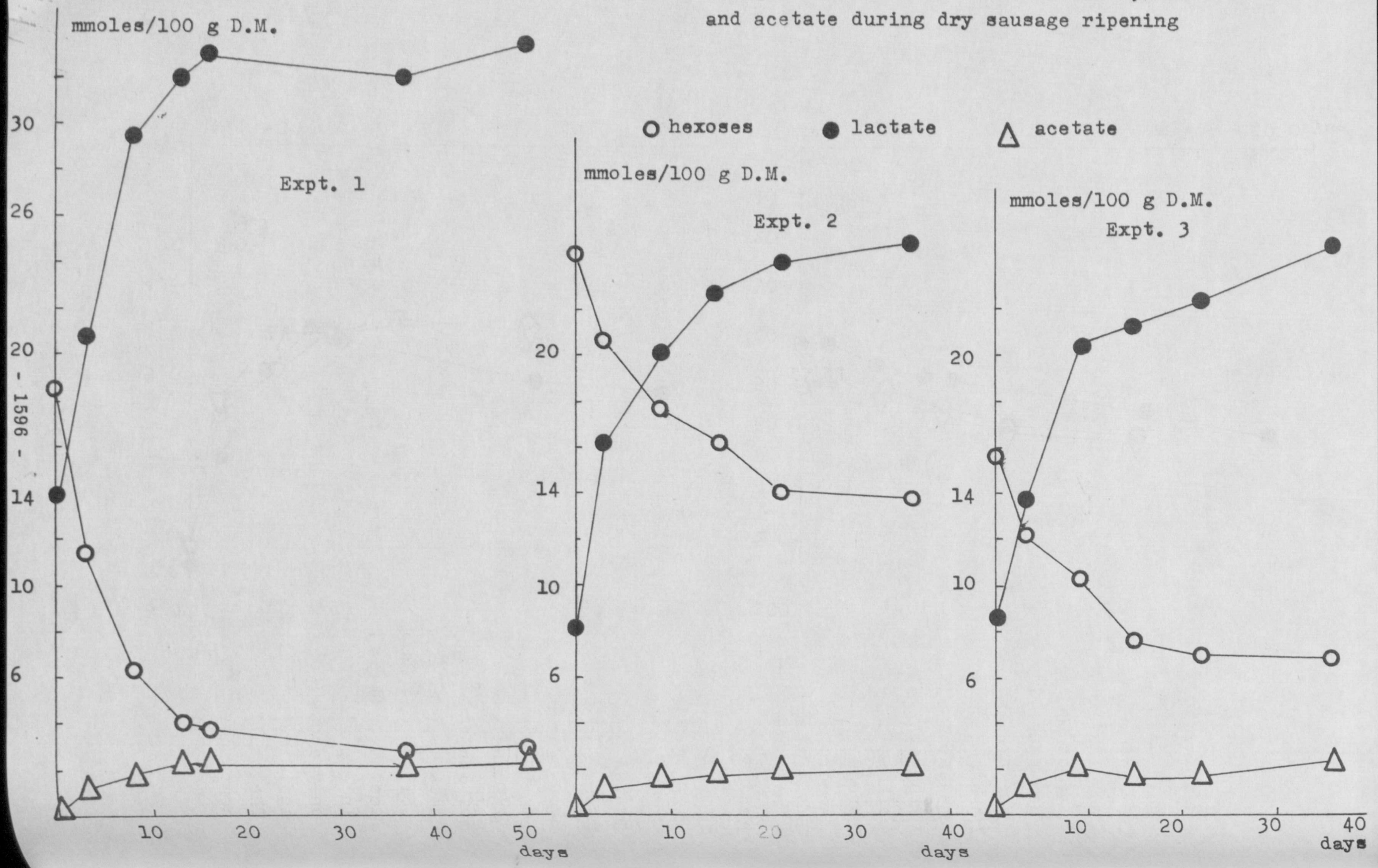


Fig. 2. Changes in concentration of hexoses, lactate and acetate during dry sausage ripening



Together with lactic acid, smaller amounts of acetic acid are formed (fig. 2) and very small, but significant amounts of propionic and bytric acids (10 - 20 µmoles/100 g D.M.). No α-keto acids could be detected by the method used, whereas total carbonyl concentration never exceded 0.5 mmole/100 g D.M.
The presence of a starter culture in expt. 3 did not produce outspoken changes for any of the characteristics measured (fig.1, fig. 2)
From the amounts of carbohydrates, expressed as mmoles of hexose, and the amounts of lactate and acetate produced, fermentation balances can be calculated, according to the reactions :

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

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

It is clear from these reactions, that for each mole of hexose disappearing, two moles of lactate and/or acetate should be formed. The theoretical amounts of these acids, calculated from hexose metabolized, are compared to the amounts acutally found for the different periods of the ripening process, as well as for the whole period, in table 2. It can be seen that for the whole period, in experiments 2 and 3, the amounts of lactate and acetate found, correspond to the amounts calculated, indicating that all hexose metabolized was anaerobically converted to lactate and acetate, the former being the major end-prodtuct.
In experiment 1 however, 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.

Table 2. Fermentation Balances, calculated at various stages of Dry Sausage Ripening.

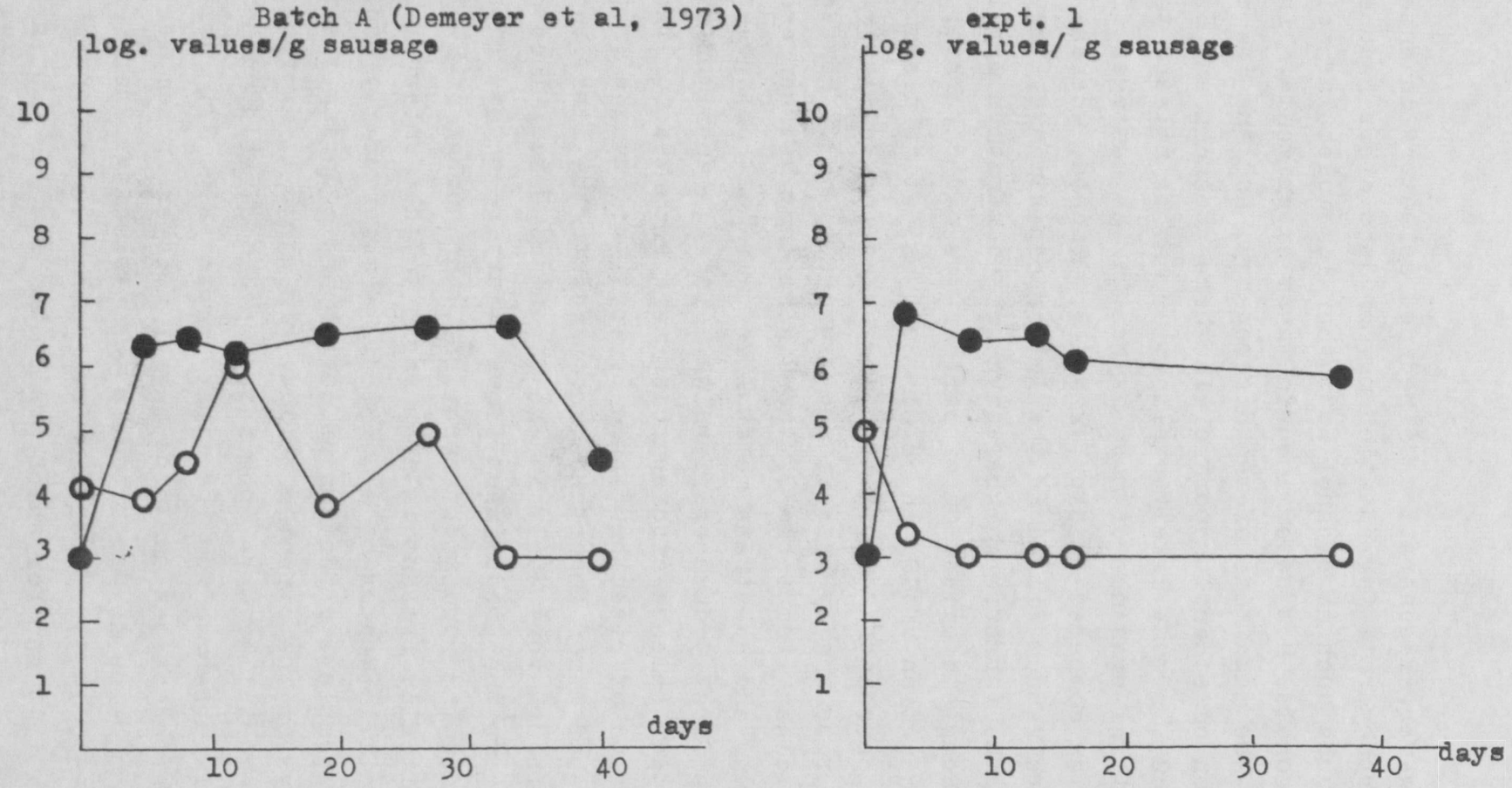
Period (days)	Hexose fermented[a]			Lactate + Acetate formed[a]		
	Expt.1	Expt. 2	Expt. 3	Expt.1	Expt. 2	Expt. 3
0-3	7.17	3.83	3.56	7.82 (14.34)[b]	9.07 (7.66)	6.01(7.12)
3-15		4.30	4.56		7.17 (8.60)	8.33(9.12)
3-16	7.68			13.12 (15.36)		
15-36		2.38	0.75		2.42 (4.76)	3.88(1.50)
16-50	0.84			0.61 (1.68)		
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/100 g of D.M.

b= ( ) = theoretical value calculated from glucose fermented.

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 Fiszner (1966). However, as is clear from table 2, the discrepancy between end products found and substrate metabolized, is most outspoken for the first 3 days of ripening. In all experiments, fermentation balance discrepancies were observed for the last period of ripening (table 2), 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, ripened under the conditions described, the number of Micrococci initially present in comparable to the number of Lactobacilli (Reuter et al, 1968). The former 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. 3). Numbers of Micrococci tended to be higher however in samples obtained from batch A, described in the preceding paper (Demeyer et al, 1973), ripened under similar conditions as batch B (expt.1) and for which preliminar results on carbohydrate metabolism indicated even more outspoken fermentation balance discrepancies.

Fig. 3 Counts of Lactobacilli (●) and Micrococci (○)
at different stages of ripening.



Although stoichiometry clearly indicates a different pattern of carbohydrate metabolism in expt.1, compared to expt. 2 and 3, the absolute amounts of lactic and acetic acids formed in all experiments are similar (table 2). Also, the final concentrating of these acids, as well as other characteristics measured, are similar to the mean values calculated for nine samples obtained commercially (table 3). Individual values of pH for these samples were found to be inversely related to the concentration of lactic acid, expressed per, 100 g of crude protein, as suggested by Andersen and Ten Cate (1965) (Fig. 4).

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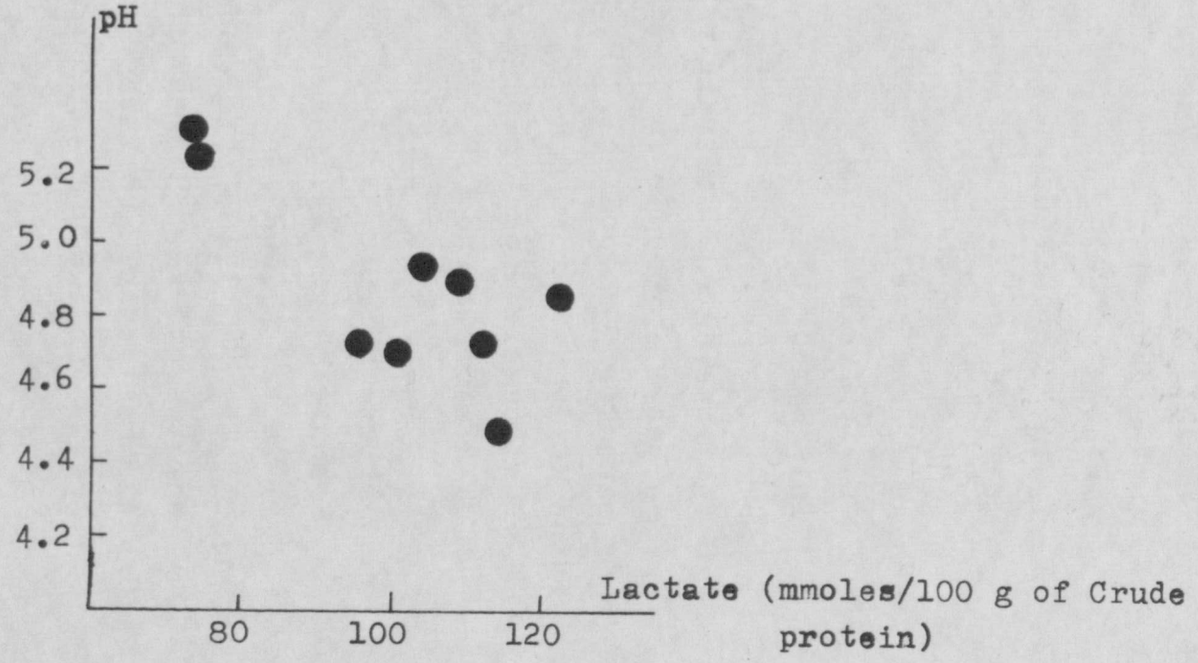
Table 3. Composition of Dry Sausages.

Brand	1	2	3	4	5	6	7	8	9	Mean ± S.E.
D.M. (%)	58.6	62.0	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
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/100 g of D.M.

[b] μmoles/100 g of D.M.

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



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