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GAS CHROMATOGRAPHIC ANALYSIS OF AMINES IN VOLATILE SUBSTANCES OF *STREPTOCOCCUS LACTIS**

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SUMMARY

The qualitative composition and quantitative relationships of amines in the volatile components of 6-, 24-, and 30-h cultures of a strain of *Streptococcus lactis*, in the logarithmic growth phase, was studied chromatographically using retention indices.

The 21 amines found were identified as follows: primary amines—methylamine, ethylamine, isopropylamine, butylamine, isobutylamine, *tert.*-butylamine, amylamine, isoamylamine; secondary amines—dimethylamine, diethylamine, dipropylamine, diisopropylamine, diisobutylamine, *n*-butylisopropylamine, pyrrolidine, piperidine; tertiary amines—trimethylamine, triethylamine, tripropylamine. The qualitative composition of amines remained constant as the culture grew, but a sharp change in the quantities of the individual amines in the 6-, 24-, and 30-h cultures was observed. This pointed to the formation of amines during the metabolism of the bacterial cells and suggested the existence of special fermentative systems involved in the synthetic production of amines. Thus a fundamentally new phenomenon that of synthesis of secondary and tertiary amines by microorganisms has been discovered.

INTRODUCTION

It is generally agreed that aliphatic amines are formed on decarboxylation of amino acids[1-3]. This is true, however, only for primary amines, whereas secondary and tertiary amines can be formed only through alkylation processes that are as yet unknown with the exception of methylation. We have previously shown[4-8] that the volatile components of food products contain a great number of organic bases many of which were rather unexpectedly found to be secondary and tertiary aliphatic amines.

It was thus considered of interest to determine whether the formation of these amines was due to vital functions or was the result of disintegration of decayed organisms. A study of the composition and kinetics of amine accumulation in the volatile components of quickly growing organisms was therefore undertaken; the

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strain of *Str. lactis* being chosen for the investigation. The organic bases were identified by gas chromatography by a procedure previously described[9,10].

MATERIAL AND METHODS

The strain of *Str. lactis* was maintained on wort agar slants at 37°. Cultivation took place in twenty-four 2 l conical flasks each containing 400 ml of sterilised skim milk, used as nutrient, inoculated with two agar slants of microorganisms.

At intervals of 6-, 24-, and 30- h (logarithmic phase of growth), fermentation was stopped by means of a 5 % solution of mercuric chloride (5 ml per 400 ml of culture medium). The 24- and 30-h portions were centrifuged and precipitated to yield 535 and 545 g, respectively. The 6-h portion looked like milk and 500 ml of it was investigated without centrifugation. The volatile compounds were isolated *in vacuo* as previously described at 30–33° under 3–4 mm pressure[4]. With the 6-h culture, the volatile compounds were trapped under 15 mm pressure. The compounds removed, together with water vapour, were condensed in three traps containing a 10 % solution of trichloroacetic acid in absolute ethanol. The traps were cooled with dry-ice and acetone. The volatile components of the 24- and 30-h cultures were allowed to distil over for 24 h, those of the 6-h specimen for 8 h. Special investigation showed it not expedient to prolong the time of isolation. After distillation under vacuum the amount remaining was 250, 152, and 159 g for the 6-, 24-, and 30-h cultures, respectively.

After each run the contents of the traps were combined and vapour-distilled under nitrogen to remove alcohol, neutral and acidic compounds. The distillate (about 1 l) was discarded, the contents of the traps treated with KOH solution up to pH 10, the organic bases were distilled under nitrogen and were absorbed by 0.2 *N* HCl. The hydrochloric solution (*ca.* 1 l) was evaporated to dryness at 30–35° in a rotary evaporator and the remaining hydrochloric salts were dried under vacuum to yield 6.7, 10.0 and 10.6 mg of 6-, 24-, and 30-h specimens, respectively. The free organic bases were analysed by gas chromatography, each sample consisting of 4.5 mg of hydrochloric amine salts, 6 μl of distilled water, 50 μl of chromatographically pure dodecane and a small piece of solid KOH[11].

Analyses of the amine solutions in dodecane were made at 100° with a gas chromatograph equipped with a flame ionisation detector and nitrogen flow rate of 20–60 ml/min using four 1.5 m × 4 mm glass columns packed with the detergent 'Novator'[9] coated with liquid phases of various polarity: 10 % of tristearin; 5 % of liquid paraffin with 2 % of KOH, 10 % of Tween-80 and 10 % of polyethylene glycol 1000.

The sample (5 μl) was injected into the column with a Hamilton syringe (10 μl). Gas chromatographic parameters were computed in terms of retention indices[12].

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms obtained on a column with liquid paraffin for amine mixtures isolated from the volatile components formed during the logarithmic phase of growth of *Str. lactis* for 6-, 24-, and 30-h. Comparative data demonstrate that the volatile components of these bacteria contain a large amount of organic bases. It is also quite evident that their proportions change with time (compare peaks 4, 12, and 17).

The amine content was estimated in terms of the peak area calculated according to the formula $S = ht$, where h is the height of the peak and t the retention time.

In the absence of absolute calibration this method fails to determine the true content of every amine in the mixture, but it can be used to follow changes in concentration of a particular amine in the sample under examination and, in our case, to obtain kinetic evidence concerning the character of the change in the composition of the amines as the *Str. lactis* grows.

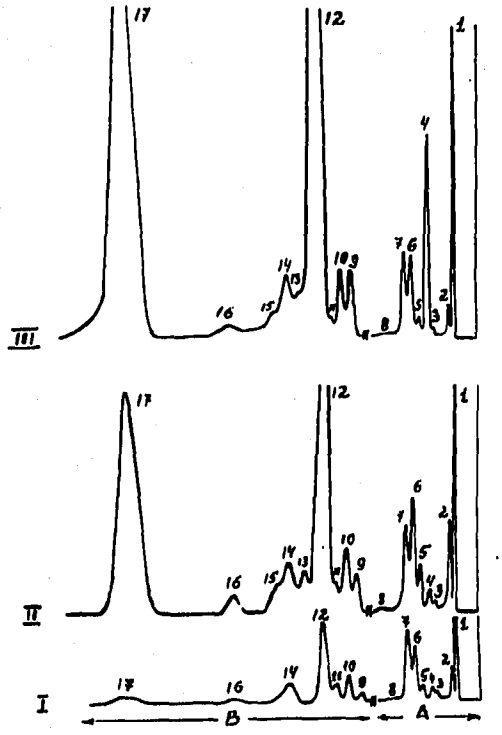

Fig. 1. Chromatogram of amines found in the volatile products resulting from the metabolism of *Str. lactis* on the 150 × 0.4 cm column packed with detergent 'Novator'; liquid phase: 5% liquid paraffin with 2% KOH. Temperature: 100°; nitrogen flow rate: 26 ml/min (section A) and 55 ml/min (section B). I = the 6-h culture; II = the 24-h culture; III = the 30-h culture. 1 = trimethylamine; 2 = dimethylamine + methylamine; 3 = ethylamine; 4 = diethylamine + isopropylamine; 5 = not identified; 6 = diisopropylamine; 7 = triethylamine, 8 = not identified; 9 = isobutylamine; 10 = dipropylamine; 11 = butylamine; 12 = not identified + pyrrolidine, 13 = isoamylamine; 14 = diisobutylamine + piperidine; 15 = amylamine; 16 = tripropylamine; 17 = not identified.

Table I lists the amines identified in the volatile compounds of these bacteria after 6-, 24-, and 30-h (logarithmic growth phase) as well as the change in their composition in the mixture during the above periods of time.

It will be seen from Table I that the mixture contains 21 amines. However, their actual number might prove to be much higher because the analytical conditions used permitted the chromatograms to register only those amines whose retention index did not exceed 1200 units. This is the index of dodecane that was used as a solvent for the introduction of the amines into the chromatograph.

Chromatograms were analysed by the method already described involving the equations derived for correlating the retention index with the number of carbon

TABLE I

CONCENTRATION CHANGES OF THE AMINES IN THE VOLATILE COMPOUNDS OF *Str. lactis*

Amine concentration is given as relative percentage.

No.	*Amines*	*Culture growth period (hours)*		
		6	*24*	*30*
Primary				
1	Methylamine	5.2	0.7	0.1
2	Ethylamine	0.3	0.1	0.1
3	Isopropylamine	traces	0.2	0.2
4	Butylamine	1.5	0.2	0.1
5	Isobutylamine	0.8	0.7	0.7
6	Amylamine	—	0.4	0.4
7	Isoamylamine	0.7	0.7	0.1
8	Aliphatic amine	1.9	1.3	0.2
Secondary				
9	Dimethylamine	0.1	1.2	0.1
10	Diethylamine	2.5	0.5	3.6
11	Dipropylamine	4.0	1.2	0.7
12	Diisopropylamine	9.4	2.8	1.3
13	Diisobutylamine	—	1.2	0.6
14	Aliphatic amine	15.9	50.6	56.1
15	Pyrrolidine	2.9	0.4	0.1
16	Piperidine	5.6	traces	0.1
17	Not identified	0.2	0.5	traces
Tertiary				
18	Trimethylamine	25.7	7.2	2.8
19	Triethylamine	18.3	2.0	1.7
20	Tripropylamine	0.7	1.3	0.3
21	Aliphatic amine	4.1	27.0	30.9

atoms and the boiling point of the substance[9,10]. This method led to the identification of seventeen of the organic bases of the 21 amines listed in Table I. Regularities, previously discovered, made it possible to determine the character of the functional group and to estimate the boiling point of three of the unidentified amines (Table I, Nos. 8, 14, 21; Fig. 1, peaks 5, 12, 17, respectively).

This was done by determining the value of ΔI that is specific and rather constant for primary, secondary, and tertiary amino groups. The data used for the identification of these three amines are given in Table II. The values of ΔI presented in Table II characterise No. 8 as a primary, No. 14 as a secondary, and No. 21 as a tertiary aliphatic amine. Knowing the character of amine it is possible to estimate its boiling point from corresponding equations for primary, secondary, and tertiary amines. These equations hold for aliphatic amines with a normal or isocarbon chain.

Table II (column 8) shows the calculated boiling points.

The data on the character of the carbon chain of the amines under investigation were obtained by equations correlating the index value with the number of carbon atoms in the molecule derived by us for *n*-aliphatic primary, symmetrical secondary and tertiary amines. The experimental values for the retention indices of the amines being investigated were substituted into these equations. The deficit in the number of carbon atoms points to branched carbon radicals.

TABLE II

DATA USED TO DECIPHER UNIDENTIFIED AMINES

I^{TS}_{100}, $I^{L.P.}_{100}$, I^{TW}_{100}, I^{PEG}_{100} = The indices of the amines at the temperature of 100° on columns with triste

No. of amine in Table I and standards	I^{TS}_{100}	$I^{L.P.}_{100}$	I^{TW}_{100}	I^{PEG}_{100}	$\Delta I^{L.P.-TS}$	ΔI^{PEG-TW}
8-Unidentified	541	645	611	784	104	173
tert.-Butyl amine	550	647	598	780	97	182
14-Unidentified	822	822	909	966	0	57
Isopropyl-*n*-butylamine	821	820	904	963	−1	59
n-Propyl-isobutylamine	823	819	897	952	−4	55
21-Unidentified	984	981	1007	1025	−3	17

It can be deduced from Table II that the three amines all contain isocarbon radicals. It was also suggested that amine No. 8 could be *tert.*-butylamine, as the retention index values for isopropyl- and isobutylamines are different[10]. To prove this suggestion a study was undertaken of the behaviour of a standard of *tert.*-butylamine in all the liquid phases; the values of the retention indices are given in Table II. The agreement between the values of indices and boiling points permitted amine No. 8 to be identified as *tert.*-butylamine.

Amine No. 14 was identified structurally by referring to the calculated retention indices of some isomers with seven atoms. It was found in this way that the closest values to that of amine No. 14 were those of *n*-butylisopropylamine and *n*-propyl-isobutylamine. The two amines were synthesised and their indices were measured on the four columns. The retention values and boiling points showed amine No. 14 to be *n*-butylisopropylamine. The tertiary amine No. 21, boiling at 166° and having ten carbon atoms might have one or several isocarbon chains. It was not possible to identify this amine because it has many isomers and, according to literature, at least four of them boil at about 166° (ref. 13).

It is evident from Table I and chromatograms on Fig. 1 that the qualitative composition of organic bases isolated from the volatile products of *Str. lactis* metabolism was constant during the whole period of growth investigated. On the other hand, a sharp change in the quantitative relationships was observed over the same period. It was of interest to follow these changes during the growth of *Str. lactis*. In the 6-h culture, taken as a reference point, about one-half of the total amines was represented by trimethylamine and triethylamine; in the 30-h culture their percentage was drastically lowered (almost by ten times) with a corresponding increase of *n*-butylisopropylamine and a tertiary amine with 10 carbon atoms whose total percentage rose up to 87 (Table I).

It is of interest compare our results with the data reported by WEURMAN[14] on the composition of volatile amines in milk. Using paper chromatography he found

liquid paraffin, Tween-80 and polyethylene glycol 1000, respectively.

B.p. of amine calc. according to formula:	*Lit b.p.*	*The number of carbon atoms (n) according to formula:*	*Identity*
b.p. $= \frac{I^{L.P.}_{100}}{4.0} - 116 = 45$	45	$n = \frac{I^{L.P.}_{100} - 386}{100} = 2.6$	*tert.*-Butylamine
b.p. $= \frac{I^{L.P.}_{100}}{4.0} - 85 = 121$	124 126	$n = \frac{I^{L.P.}_{100} - 176}{100} = 6.5$	Isopropyl *n*-butylamine
b.p. $= \frac{I^{L.P.}_{100}}{4.0} - 79 = 166$		$n = \frac{I^{L.P.}_{100} - 146}{86} = 9.7$	Tertiary amine with 10 carbon atoms

methylamine, ethylamine, butylamine, and dimethylamine and suggested the presence of pyrrolidine. Our experiments showed a rapid decrease in the amount of pyrrolidine during the growth of the bacteria. It is therefore thought that the presence of this amine was due to its availability in the milk initially. The data presented in Table I and Fig. 1 cannot be used to draw any conclusions regarding the mechanism of formation of secondary and tertiary amines. It can only be said that the monoalkylamines do not undergo a gradual (stepwise) alkylation to tertiary amines as shown by the differences in, say, the concentrations of diethylamine and triethylamine. The same is observed to be true for isobutylamines and diisobutylamines.

The qualitative composition of the amines isolated from the volatile products of *Str. lactis* metabolism is quite varied involving primary, secondary, and tertiary amines with a normal or isocarbon chain, pyrrolidine and piperidine.

An interesting peculiarity of the amine composition is its relatively high content of various secondary and tertiary amines such as diisopropylamine, diethylamine, diisobutylamine, *n*-butylisopropylamine, and a tertiary amine with ten carbon atoms.

Decarboxylation of free amino acids can account for such primary aliphatic amines as methylamine, ethylamine, isobutylamine, and isoamylamine whose precursors may be, correspondingly, glycine, alanine, valine and leucine. Pyrrolidine may result, similarly, from proline. However, the mechanism of the formation of most of the amines is not clear. The presence of such structurally simple primary amines as *n*-butylamine, *n*-amylamine, isopropylamine and, even more so, of secondary and tertiary amines cannot be explained solely by decarboxylation of amino acids.

The presence of secondary and tertiary amines of varied structure and their increasing percentage during the growth of the lactobacilli proves that these amines result from the metabolism of these bacteria. One might, therefore, suggest the existence of special enzymes involved in the synthesis of these amines.

It is to be noted that the qualitative composition of the amines in volatile

products of *Str. lactis* metabolism differs from that found by us in other food products[4-8].

Both the origin of the amines and the role they play in the vital activity of the organisms are not clear. It could be suggested that the amines provide a peculiar defence for the microorganisms; in which case the qualitative and quantitative composition of the amines in the volatile components of various microorganisms could be specific for every particular species.

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