Mechanism of Major Congener Formation in Alcoholic Grain Fermentations

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The biochemical mechanisms for congener production were studied by adding glucose-U-^{14}C, leucine-U-^{14}C, and isoleucine-U-^{14}C to bourbon fermentations. Glucose was found to form acetic acid and fusel oil; leucine formed butyric and acetic acids and 3-methyl-butanol; isoleucine produced 2-methyl-butanol. All three formed ethyl acetate, apparently via acetyl CoA condensation with ethanol. Volatile acid and fusel oil formation are interrelated through common keto acid intermediates. Higher alcohols are produced either as a byproduct of amino acid biosynthesis from carbohydrate or directly from existing amino acids; the amount produced is governed in part by the yeast's ability to transform carbohydrate. Early in the fermentation fusel oil is produced mostly from amino acids; in later stages both amino acids and carbohydrates are substrates. Addition of ammonium ion reduces fusel oil formation by blocking its synthesis from carbohydrate through feedback mechanisms.

The fermentation of grain mashes by yeast results in the formation of small amounts of congeners in addition to ethyl alcohol. These congeners contribute to the characteristic flavor and odor of the alcoholic beverage. It was once believed that fusel oil was produced only from amino acids, while esters and acids were formed from mash carbohydrates. These concepts originated from observations that fusel oil (higher alcohols) increased when either albumin digests or casein hydrolyzate was added to a fermentation (Ehrlich, 1907; Thorne, 1945, 1950) and that ester and acid formation was dependent upon the aerobic fermentation of mash carbohydrates (Kayser and Demolon, 1909; Tabachnick and Joslyn, 1953). Current information on the intracellular metabolism of yeast has provided biochemical mechanisms, indicating that the major whiskey congeners can be produced from both amino acids and carbohydrates. Most of the studies concerned with the biochemistry of congener formation were made using either mutant yeast and/or synthetic media (Ayrapaa, 1967; Guymon, 1966; Ingraham *et al.*, 1961; Nordstrom, 1961; Ylamen, 1966).

The data to be presented were obtained from fermentations of bourbon mash with distillers yeast, *Saccharomyces cerevisiae*, to determine how these biochemical systems function under conditions typical of the production process. The experimental fermentations consisted of bourbon mash containing added glucose or amino acids uniformly labeled with radioactive carbon-14. The higher alcohols, ethyl acetate, and volatile acids were isolated and the amount of radioactivity in each fraction determined. By comparing the distribution of radioactive carbon among the major congeners, the role of the above substrates in congener production was determined.

MATERIALS AND METHODS

Fermentation Conditions. Approximately 6 μCi of radioactive substrate were added to 1.5 l. of a laboratory prepared bourbon mash (75% corn, 13% rye, and 12% barley malt). The following radioactive materials were used individually in fermentations: D-glucose-U-^{14}C, L-leucine-U-^{14}C, and L-isoleucine-U-^{14}C, obtained from New England Nuclear Co. The fermentation was controlled at 30° C for a period of 3 days.

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Congener Analysis of Fermented Mash. A 100-ml portion of the fermented mash was distilled, using a Kjeldahl unit, into a 100-ml receiver, and the distillate used for the usual distillers congener analysis. Fusel oil was determined by a Komarowsky colorimetric procedure (Methods of Analysis, A.O.A.C., 1965) and total esters by the color reaction with hydroxylamine, modified from Hestrin (1949). The concentration of the individual volatile acids and higher alcohols was determined by gas liquid chromatography.

Congener Fractionation. The remaining 1400 ml of fermented mash was Kjeldahl distilled in 200 ml portions and the distillates combined. Five milliliters of 5% sodium bicarbonate solution were added and the product was redistilled. The volatile acids remained in the residue and the distillate contained the esters, ethanol, and fusel oil.

The residue was acidified and the volatile acids separated by steam distillation. This distillate was made alkaline and concentrated by evaporation on a steam bath to a volume of 10 ml. The various acids present were quantitated by analytical gas chromatography and then separated into acetic, propionic, butyric, and isovaleric acids by preparative gas chromatography.

Esters and fusel oil were separated from the distillate in a 48 in. × 25 mm Podbielniak fractionation still operated with a boilup rate of 300 ml per hr and a reflux ratio of 15:1. The isoamyl, *d*-amyl, isobutyl, and *n*-propyl alcohols in the fusel oil fraction were quantitated and collected by analytical and preparative gas chromatography, respectively. The ethyl acetate in the ester fraction was treated in a similar manner.

Gas Liquid Chromatography. An Aerograph Model 200 gas chromatograph with dual hydrogen flame ionization detectors was used. A stainless steel column 10 ft × $^1/_8$ in. o.d. packed with 5% FFAP on 60/80 mesh acid washed Chromosorb G was employed for analytical determinations of acids. Fusel oil alcohols were analyzed by means of a 10 ft × $^1/_8$ in. o.d. stainless steel column packed with 20% glycerol on 60/80 mesh acid washed firebrick connected in series with a 10 ft × $^1/_8$ in. o.d. column packed with 20% Carbowax 1540 on 60/80 mesh acid washed Chromosorb P. The concentration of the materials present was determined by peak areas, using as the internal standard valeric acid in the acid analysis and *n*-butanol in the fusel oil analysis.

The various congener groups studied were fractionated by preparative gas chromatography with the same packings as used in the analytical gas chromatography studies. However,

Table I. Distribution of Carbon-14 in Volatile Acids from Fermentations with Radioactive Glucose and Leucine[a]

Acid	Composition, %	Glucose-U-^{14}C		Leucine-U-^{14}C	
		mμc	%	mμc	%
Acetic	88.5	0.324	76.6	0.034	1.7
Propionic	1.8	0.006	1.4	0.008	0.4
Isobutyric	4.6	0.045	10.6	0.045	2.2
n-Butyric	0.5	0.000	0.0	0.582	28.2
Isovaleric	4.6	0.048	11.4	1.392	67.5

[a] Fermentations contained 6.29 μc glucose-U-^{14}C or 4.75 μc leucine-U-^{14}C per l. of mash.

Table II. Radioactivity of Ethyl Acetate from Fermentations Containing Radioactive Glucose and Amino Acids

Radioactive Substrate	Ethyl Acetate		
	Concentration ppm	Radioactivity mμc/1.5 l. mash	Specific activity mμc/mg of carbon
Glucose[a]	9.1	2.07	0.279
Isoleucine[b]	8.5	4.60	0.662
Leucine[c]	9.0	9.00	1.226

[a] 6.29 μc glucose-U-^{14}C added per l. of mash: specific activity was 0.288 mμc per mg of carbon. [b] 6.37 μc isoleucine-U-^{14}C added per l. of mash. [c] 4.75 μc leucine-U-^{14}C added per l. of mash.

Table III. Radioactivity of Higher Alcohols from Fermentations Containing Radioactive Glucose and Amino Acids

Radioactive Substrate	Radioactivity in Higher Alcohol, mμc/1.5 l. Fermentations			
	n-Propyl	Isobutyl	*d*-Amyl	Isoamyl
Glucose[a]	0.9	4.0	4.7	6.6
Isoleucine[b]	0.0	0.0	1607.0	225.0
Leucine[c]	0.0	19.9	68.5	1249.0

[a] 6.79 μc glucose-U-^{14}C added per l. of mash. [b] 6.37 μc isoleucine-U-^{14}C added per l. of mash. [c] 4.75 μc leucine-U-^{14}C added per l. of mash.

in this case, the column was 1/4 in. o.d. stainless steel. A stream splitter, 10:1 ratio, was inserted between the column and detector. The effluent was condensed in Aerograph collection tubes submerged in a methyl Cellosolve bath at −60° C. One milliliter of scintillation fluid, used in radioactivity determinations, was placed in each tube to aid in trapping the congener. After each compound was collected, the contents of the collection tube were analyzed for radioactivity, as described below.

Operating conditions of the gas chromatograph for analysis and collection of acids were: oven temperature 135° C, detector temperature 275° C, and injection temperature 250° C. Operating conditions for analysis and collection of the higher alcohols were: oven temperature 85° C, detector temperature 175° C, and injection temperature 125° C. The carrier gas in all cases was nitrogen (prepurified grade) at 30 ml per min flow rate.

Ethyl acetate was isolated from the ester fraction using the same preparative series column as was used for fusel oil.

Radioactivity Measurements. The radioactivity of the isolated congeners was determined in a Packard Model 314 Tri-Carb liquid scintillation spectrometer. A channel ratio method was used to correct for sample quenching. The scintillation fluid, 20 ml dispensed into 25 ml polyethylene screw-cap vials (Packard Instrument Co.), consisted of a mixture of toluene and Cellosolve containing PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-2-(5-phenyl-oxazolyl)-benzene] as described by Bruno and Christian (1961). All materials were of scintillation and spectroscopic quality.

RESULTS

As shown in Table I, 89% of the volatile acidity formed during fermentation is acetic acid. Isobutyric and isovaleric acids were next in concentration, while propionic and *n*-butyric acids were presented in the smallest amounts.

When radioactive glucose was added to the mash, 77% of the radioactivity in the volatile acid fraction was found in the acetic acid and the remaining 23% in propionic, isobutyric, and isovaleric acids. No radioactivity was found in the *n*-butyric acid fraction. When radioactive leucine was added to the mash, isovaleric and *n*-butyric acids contained most of the radioactive carbon, while the acetic acid contained very little. These data indicate that volatile acids are products of both carbohydrate and amino acid metabolism, but that the individual acids may be formed primarily from specific mash components, *e.g.*, acetic acid from the mash carbohydrates; isovaleric acid from leucine.

The radioactivity found in the ethyl acetate is shown in Table II. Each fermentation produced the same amount of ester and each of the three radioactive substrates were converted into this ester.

The relative role of these substrates in the production of esters was determined by calculating the ratio of radioactive carbon to total carbon (specific activity) of the ester molecule. Since the glucose pool within the yeast exists as a mixture of glucose from mash carbohydrate and from radioactive glucose, the specific activity of the pool was estimated from the glucose radioactivity added (radioactive carbon) and the initial amount of fermentable carbohydrate in the mash (total carbon). The specific activity of the yeast glucose pool was estimated to be 0.288 mμc per mg of carbon. This specific activity was very close to that of the ethyl acetate, 0.279 mμc per mg of carbon, indicating that mash carbohydrate was the primary substrate for ethyl acetate production.

Since the concentration of the individual amino acids in the mash was not determined, similar calculations relating ester specific activity to amino acid specific activity were not made. Therefore, the significance of the other two values is not yet known. It would appear from other data that the specific activity of the respective amino acids is considerably higher than that of the ester molecule. Therefore, these amino acids probably are a minor source of ethyl acetate.

Radioactivity data were also obtained for the individual higher alcohols of fusel oil, Table III. When radioactive glucose was present in the mash, isoamyl alcohol had the highest radioactivity, followed by *d*-amyl, isobutyl, and *n*-propyl alcohol. When radioactive isoleucine was added, only *d*-amyl and isoamyl alcohols became radioactive. About 90% of the radioactivity was found in the *d*-amyl alcohol fraction. When radioactive leucine was added, 95% of the tracer was found in the isoamyl alcohol.

The specific activity of each higher alcohol, determined by dividing its radioactivity by the mg of carbon represented by the concentration of higher alcohol, is shown in Table IV. In each case the alcohol specific activity is less than that of the glucose, which was 0.141 mμc per mg of carbon. By determining the ratio of higher alcohol to glucose specific activity, the percent of higher alcohol produced from mash carbohydrate was about 25% of *n*-propyl alcohol, 43% of isobutyl alcohol, and 35% of each amyl alcohol. It is estimated that approximately 35% of the total fusel oil produced was derived from mash carbohydrates.

Having conducted experiments relating to the types of congeners produced from various mash components, additional experiments were performed to determine the effect

Table IV. Specific Activities of Higher Alcohols Produced in Fermentations Containing Radioactive Glucose

Higher Alcohol	Concentration ppm	Total Radioactivity mμc/1.5 l.	Specific Activity[a] mμc/mg of carbon	Amount from Glucose, %
n-Propyl	27.3	0.85	0.035	24.8
Isobutyl	67.8	3.98	0.060	42.6
d-Amyl	91.4	4.71	0.050	35.4
Isoamyl	134.0	6.59	0.048	34.0
Fusel Oil	320.5	16.13	0.050	35.4

[a] Specific activity of glucose estimated to be 0.141 mμc/mg of carbon.

Table V. Fusel Oil Production by Different Yeast Strains in Fermentations with Added Radioactive Amino Acids

Yeast Strain	Fusel Oil Produced, ppm	Amount of Radioactive Amino Acid Converted into Higher Alcohol[a]	
		Leucine-U-^{14}C, %	Isoleucine-U-^{14}C, %
A	325	16.0	10.2
B	327	16.0	12.5
C	297	16.0	10.8
D	245	19.3	16.8
E	239	16.1	12.5
F	179	20.0	15.3
G	158	20.3	15.1
H	108	12.3	9.6
I	107	15.5	6.7

[a] Amount of radioactive amino acid added was constant for each series of fermentations.

of certain fermentation variables on the relative utilization of these substrates. Yeast strains are known to differ in the amount of fusel oil they produce; therefore, it was of interest to determine if they also differ in their ability to produce higher alcohols from mash amino acids and carbohydrates. Since radioactive leucine and isoleucine were shown to be transformed mostly into their respective higher alcohol, the percentage of radioactivity found in the distillate will indicate the amount of amino acid used in the production of the higher alcohol. Table V summarizes the results obtained with nine yeast strains and shows that the percent of radioactive leucine and isoleucine converted into fusel oil remains more or less constant, even though the total amount of fusel oil produced varies widely. This suggests that the activity of the carbohydrate-fusel oil system varies, while the activity of the Ehrlich amino acid-fusel oil system is more or less constant. Thus, those yeasts that produce large amounts of fusel oil appear more able to transform glucose into fusel oil, or into amino acids, than those producing low amounts of fusel oil.

In another experiment, using radioactive leucine, changes in fusel oil production and specific activity were followed during the course of a fermentation (Figure 1). In 12 hr, about 82% of the α-amino nitrogen initially present was used, while only 25% of the total fusel oil had been produced. The specific activity of the fusel oil remained constant to the eighth hour then decreased throughout the rest of the fermentation. These data show a shift in the mechanisms for producing fusel oil. If fusel oil formation from both the mash carbohydrates and amino acids had remained constant during the fermentation, the specific activity of the fusel oil would have remained constant, even though the amount of fusel oil present increased. This did not occur. The data suggest that, during the first 8 hr of fermentation, fusel oil was produced from the mash amino acids, while after this time fusel oil was also produced from mash carbohydrates

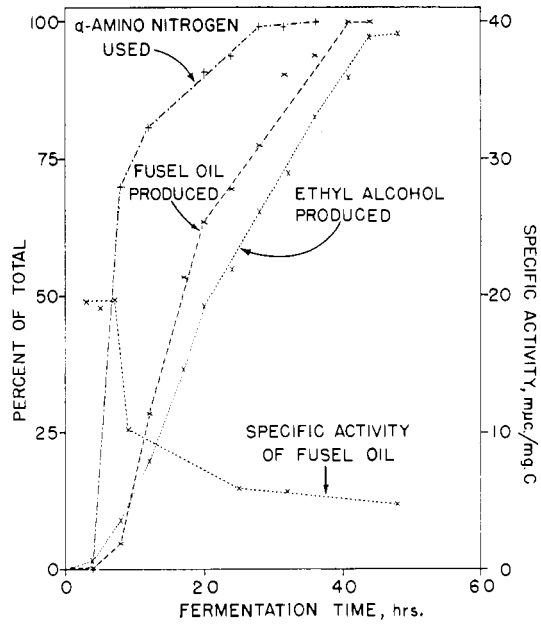

Figure 1. Changes in fusel oil specific activity and α-amino nitrogen during bourbon mash fermentation

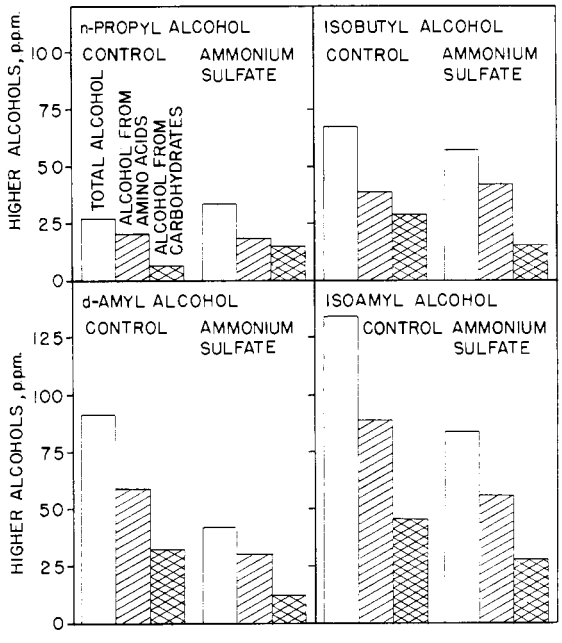

Figure 2. Effect of ammonium ion on proportion of higher alcohols originating from mash carbohydrates and amino acids

at an increasing rate. It should be pointed out that, even though the specific activity decreased in the latter stages of fermentation, fusel oil is still being produced from the mash amino acids, some of which are formed during fermentation. This is demonstrated by the fact that more fusel oil originates from mash amino acids than from carbohydrates (Table IV).

The effect of adding ammonium ion to the mash was also determined. 260 ppm nitrogen was added in the form of ammonium sulfate, along with radioactive glucose. The higher alcohols were isolated and their specific activities determined. By comparing these values with the specific activity of the original glucose, the percent of higher alcohol originating from carbohydrate and from amino acids (by difference) can be estimated. As shown in Figure 2, there was a selective effect on higher alcohol production but, in general, a decrease resulted.

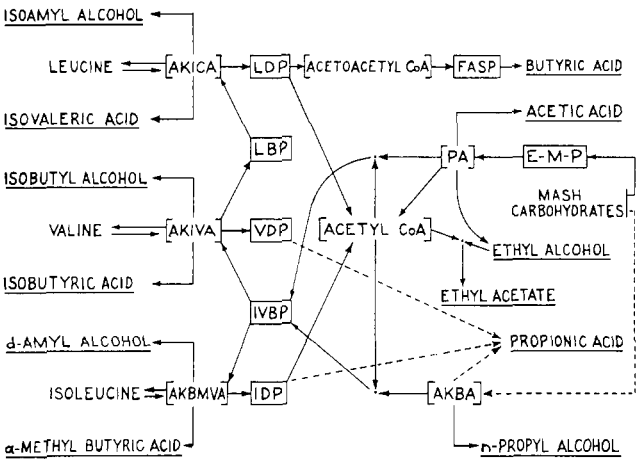

Figure 3. Proposed metabolic pathways for the synthesis of whiskey congeners

— synthetic and degradative pathways: - - - hypothetical pathways. LBP—Leucine biosynthesis: IVBP—Isoleucine-valine biosynthesis; FASP—Fatty acid biosynthesis; LDP—Leucine degradation; VDP—Valine degradation; IDP—Isoleucine degradation; EMP—Embden-Meyerhof-Parnas fermentation pathway

In the case of *n*-propyl alcohol, both the total amount and that produced from mash carbohydrates were greater in the presence of ammonium ion than in the control fermentation. That produced from mash amino acids did not change. In the case of isobutyl alcohol, both the total and that produced from mash carbohydrates was less with ammonium ion than was found for the control fermentation. That from amino acids did not change. Ammonium sulfate caused a reduction in isoamyl and *d*-amyl alcohol production from both amino acids and carbohydrates.

DISCUSSION

Since it has been demonstrated that most of the congeners studied can be produced from both carbohydrates and amino acids, it may be that there are common intermediates in the metabolic pathways. The various metabolic systems that could be involved in these transformations are shown in Figure 3. It is suggested that the various keto acids have a common role in the production of the volatile acids and alcohols. For example, the keto acid, α-ketoisocaproic acid (AKICA), could undergo any or all of the following reactions:

1. Transamination into leucine.
2. Decarboxylation and reduction into isoamyl alcohol.
3. Decarboxylation and oxidation into isovaleric acid.
4. Degradation by the "leucine degradative pathway" (Coon *et al.*, 1955) into acetyl CoA and acetoacetyl CoA.

α-Keto isovaleric acid (AKIVA) could undergo a similar set of reactions. This is the keto acid intermediate between isobutyl alcohol and isobutyric acid and is also degraded into propionyl CoA (Coon *et al.*, 1955). α-Keto β-methyl valeric acid (AKBMVA) is the keto intermediate between isoleucine and 2-methyl-butanol. α-Methyl butyric acid, the oxidation product of AKBMVA, recently was reported present in alcoholic distillates (Conner *et al.*, 1969). The products of the "isoleucine degradative pathway" are propionyl CoA and acetyl CoA (Coon *et al.*, 1955).

The analogy in the case of propyl alcohol is not as apparent. In this case, α-keto butyric acid (AKBA) is the keto acid intermediate (Guymon *et al.*, 1961) which would be expected to produce propionic acid and α-amino butyric acid. However, the latter acid apparently exists only as an intermediate of threonine metabolism. Pending further investigation, the same general pattern would appear to apply.

This concept of the keto acids may be extended to include the production of ethanol. In this case pyruvic acid (PA) is the keto acid intermediate, ethanol the reduced product, acetate the oxidized product, and alanine the related amino acid.

It may eventually be found by *in vitro* studies that one basic enzyme system, possibly alcohol dehydrogenase, is responsible for transforming these keto acids into these congeners. Differences in the production of volatile acids and alcohols would then be due to the ability of the yeast to supply the respective keto acids.

It has been proposed that ester production is the result of a condensation of fatty acid CoA complexes with alcohol (Nordstrom, 1962). Those metabolic pathways that produce CoA complexes should have the potential for ester production. The formation of radioactive ethyl acetate in fermentations containing either radioactive leucine or isoleucine suggests that leucine and isoleucine are degraded into acetyl CoA, which is transformed into ethyl acetate. Other degradative products of leucine and isoleucine metabolism are acetoacetyl CoA and propionyl CoA, respectively. The acetoacetyl CoA, an intermediate in fatty acid metabolism, can be converted into butyryl CoA, which may form either butyric acid or ethyl butyrate. This probably explains the substantial amount of radioactive butyric acid produced in the fermentations containing radioactive leucine. Propionyl CoA would be expected to produce ethyl propionate. Nordstrom (1964, 1964) reported that this reaction does not occur in yeast and, for this reason, it is tentatively suggested that propionyl CoA is transformed into propionic acid.

The mechanism by which carbohydrates are transformed into these congeners may be summarized as follows. Pyruvic acid produced by the Embden-Meyerhof-Parnas (EMP) fermentation pathway may either be reduced into ethanol, oxidized into acetate, or combined with another molecule of pyruvic acid to form acetolactic acid. The acetolactic acid is transformed by the "valine-isoleucine biosynthetic pathway" (Strassman *et al.*, 1956) into AKIVA and its related congeners. The AKIVA may combine with acetyl CoA to produce AKICA, (Strassman and Cici, 1963) the precursor of isoamyl alcohol, isovaleric acid, and leucine. The same set of enzymes that produced AKIVA can initially condense AKBA and acetyl CoA to produce the analogous keto acid of isoleucine, which is transformed into *d*-amyl alcohol. That these systems proposed by Strassman are involved in fusel oil formation has been demonstrated by other workers (Ayrapaa, 1967; Guymon, 1966; Ingraham *et al.*, 1961; Reazin, 1960).

The failure to convert mash carbohydrates into fusel oil in the early stages of fermentation is attributed to feedback inhibition of the carbohydrate system by the amino acids. Valine, leucine, and isoleucine have been shown to be inhibitors of acetolactic acid formation (Armstrong and Wagner, 1963; Bauerle *et al.*, 1964) and, as a result, isobutyl and amyl alcohols produced from mash carbohydrates would be diminished. After the eighth hour, however, the amino acid concentration in the mash may no longer be high enough to inhibit this system, and fusel oil can be produced from sugar.

The data obtained in the experiments where ammonium ions were added to the fermentation also suggest, except for *n*-propyl alcohol, a feedback inhibition of the carbohydrate system. Excess ammonium ions will cause an accumulation

of amino acids by promoting the amination of keto acids (Thorne, 1950). An excess of leucine, isoleucine, and valine would then block the formation of acetolactic acid, causing less sugar to be transferred into fusel oil. The precursor for *n*-propyl alcohol, AKBA, is produced ahead of the enzyme blockage. This keto acid normally forms isoleucine and *d*-amyl alcohol as well as *n*-propyl alcohol. However, since the enzyme system is now blocked, the AKBA is shunted into *n*-propyl alcohol, thereby causing its concentration to increase.

The major congeners are produced by dynamic and interrelated biochemical mechanisms which are influenced in different ways by the manner in which the fermentation is conducted. As more knowledge becomes available regarding environmental effects and, in particular, the role of the mash amino acids in regulating the synthesis of congeners from carbohydrates, the mechanisms controlling the production of the major flavor components will become better understood.

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Received for review October 16, 1969. Accepted April 27, 1970. Presented at the Division of Microbial Chemistry and Technology, 158th Meeting, ACS, New York, N.Y., September 1969.