Nonenzymatic Browning in a Lactose-Casein Model System

Aldo Ferretti,[1] V. P. Flanagan,[1] and J. M. Ruth[2]

Forty compounds were isolated and identified from a dichloromethane extract of a lactose–casein browning system in the "dry" state. They comprise 13 furanics, nine lactones, five pyrazines, two pyridines, 2-acetyl pyrrole, two amides, pyrrolidinone, succinimide, glutarimide, two carboxylic acids, acetone, 2-heptanone, and maltol. They were separated by gas–liquid chromatography and identified on the basis of gas chromatographic, infrared, and mass spectroscopic data in comparison with authentic samples. Many of the identified compounds are easily accountable solely on the basis of a Maillard-type lactose–casein interaction. The presence of D-galactose, D-tagatose, and lactulose in the browned mixture has also been established. Possible relevance of the browning reaction in off-flavor development in milk and its manufactured products is discussed.

The flavor of dried and sterile concentrated milks deteriorates under normal storage conditions. This results in consumer's rejection, and is probably the main reason for the limited acceptance of such products. Considerable research has already been done on causes and mechanisms of off-flavor formation, but to date most work leading to isolation and identification of off-flavor precursors and constituents has been limited to the lipid phase of milk (Parks *et al.*, 1963; Parks, 1965, 1967).

It has been known for a long time that browning is a concomitant feature of milk deterioration, and considerable evidence has already been presented in support of the view that browning in milk is caused by a Maillard-type interaction between lactose and milk protein (Coulter *et al.*, 1951; Patton, 1955). Early formation of a lactose-casein complex has been postulated (Goulden, 1956; Patton and Flipse, 1953). The major involvement of the ϵ-amino group of lysine—which represents about 90% of total free amino groups in casein—was clearly demonstrated by the classical experiments of Henry *et al.* (1948) and Lea and Hannan (1950), and, more recently, by Finot *et al.* (1968) and Brueggemann *et al.* (1968) who isolated a lysine derivative from heated milk powders. Participation of other basic amino acids, such as arginine and histidine, has also been extensively documented (Baba and Niizeki, 1964; Evans and Butts, 1951; Lea and Hannan, 1950; Patton *et al.* 1948, 1954).

Previous investigators (Patton, 1950a,b; Patton and Josephson, 1949) have isolated and identified in heated skim milks typical products of browning, such as maltol, 5-hydroxymethyl-2-furaldehyde, and furfuryl alcohol, which were proved to arise from the casein-catalyzed degradation of lactose (Patton, 1950c). It is also generally admitted that a major cause of flavor deterioration in dairy products (resulting in stale and other off-flavors) is the interaction between lactose and milk protein (Coulter *et al.*, 1951; Henry *et al.*, 1948; Patton, 1955; Patton and Keeney 1957; Richards, 1963). However, this has not yet been convincingly established. In fact, concomitance of off-flavor development with typical manifestations of the Maillard reaction—such as an increase of the undialyzable fraction of milk and combined sugars, a decrease of the free amino (Van Slyke) nitrogen, discoloration—cannot be regarded as conclusive proof of relevance of the Maillard browning in production of off-flavors in milk and its manufactured products.

We believe that the study of a model lactose–casein browning system can be a first important step toward establishing such relevance. In the present work we report isolation and identification of the products arising from the browning reaction of casein–lactose in the dry state. Flavor evaluation of the identified materials will be the object of study at a later date.

EXPERIMENTAL

Chromatographic Methods. Gas–liquid chromatography (GLC), using stainless steel columns with a glass liner at the injection port, was carried out with the following conditions. Column A: 14-foot × 0.25-inch (O.D.) packed with 15% Triton X305 on 60/80 Chromosorb W AW/DMCS treated. Temperature programming was from 70° to 200° C. at 4° per minute from the air peak. Column B: 8-foot × 0.25-inch (O.D.) packed with 20% Silicone SE-30 on 60/80 Chromosorb W AW/DMCS treated. Temperature programming was from 75° to 200° C. at 2° per minute from air. Column C: 6-foot × 0.25-inch (O.D.) packed with 10% Carbowax 20M on 60/80 Diatoport S. Temperature programming was varied, depending upon the particular cut being analyzed.

For all three columns above, the carrier gas was helium at a flow of 75 ml. per minute, the injection port temperature 250° C.. and detector temperature 250° C. The instrument employed was a 5750 Hewlett-Packard, equipped with a thermal conductivity detector. Column D was a 24-foot × 0.125-inch, stainless steel, 1% Carbowax 20M High-Efficiency packed column (Hi-Pak) by Hewlett-Packard. This was placed in the oven of an LKB-9000 gas chromatograph mass spectrometer whereby the effluent was directly scanned. The carrier flow (He) was 22 ml. per minute and temperature programming from 75° to 150° C. at 2° per minute; injection port temperature 200° C.

The analytical chromatogram shown in Figure 1 was obtained with a 14-foot × 0.15-inch (I.D.) glass column packed with 15% Triton X305 on Chromosorb W AW/DMCS treated; flow, 75 ml. of helium per minute with effluent split 5 to 1; temperature programming from 70° to 200° C. at 4° per minute; instrument 5750 Hewlett-Packard, with flame ionization detection; sample size 6 μl.

Paper chromatography (descending) was carried out overnight on Whatman No. 4 paper using *n*-butanol:ethanol:water (40:11:19) as the solvent; spray reagent:0.6% silver nitrate in acetone, followed by 2% ethanolic sodium hydroxide.

[1] Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C. 20250

[2] Pesticide Chemicals Research Branch, Entomology Research Division, U. S. Department of Agriculture, Beltsville, Md. 20705

Table I. Data on Volatile Compounds Identified in a Lactose–Casein Browning System in the Dry State[a]

	Compound	R_T[a]	R_T[b]	Mass Spectrum, *m/e*	Infrared
1	Furan[b]	2.1	0.7	39(off scale), 68M$^+$(100), 38(21), 29(16)	
2	Acetone[b]	2.5	0.8	43(100), 58M$^+$(33), 42(7), 27(5)	
3	2-Methyl furan[b]	3.4	1.9	82M$^+$(100), 53(75), 81(61), 39(49)	
4	2-*n*-Heptanone[b]	13.2	10.3	43(100), 58(66), 71(18), 114M$^+$(7)	
5	Pyridine[b]	13.5	4.9	79M$^+$(100), 52(71), 51(33), 50(24)	
6	Pyrazine[b]	13.6	4.2	80M$^+$(100), 26(64), 53(54), 52(14)	
7	2-Methyl pyridine[b]	14.9	7.6	93M$^+$(100), 66(39), 39(26), 92(22)	
8	2-Methyl pyrazine[b]	16.0	8.3	94M$^+$(100), 67(60), 26(41), 39(36)	
9	2,5-Dimethyl pyrazine[c]	18.5	13.1	108M$^+$(100), 42(92), 39(50), 40(49)	
10	2,3-Dimethyl pyrazine[b]	18.9	12.7	67(100), 108M$^+$(96), 42(44), 40(31)	
11	2,6-Dimethyl pyrazine[c]	19.1	12.9	108M$^+$(100), 42(92), 39(50), 40(49)	
12	3-Butenoic acid-γ-lactone[d]	19.6	5.6	55(100), 27(70), 84M$^+$(57), 26(40)	1792, 1029, 1103, 1145, 975, 837, 1253, 1613
13	Acetic acid[b]	20.2	2.1	43(100), 45(93), 60M$^+$(64), 42(21)	1715, 1290, 1412, 3000, 2500–3000, 813, 936, 1010
14	2-Furfuryl methyl sulfide[b]	23.0	17.7	81(100), 128M$^+$(42), 53(36), 45(15)	
15	Propionic acid	23.1	5.3		1709, 1233, 3000, 1414, 1466, 1078, 2500–3000, 924
16	Furfuryl acetate	24.4	16.9		1736, 1235, 1015, 1024, 1370, 1149, 917, 1355
17	5-Methyl-2-acetyl furan[b]	24.4	20.8	109(100), 124M$^+$(43), 53(20), 43(18)	
18	2-Acetyl furan	24.5	11.8		1686, 1468, 1282, 1567, 1164 1024, 884, 905
19	2-Furfuryl methyl ketone[b]	24.8	13.3	81(100), 43(97), 53(44), 124M$^+$(33)	
20	Protoanemonin[d]	26.0	10.5	42(100), 96M$^+$(83), 54(59), 68(54)	1776, 969, 1745, 1124, 873, 1280, 1064, 1650
21	5-Methyl-2-furaldehyde[b]	26.6	15.3	110M$^+$(100), 109(88), 53(44), 27(16)	
22	Furfuryl alcohol	28.0	9.0		1005, 1147, 909, 1227, 883, 1374, 3610, 3436
23	Butyrolactone	28.0	11.7		1767, 1167, 1036, 992, 1374, 1229, 926, 866
24	2-Propionyl furan[b]	28.0	17.7	95(100), 124M$^+$(27), 39(15), 96(6)	
25	2,2′-Difurylmethane[d]	28.0	24.5	91(100), 39(54), 148M$^+$(43), 65(26)	
26	[2-Furyl-(2′-methyl-5′-furyl)]methane[b]	30.1	30.8	162M$^+$(100), 91(48), 43(28), 147(25)	
27	Acetamide[d]	30.9	9.7	59M$^+$(100), 44(63), 43(41), 42(21)	1661, 1377, 1592, 1333, 3413, 3509, 1229, 3175
28	2-Butenoic acid-γ-lactone[d]	31.2	11.8	55(100), 84M$^+$(78), 54(20), 27(17)	1779, 1748, 1094, 1156, 1038, 880, 1348, 1447
29	Propionamide[b]	31.7	12.1	44(100), 73M$^+$(52), 29(19), 57(18)	1672, 1587, 1227, 1380, 3413, 3509, 1198, 1460
30	2-Acetyl pyrrole[d]	36.9	22.2	94(100), 43(62), 66(54), 109M$^+$(35)	
31	Maltol[b]	38.8	26.2	126M$^+$(100), 71(47), 43(36), 55(28)	1618, 1264, 846, 1188, 1458, 1563, 917, 3257
32	Pyrrolidinone[d]	41.1	23.4	30(100), 85M$^+$(92), 42(89), 41(74)	1689, 1287, 1261, 1232, 1425, 3460, 3236, 1464
33	Glutarimide[b]	57.5	29.7	42(100), 70(44), 113M$^+$(38), 39(17)	
34	2-Furoic acid	60.5	25.2		1695, 1475, 1290, 1582, 1176, 1130, 1014, 3000
35	β-Acetyl butyrolactone[d]	[e]	25.7[f]	85(100), 43(86), 98(23), 128M$^+$(2)	1776, 1736, 1232, 1163, 1042, 1078, 1370, 942
36	Succinimide[b]	71.7	30.1	27(100), 99M$^+$(74), 56(58), 26(16)	1718, 1159, 1754, 1230, 1346, 1779, 3195, 3436

Table I. (Continued)

	Compound	R_T[a]	R_T[b]	Mass Spectrum, *m/e*	Infrared
37	4,5-Dihydroxyvaleric acid-γ-lactone[d]	72.7	32.0	85(100), 31(16), 56(9), 116M$^+$(2)	1770, 1179, 1064, 1092, 1020, 1230, 930, 3436
38	β-Formyloxybutyrolactone[d]	[g]	24.3	55(100), 84(82), 27(52), 130M$^+$(1)	1163, 1783, 1721, 1081, 1049, 1008, 960, 1227
39	β-Acetoxybutyrolactone[d]	[g]	31.1	85(100), 31(9), 56(7), 144M$^+$(0.04)	
40	β-Hydroxybutyrolactone[d]	87.3	31.3	44(off scale), 43(100), 55(88), 102M$^+$(10)	1167, 1783, 1087, 1050, 995, 3448, 1227, 966

[a] R_T[a] and R_T[b] refer to column A and column B, respectively; for conditions, see Experimental. The order of listing approximately follows the order of elution from the first polar column (Triton). [b] Mass spectrum determined with the LKB-9000 instrument. [c] 2,5- and 2,6-Dimethylpyrazine do not separate completely on the Hi-Pak column. Their mass spectra are virtually identical and the data reported here are those of a typical scanning (LKB instrument) in the area corresponding to the elution time of these two compounds. [d] Mass spectrum determined with the CEC instrument. [e] An accurate R_T[a] value for this compound could not be obtained because pure authentic material was not available. [f] This value was obtained under the following programming conditions: 100 to 200° C. at 2° per minute. [g] An accurate retention time on Triton column could not be determined because the compound undergoes partial decomposition during chromatography.

Spectrometric Methods. The mass spectra were determined on a CEC 21-110B spectrometer (ionizing voltage 70 e.V.), or on an LKB-9000 spectrometer linked with a GLC column (ionizing voltage 70 e.V., source temperature 290° C., separator temperature 230° C.). The IR spectra were determined in $CHCl_3$ solution with a Beckman IR 5A instrument employing a beam condenser and an ultramicro cell (0.111 mm. thickness); spectra were calibrated with polystyrene. Unless otherwise specified, the NMR spectra were run in 5 to 7% deuterochloroform solution, in a 70-μl. microcell, on a Varian A60 spectrometer, and data reported in parts per million (δ) from tetramethylsilane (internal reference); probe was at ambient temperature.

Preparation of Dichloromethane Extract and Neutral Fraction. Two-hundred grams of Vitamin Free Casein (Nutritional Biochemicals Corp., Cleveland, Ohio) was extracted in a Soxhlet with dichloromethane for 32 hours, air dried, and then extracted twice with 80% aqueous ethanol at room temperature. The washed casein was then suspended in 1 liter of deionized water, the pH brought to 6.4 by slow addition of 0.1*N* sodium hydroxide with stirring, and the resulting colloidal solution treated with 44 grams of α-lactose (Fisher certified). The employed ratio lactose to casein corresponds to 1 mole of lactose per free amino group in casein. The mixture, *ca.* 2200 ml., was freeze-dried. The solid residue was kept in an atmosphere of 75% relative humidity (R.H.) at 80° C. constant temperature for 8 days. Discoloration proceeded uniformly throughout the entire mass, and, after 8 days, the resulting brown cake was broken up and extracted in a Soxhlet for 22 hours with dichloromethane (analytical grade and previously rectified). The CH_2Cl_2 extract was concentrated at normal pressure under a 33-cm. helices-packed column down to 40 ml. The residue was then pumped with a water aspirator at room temperature until removal of solvent was essentially complete. The residual reddish brown liquid, which on standing in a refrigerator yielded several milligrams of crystalline maltol, had a characteristic, pleasant aroma; yield 2.03 grams. This crude, which will be referred to as the dichloromethane extract, was analyzed by gas–liquid chromatography as indicated below.

The brown solid, after CH_2Cl_2 extraction and air drying, was extracted twice with a total of 5 liters of 80% aqueous ethanol at room temperature. The solvent was removed under reduced pressure to a final volume of *ca.* 30 ml. (A small amount of precipitate which separated during concentration, and which appeared to be sodium caseinate, was filtered off.) The residue was taken up with 400 ml. of deionized water, the solution clarified by filtration through celite, and then passed through a 200-gram column of Amberlite IR-120 (H^+) resin. The column was then thoroughly washed with water. The combined effluent and washings, 1500 ml., were stirred for 20 hours with 100 ml. of AG-1-X2 (Bio-Rad Corp., Cleveland, Ohio) (200- to 400-mesh) anion exchange resin in the carbonate form. The resin was then transferred to a column and washed extensively with water. The combined eluate and washings, containing the neutral products, were evaporated to dryness. The resulting thick syrup, 1.30 grams, which will be referred to as the neutral fraction, was analyzed by partition chromatography over cellulose.

Analysis of Dichloromethane Extract. The crude dichloromethane extract was fractionated into 10 cuts by repeated 45-μl. injections on column A, and trapping of the effluent in semicapillary tubes, cooled with dry ice, at time-measured intervals. Such cuts were numbered 1 to 10. The content of cuts 4 through 10 was analyzed with columns B and C. The compounds isolated were considered chromatographically pure when they gave single peaks in both such columns. They were then identified by comparing their retention times, mass spectra, and/or infrared spectra with those of authentic samples (except in the case of β-acetyl butyrolactone whose identification is therefore tentative). Namely, when the identity of a given compound could be firmly established from its infrared spectrum, the mass spectrum was not determined. On the other hand, when there was not enough material for determination of an infrared spectrum, identification was based solely on the mass spectrum and retention time. The products present in cuts 1 through 3 were analyzed with column D, and identified by their mass spectrum and retention times in comparison with authentic samples. The compounds identified are reported in Table I where the retention times R_T[a] and R_T[b], expressed in minutes, refer to columns A and B, respectively, under the conditions specified above. The four strongest mass spectral peaks (*m/e*) are reported; their intensities relative to that of the base peak (100%) are given in parentheses. The molecular ion (M^+) peak is reported in all cases, even if it was not among the four most intense peaks. The frequencies ($cm.^{-1}$) of the eight most intense infrared bands (between 4000 and 820 $cm.^{-1}$) are also reported in order of decreasing intensity. We are reporting in Table I infrared and mass spectral data only for those compounds whose such spectra were actually used in the identification process. The authentic materials were obtained from commercial sources;

few had to be synthesized as described below. In either case they were purified by GLC before determination of their spectral properties.

Authentic β-acetyl butyrolactone could not be made available, and the unknown was identified by correlating its mass and infrared data with those of the isomeric α- and γ-acetyl butyrolactones; the α-isomer is commercially available, and the γ-isomer was prepared as described below. The mass and infrared spectral data given for β-acetyl butyrolactone in Table I are those found for the unknown. The 2,5- and 2,6-dimethylpyrazine could not be completely separated on column D, the 2,5-isomer appearing as a shoulder on the peak corresponding to the 2,6-isomer. According to published data (Bondarovich *et al.*, 1967), which agree with our data obtained with the LKB spectrometer, the mass spectra of 2,5- and 2,6-dimethylpyrazine are virtually identical; continuous scanning of the composite peak does not produce any significant change in the mass spectrum, and the data reported in Table I were derived from a typical scan.

The structure of compounds 12, 20, and 40 was confirmed also by their NMR spectrum. In the spectrum of 3-butenoic acid-γ-lactone the methylene protons appear as a three-line signal at 3.17 δ; the vinylic proton at C-3 gives rise to a complex multiplet centered at 5.60 δ, and consisting of two overlapping triplets; the signal of the vinyl proton at C-4, also consisting of two overlapping triplets, is centered at 6.85 p.p.m. In the spectrum of β-hydroxybutyrolactone, the C-2 and C-4 methylene protons give complex multiplets at 2.63 and 4.38 p.p.m., respectively; the methine proton at C-3 absorbs at *ca.* 4.66 δ (complex multiplet), and the hydroxyl proton appears at 3.40 δ. In the spectrum of protoanemonin, determined in acetone-d_6 in a capillary tube, with a Varian HA-100, the terminal methylene protons absorb at 5.10 δ; the olefinic protons at C-2 and C-3 show up at 6.40 and 7.70 δ, respectively; all three signals are complex multiplets.

Synthesis of Authentic Materials. β-Hydroxybutyrolactone, 2- and 3-butenoic acid-γ-lactone. Alkaline potassium permanganate oxidation of vinyl acetic acid (Glattfeld and Miller, 1920) resulted in formation of β-hydroxybutyrolactone which was purified by GLC. However, when the crude sample was heated for 1 hour on a hot water bath prior to injection into the gas chromatograph, presence of 2- and 3-butenoic acid-γ-lactone was also observed in quantities sufficient for trapping.

2-Furfuryl methyl sulfide was prepared by methylation of 2-furfuryl mercaptan with dimethyl sulfate (Gianturco *et al.*, 1964).

5-Methyl-2-acetylfuran was obtained by acetylation of 2-methyl furan with acetic anhydride in the presence of boron trifluoride etherate.

2-Furfuryl methyl ketone was prepared by sodium ethoxide-catalyzed condensation of 2-chloropropionate with furfural, followed by alkaline hydrolysis and concomitant decarboxylation of the resulting glycidic ester, according to the procedure of Darzens (1906).

Protoanemonin. This compound was synthesized by acid-catalyzed lactonization of acetylacrylic acid (Shaw 1946) which was prepared by dehydrobromination of bromolevulinic acid (Overend *et al.*, 1950).

2,2′-Difurylmethane. This compound was prepared by acid-catalyzed condensation of furfuryl alcohol and furan according to the procedure of Brown and Sawatzky (1956).

[2-Furyl-(2′-methyl-5′-furyl)] methane was prepared by condensation of furfuryl alcohol and 2-methyl furan by the same procedure used for 2,2′-difurylmethane.

2-Acetylpyrrole was prepared by acetylation of pyrrole as described by Hess (1915).

Glutarimide was obtained by distillation of the dry ammonium salt of glutaric acid as described by Sircar (1927).

γ-Acetylbutyrolactone. Oxidation of 3-acetoxy-1-butene with manganese(III) acetate, according to the procedure of Bush and Finkbeiner (1968), followed by distillation, led to the isolation of 4,5-dihydroxy-*n*-hexanoic acid-γ-lactone acetate which was readily hydrolyzed and lactonized. The resulting 4,5-dihydroxyhexanoic acid-γ-lactone was finally oxidized with chromic acid to γ-acetylbutyrolactone, whose structure was confirmed by NMR spectroscopy.

4,5-Dihydroxyvaleric acid-γ-lactone. This compound was prepared by alkaline potassium permanganate oxidation of allylacetic acid according to the same procedure used for the preparation of β-hydroxybutyrolactone.

β-Formyloxybutyrolactone was prepared by zinc chloride-catalyzed formylation of β-hydroxybutyrolactone according to a previously reported procedure (Pickard *et al.*, 1923).

β-Acetoxybutyrolactone was prepared by acetylation of β-hydroxybutyrolactone (Ducher, 1962).

Analysis of Neutral Fraction. The syrupy crude product (1.30 grams) was stirred with 3 ml. of water and crystallization started by rubbing the walls of the flask. Several crops of pure lactose were obtained by repeated dilution with aqueous alcohol and chilling; yield *ca.* 600 mg. When no more lactose could be crystallized out, the mother liquor was evaporated to dryness. Paper chromatography of the syrupy residue showed presence of five compounds giving positive reaction with silver nitrate/sodium hydroxide reagent. Five-hundred milligrams of this mixture was fractionated by partition chromatography on 50 grams of degassed CF-11 Whatman standard grade cellulose (Reeve Angel and Co., Clifton, N. J.). The size of the packed column was 530 × 22 mm. and the solvent *n*-propanol/water 4 to 1. The crude mixture of carbohydrates was dissolved in 1 ml. of water, and 1.5 ml of *n*-propanol was added to incipient cloudiness. This solution was deposited on the cellulose column, and development started immediately. After an amount of effluent had emerged from the column, equivalent to the dead space volume (determined by passing through the column a few milligrams of Sudan III), 10-ml. fractions were collected, and their content monitored by paper chromatographic analysis. Fractions of identical composition were pooled and evaporated to dryness under reduced pressure. The following chromatographically pure sugars were obtained (chromatographic mobility relative to galactose in parenthesis): D-tagatose, 23 mg. (R_{gal} 1.22), D-galactose, 60 mg. (R_{gal} 1.00), lactulose, 85 mg. (R_{gal} 0.75), and lactose, 50 mg. (R_{gal} 0.59). Intermediate fractions consisting of mixtures of the above were not further purified.

D-Tagatose was identified by its chromatographic behavior and conversion to galactosazone. D-Galactose was identified by comparison of the infrared spectrum of the recrystallized (abs. ethanol) unknown with that of an authentic sample. Identity of lactulose was established by its chromatographic behavior and by its ready conversion to lactosazone; furthermore, on acid hydrolysis with 1% sulfuric acid at 100° C. for 24 hours, it yielded galactose and fructose. Lactose was converted into mucic acid by nitric acid oxidation. The identity of the substance responsible for the fifth and most mobile spot (R_{gal} 1.40) that appears in the paper chromatogram of the crude mixture of carbohydrates was not determined.

Lactulose, tagatose, and galactose, as well as 5-hydroxy-

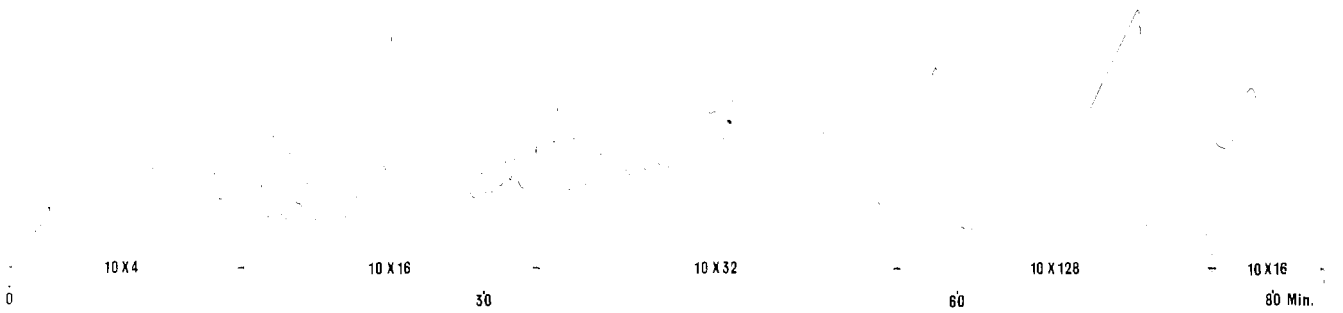

Figure 1. Gas chromatogram (time *vs.* instrument response) of a dichloromethane extract of a browned lactose-casein mixture

For conditions, see Experimental

methyl-2-furaldehyde, have been previously identified by Richards (1965) in the neutral fraction of an extract of a browned lactose–casein mixture prepared by a procedure similar to ours. In an earlier paper the same author (Richards, 1963) discussed also a possible pathway for their formation. However, we were unable to confirm the presence of 5-hydroxymethyl-2-furaldehyde in our neutral fraction.

RESULTS AND DISCUSSION

As a result of the present investigation, 40 compounds were isolated and identified in the dichloromethane extract of the browned mixture. They are listed in Table I which also shows gas chromatographic and spectral data. Figure 1 shows the chromatogram of the total dichloromethane extract on a Triton column.

Among the furanic compounds listed in Table I, only furfuryl alcohol, 5-methyl-2-furaldehyde, and 2-acetyl furan were previously identified in casein–lactose model systems (Langner and Tobias, 1967). Particularly noteworthy is the absence, from Table I, of 5-hydroxymethyl-2-furaldehyde which, like maltol, is considered a typical, and nearly ubiquitous, product of browning in model systems and heated milks as well. Occurrence of furanic compounds with a number of carbon atoms greater than 6, such as 17, 19, 24, 25, and 26 are of interest. At least two of them, 2,2′-difurylmethane and [2-furyl-(2′-methyl-5′-furyl)]methane, might have arisen from a polymerization reaction of two furanic precursors, and are almost certainly products of degradation and dehydration of lactose.

Compound 38 is the lactone of the well known C_4 saccharinic acid (3,4-dihydroxybutyric acid) which forms on alkaline degradation of carbohydrates (Sowden, 1957), particularly of 4-*O*-substituted glucose derivatives (Blears *et al.*, 1957); this lactone was previously identified by Richards (1965) in a lactose–casein browning system similar to ours. The 2- and 3-butenoic acid-γ-lactones are probably gas chromatographic artifacts. Their formation was observed, in our laboratory, when a sample of synthetic 3,4-dihydroxybutyric acid was chromatographed, after heating, on a silicone rubber (SE-30) or a Carbowax 20M column, and during chromatography, on a Triton X305 column, of β-acetoxy- and β-formyloxybutyrolactone; in addition, each one of the unsaturated lactones could be converted into a mixture of both by injection in any of the above columns. Formation of β-hydroxybutyrolactone and 2-butenoic acid-γ-lactone by distillation of 3,4-dihydroxybutyric acid was first observed by Glattfeld *et al.* (1931). However, in view of the relatively facile mutual interconversion of the α- and β-butenolide systems (Cocker and Hornsby, 1947; Filler *et al.*, 1961; Newman and VanderWerf, 1945; Thiele, 1901) and of the conditions under which the browning reaction was carried out, the possibility that the unsaturated lactones 12 and 28 were present in the original dichloromethane extract cannot be ruled out.

β-Acetyl butyrolactone and 4,5-dihydroxyvaleric acid-γ-lactone do not seem to be directly related to the saccharinic acids, but are probably degradation products of lactose.

Pyrazine and alkyl-substituted pyrazines have not been previously reported in casein–lactose systems, but simple pyrazines have been found in sugar–amino acids (Dawes and Edwards, 1966; Newell *et al.*, 1967; van Praag *et al.*, 1968) and sugar–ammonia browning mixtures (Hough *et al.*, 1952; Jezo and Luzak, 1966); Koehler *et al.* (1969) suggested a possible pathway for their formation. On the contrary, no pyridine compound has been previously reported in browning systems involving casein; pyridine and 2-methyl pyridine, found in our flavor extract, probably are formed in a way analogous to the one leading to formation of pyrazines. 2-Acetyl pyrrole has been recently reported in lactose–amino acids and lactose–casein heated systems (Langner and Tobias, 1967).

2-*n*-Heptanone has been consistently associated with heated milk fat (Parks, 1967; Scanlan *et al.*, 1968) and, also in our case, it probably originated from traces of residual fat present in the casein. Acetic acid (Keeney *et al.*, 1950) and propionic acid (Kern *et al.*, 1954) have also been previously reported in heated milks, and they are probably the precursors of acetamide and propionamide. Pyrrolidinone, succinimide, and glutarimide are less readily accountable on the basis of the accepted pathways of the Maillard reaction; it is noteworthy, however, that earlier investigators have reported formation of succinic acid during alkaline degradation of lactose (Cuisinier, 1882; Hlasiwetz and Barth, 1866; Hoppe-Seyler, 1871; Nencki and Sieber, 1881).

D-Galactose, lactulose, and D-tagatose, along with intact lactose, were the only carbohydrates identified in the neutral fraction of the aqueous alcoholic extract of the browned mass. Lactulose and D-tagatose have been previously found in heated and/or stored dairy products (Adachi and Patton, 1961; Adachi, 1952, 1958).

In two separate blanks, samples of casein and lactose were allowed to stand in the reaction chamber for the same length of time and under identical conditions of temperature and R.H. as the experimental browning mixture. No change occurred in either blank which could be detected by GLC or paper chromatography.

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