Production of Higher Alcohols from Threonine and Isoleucine in Alcoholic Fermentations of Different Types of Grain Mash

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Threonine-U-^{14}C and isoleucine-U-^{14}C were added individually to alcoholic fermentations of different grain mashes to establish the role of these amino acids in the production of specific higher alcohols. When threonine-U-^{14}C was added to malt mashes, 60% of the radioactivity of the fusel oil was recovered in 2-methyl-1-butanol and 30% was recovered in 1-propanol; for corn mashes the values were 86 and 8%, respectively. When isoleucine-U-^{14}C was added, the only radioactive product was 2-methyl-1-butanol. Less 2-methyl-1-butanol was produced from malt mashes than corn mashes, even though malt mashes contain more amino acids; also, its radioactivity was lower. Reduction of 2-methyl-1-butanol synthesis in malt mashes is attributed to amino acid feedback inhibition. Only threonine formed radioactive ethanol, amounting to 7% of the total radioactivity added.

The fermentation of grain mashes by yeast results in the formation of various substances, called congeners, which contribute to the characteristic flavor and odor of distilled alcoholic beverages. The major congener group consists of higher alcohols (fusel oil), a mixture of *n*-propyl (1-propanol), isobutyl (2-methyl-1-propanol), *d*-amyl (2-methyl-1-butanol), and isoamyl (3-methyl-1-butanol) alcohols. Ayrapaa (1967), Guymon (1966), Ingraham and Guymon (1960), Thorne (1950), and others have shown that isobutyl, *d*-amyl, and isoamyl alcohols are produced from the amino acids valine, isoleucine, and leucine, respectively. The biochemical mechanisms for these transformations were demonstrated for grain fermentations used in the production of distilled alcoholic beverages (Reazin *et al.*, 1970).

Of these mechanisms, the one for the formation of *n*-propyl alcohol is the least understood. Guymon *et al.* (1961) added α amino butyric acid (AABA) to a fermentation and found an increase in *n*-propyl alcohol production. α ketobutyric acid (AKBA) was proposed as the metabolic intermediate between AABA and *n*-propyl alcohol. Guymon tested this hypothesis by adding AABA-U-^{14}C or AKBA-U-^{14}C to fermentations and found that radioactive *n*-propyl alcohol was produced. Since AABA is not a naturally occurring substrate, it was proposed that AKBA is produced from threonine, homoserine, and/or aspartic acid.

The role of threonine as a substrate for *n*-propyl alcohol formation was investigated by Guymon *et al.* (1961) in experiments using three threonine-requiring mutant yeasts. Fermentations were with resting cells in a synthetic medium containing glucose but no threonine. One strain did not produce *n*-propyl alcohol; the other two produced large amounts of this alcohol and small amounts of *d*-amyl alcohol. These results suggest a complex mechanism for formation of *n*-propyl alcohol.

The data to be presented were obtained from conventional fermentations of grain mashes with distillers yeast, *Saccharomyces cerevisiae*. Different types of mash were used to determine the effect of substrate composition on the production of higher alcohols. The fermentations contained either threonine-U-^{14}C or isoleucine-U-^{14}C. The higher alcohols and ethyl alcohol produced were isolated and the amount of radioactivity present was determined. By comparing the distribution of radioactive carbon among these products, the role of these two amino acids in higher alcohol production was determined.

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MATERIAL AND METHODS

Fermentation Conditions. The following types of mash were used: 100% barley malt, 100% rye malt, bourbon (60% corn, 28% rye, 12% barley malt), corn (90% corn, 10% barley malt), and corn converted with Oloclast (98% corn, 2% malt, 0.04% Oloclast). Oloclast is an α amyloglucosidase preparation produced by Biocon Limited, Hall Lane, Rookery Bridge, Nr. Sandback, Cheshire, England. Each mash contained the equivalent of 1 bushel of grain (56 lb, as is) diluted with water to a volume of 38 gal. On a dry basis the mashes contained approximately 166 g of grain/l.

The malt mashes were cooked at 145°F for 30 min. The corn and rye in the other mashes were mixed and cooked at 212°F for 30 min, cooled to 145°F, and saccharified with malt for 30 min. The mash was cooled to 86°F for yeast inoculation and controlled at this temperature during fermentation for 3 days. In preparing the corn mash converted with Oloclast, 1% of the malt was added before cooking at 212°F. After cooking, the mash was cooled to 145°F and the remaining 1% malt was added, held at 145°F for 10 min, and then cooled for yeast inoculation. The enzyme preparation was added at 139°F during cooling. All mashes were adjusted with sulfuric acid to pH 5.6 before cooking and to pH 5.0 before yeast addition.

Each mash was subdivided into 1.5-l. portions in 2-l. sterilized Florence Flasks and inoculated with 2% v/v of yeast grown in commercial malt extract. Ten milliliters of a stock solution of either threonine-U-^{14}C or isoleucine-U-^{14}C containing approximately 10 μCi of radioactive carbon was added. The radioactive amino acids were obtained from New England Nuclear Corp., Boston, Mass. The level of radioactivity in each stock solution was determined prior to use.

Chemical Analysis and Separation of Alcohols. Samples of each mash were analyzed before yeast inoculation. After centrifugation, total nitrogen was determined on the supernatant by a micro-Kjeldahl method (AOAC, 1970), amino acid nitrogen by the ninhydrin method (Moore and Stein, 1954), and carbohydrate, after acid hydrolysis to reducing sugars, by the Somogyi-Nelson method (Neish, 1952).

A 100-ml portion of the fermented mash was distilled and analyzed for ethyl alcohol and fusel oil content by the Komarowsky colorimetric procedure (AOAC, 1970). The remaining 1.4 l. of fermented mash was distilled and a

Table I. Relationship of Mash Composition to Ethyl Alcohol and Fusel Oil Production

Mash type[a]	Mash composition: Carbohydrate, Total, g/100 ml	Mash composition: Nitrogen, Total, ppm[b]	Mash composition: Nitrogen, Free α amino, ppm[b]	Distillate composition: Ethyl alcohol, ml/100 ml	Distillate composition: Fusel oil, ppm[b]
Barley malt	10.5	4050	394	6.39	233
Rye malt	10.9	3920	406	6.39	202
Bourbon	12.0	797	87	6.92	319
Corn	12.4	692	87	7.19	338
Corn and GA[c]	11.9	554	53	6.85	327

[a]Grain composition of mash listed in text. [b]ppm = parts per million. [c]GA = glucoamylase.

crude fusel oil fraction was obtained by fractional distillation in a Podbielniak still. The fusel oil fraction was separated into individual higher alcohols by preparatory gas chromatography, as outlined in a previous publication (Reazin *et al.*, 1970).

Radioactive measurements were made with a liquid scintillation spectrometer in the customary manner.

RESULTS

Fusel Oil Production in Different Mashes. The 100% barley and 100% rye malt mashes were found to contain more nitrogen and less carbohydrate than the bourbon, corn, and corn converted with Oloclast mashes (Table I). Therefore, for discussion, the five different mashes will be considered as two groups and will be referred to as "malt" and "corn" mashes. The alcoholic distillates prepared from these fermentations, to which radioactive amino acids were added, were assayed to determine the amount of radioactivity present. The data in Table II show that 1% of the added threonine-*U*-^{14}C radioactivity was recovered in the alcoholic distillate from malt mashes, while 8% was recovered in the distillate from corn mashes. The same relationship was found for isoleucine-*U*-^{14}C, *i.e.*, about 12 and 20% recovery, respectively.

Table II. Carbon-14 Recovery in Different Type Mash

Mash type[a]	Carbon-14 recovered in alcoholic distillate: Threonine-^{14}C[b] R[d]/1.5 l.	Threonine-^{14}C[b] %	Isoleucine-^{14}C[c] R[d]/1.5 l.	Isoleucine-^{14}C[c] %
Barley malt	107	1	1043	11
Rye malt	91	1	1411	14
Bourbon	768	8	1843	19
Corn	790	8	2105	22
Corn and GA[e]	721	8	1685	17

[a]Grain composition of mash type used in text. [b]Radioactivity of added threonine-9.3 μCi. [c]Radioactivity of added isoleucine-9.9 μCi. [d]R = Radioactivity in mμCi. [e]GA = glucoamylase.

The rate of fusel oil and ethanol production during fermentation was also determined. The rate of ethanol production is a measure of the rate of carbohydrate utilization. The data of Figure 1 show that ethanol is produced at a faster rate in 100% barley malt than in bourbon fermentations. In both types of mash, fusel oil production did not continue after ethanol formation had stopped. In corn mashes, the rate of carbohydrate utilization is limited primarily by a relatively slower conversion of dextrins into fermentable sugar (Pan *et al.*, 1951).

In Figure 2, data on amino acid utilization and fusel oil production in the barley malt fermentation show that the amino nitrogen decreased during ethanol and fusel oil production and increased thereafter. Fusel oil production stopped, even though an excess of amino acid substrate was present. The experiment was repeated (Figure 2) but in this case glucose was added at 17 hr in an amount to give 18% (w/v) after the original carbohydrate content of the mash was nearly exhausted. The addition of glucose caused the fusel oil to increase while the amino acid level decreased. The addition of sugar also caused a resumption of ethanol production. This experiment indicates that fermentable carbohydrate is necessary for fusel oil production and amino acid utilization. The accumulation of amino acid nitrogen after ethanol and fusel oil production had stopped was presumably caused by continued action

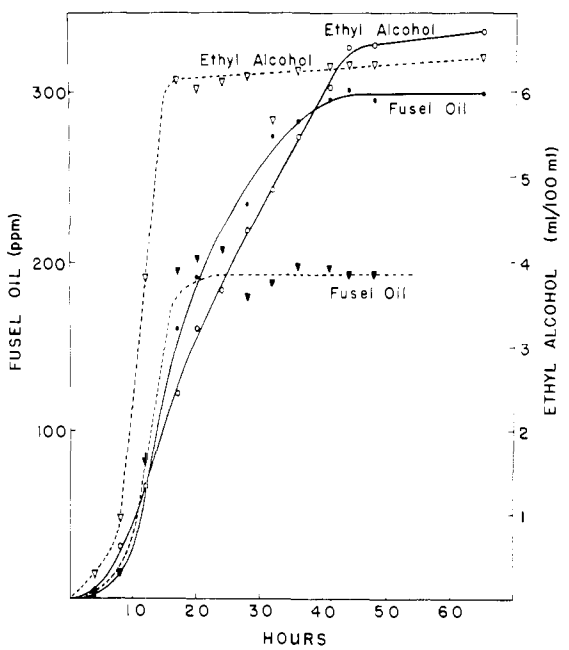

Figure 1. Rate of fusel oil and ethyl alcohol production in malt and bourbon mashes. O—O, Ethyl alcohol; ●—●, fusel oil production in bourbon mash; ▽--▽, ethyl alcohol; ▼--▼, fusel oil production in 100% barley malt mash.

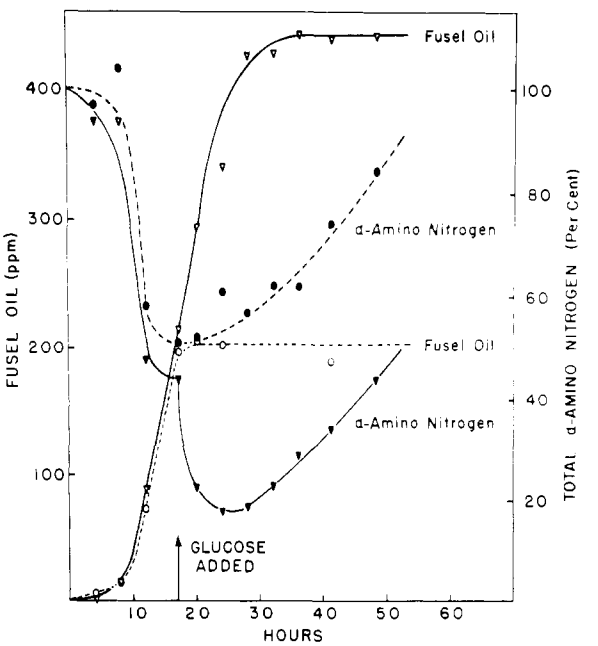

Figure 2. Effect of added glucose on fusel oil production and amino nitrogen utilization in malt mash fermentations. Control fermentation: O—O, fusel oil; ●—●, α amino nitrogen. Experimental fermentation: ▽—▽, fusel oil; ▼—▼, α amino nitrogen.

Table III. Higher Alcohol Composition of Fusel Oils Produced in Fermentations of Different Mashes

Mash type	*n*-Propyl, ppm	Isobutyl, ppm	*d*-Amyl, ppm	Isoamyl, ppm
Barley malt	20	79	37	97
Rye malt	27	52	36	87
Bourbon	16	89	85	129
Corn	21	81	92	144
Corn and GA[a]	25	83	84	135

[a]GA = glucoamylase.

of the malt proteolytic enzymes. Therefore, the lower fusel oil level in distillates from malt mash is not due to a lack of amino acids or the inability of the yeast to produce fusel oil, but must be due in large part to a depletion of fermentable carbohydrates.

Since the total amount of higher alcohols produced in malt and corn mashes differed (Table I), it was of interest to determine if there were differences in composition. Table III shows that the production of *n*-propyl and isobutyl alcohols was similar in all mashes. However, significantly less *d*-amyl and isoamyl alcohols were produced in the malt mash fermentations. Therefore, the type of mash used can affect the composition as well as the amount of fusel oil formed.

Production of Higher Alcohols from Threonine and Isoleucine. Experiments were conducted to determine the role of threonine and isoleucine in the production of the individual higher alcohols. When the distillates produced from fermentations containing either of these radioactive amino acids were analyzed for the individual higher alcohols (Table IV) most of the radioactivity was found in the *d*-amyl alcohol. Considerably less was found in the isoamyl alcohol. Only threonine contributed significantly to the production of *n*-propyl alcohol. Isobutyl alcohol contained little or no carbon-14.

The radioactivity per mg of carbon (specific activity) of the different higher alcohols was determined from the data of Tables III and IV. As shown in Table V, fermentations of malt mash containing radioactive threonine produced *n*-propyl and *d*-amyl alcohols with lower specific activities than comparable corn mash fermentations. These results could be explained by differences in the specific activity of the amino acid pool of the yeast due to the differences in the amino nitrogen content of the malt and corn mashes. However, the difference in specific activity between the two mashes is less for *n*-propyl alcohol than for *d*-amyl alcohol, even though the same amount of radioactive threonine was present. This difference in specific activity suggests that these alcohols are being produced from threonine by different metabolic systems. Proof of this could be obtained by determining the levels of the individual amino acids in each mash and comparing the amino acid specific activity to that of the various higher alcohols. However, measurement of the amino acid composition of the different mashes is beyond the scope of the present study.

When radioactive isoleucine was added to the mashes (Table IV) virtually only *d*-amyl alcohol was produced from it. Malt mashes produced *d*-amyl alcohol with a slightly higher specific activity (Table V) than was obtained from corn mashes, which is the opposite of that found for threonine. Although more *d*-amyl alcohol was produced from isoleucine in corn mashes, the results suggest that a larger proportion of *d*-amyl alcohol was produced from nonisoleucine substrates in corn mashes than in malt mashes.

Production of Ethyl Alcohol from Threonine and Isoleucine. As shown in Table VI, the ethanol from all the threonine-*U*-^{14}C-labeled mashes contained about 7% of the total radioactivity added to the mash, except when glucoamylase was used. Isoleucine was not transformed into ethanol.

DISCUSSION

Biochemical Mechanisms. The data presented indicate that *n*-propyl, *d*-amyl, and isoamyl alcohols are produced from threonine. The formation of *n*-propyl and *d*-amyl alcohols is in agreement with data of other workers (Ayrapaa, 1968; Guymon, 1966; Guymon *et al.*, 1961). The various biochemical reactions involved in threonine metabolism and their relationship to isoleucine and leucine metabolism are presented in Figure 3. Pathway A represents the reactions known to be involved in the transformation of threonine into *n*-propyl alcohol. Threonine is transformed by biodegradative threonine dehydratase (Greenburg, 1969) into α amino-2-butenoic acid, which upon deamination produces the keto acid, AKBA. The AKBA is decarboxylated and the resultant aldehyde is reduced, producing *n*-propyl alcohol.

The transformation of threonine into *d*-amyl alcohol may be explained by Pathway B. The initial step is the production of active α amino-2-butenoic acid (Greenburg, 1969; Umbarger and Brown, 1957). The enzyme responsible for this reaction is termed biosynthetic threonine dehydratase (Greenburg, 1969). It was also reported (Greenburg, 1969) that although the threonine dehydratase enzymes of Pathways A and B are similar, the products exist in different biochemical states so that the metabolic intermediates produced by one enzyme system cannot be used in the other.

The active amino-2-butenoic acid of Pathway B is deaminated to produce active AKBA, which reacts with acetyl coenzyme A to produce acetohydroxybutyl coenzyme A. This is a metabolic intermediate which is also part of the metabolic system that converts carbohydrates into *d*-amyl alcohol and isoleucine (Reazin *et al.*, 1970). The keto acid analog of isoleucine, α keto β methylvaleric acid (AKBMVA), may be the metabolic intermediate

Table IV. Distribution of Radioactivity in Fusel Oils Produced from Threonine-^{14}C and Isoleucine-^{14}C

	Radioactivity in higher alcohols							
	n-Propyl		Isobutyl		*d*-Amyl		Isoamyl	
	mμCi/1.5 l.		mμCi/1.5 l.		mμCi/1.5 l.		mμCi/1.5 l.	
Mash type	T[a]	I[b]	T[a]	I[b]	T[a]	I[b]	T[a]	I[b]
Barley malt	28	1	1	0	66	940	10	101
Rye malt	33	8	1	0	52	1205	6	133
Bourbon	58	1	3	0	669	1784	39	55
Corn	64	1	4	0	667	2015	56	88
Corn and GA[c]	53	1	2	0	635	1604	32	81

[a]T = fermentations with 9.3 μCi of threonine-^{14}C/1.5 l. of mash. [b]I = fermentations with 9.9 μCi of isoleucine-^{14}C/1.5 l. of mash. [c]GA = glucoamylase.

Table V. Specific Activities of Higher Alcohols Produced from Threonine-^{14}C and Isoleucine-^{14}C

	Specific activity[a] of higher alcohols							
	n-Propyl		Isobutyl		*d*-Amyl		Isoamyl	
Mash type	T[b]	I[c]	T[b]	I[c]	T[b]	I[c]	T[b]	I[c]
Barley malt	1.6	0	0	0	1.8	25	0.1	1.0
Rye malt	1.3	0	0	1	1.4	33	0.1	1.5
Bourbon	4.0	0	0	0	7.7	21	0.3	0.4
Corn	3.4	0	0	0	7.1	21	0.4	0.6
Corn and GA[d]	2.4	0	0	0	7.4	19	0.2	0.6

[a]Specific activity = mμCi/mgC. [b]T = fermentations with threonine-^{14}C. [c]I = fermentations with isoleucine-^{14}C. [d]GA = glucoamylase.

Table VI. Ethyl Alcohol Production from Threonine-^{14}C and Isoleucine-^{14}C

	Ethyl alcohol radioactivity			
	Threonine[a]		Isoleucine[b]	
Mash type	R[c]	%	R[c]	%
Barley malt	686	7	11	0.1
Rye malt	553	6		
Bourbon	735	8	8	0.1
Corn	512	6	8	0.1
Corn and GA[d]	382	4	8	0.1

[a]Radioactive threonine added = 9.3 μCi/1.5 l. of mash. [b]Radioactive isoleucine added = 9.9 μCi/1.5 l. of mash. [c]R = radioactivity in mμCi/1.5 l. of distillate. [d]GA = glucoamylase.

linking the transformation of threonine and isoleucine into *d*-amyl alcohol.

In Pathway C, threonine dehydrogenase (Green and Elliott, 1964) oxidizes the hydroxyl group of threonine to form 2-amino-3-ketobutyric acid. This substance is unstable and a decarboxylation occurs, producing amino acetone. The amino acetone is next converted into methyl glyoxal, which may be oxidized into pyruvic acid. The pyruvic acid may be transformed into ethyl alcohol or, by combination with acetyl coenzyme A, into α acetolactate, an intermediate in the metabolic system for the formation of leucine and isoamyl alcohol from carbohydrates. The presence of this system may explain how some of the threonine-*U*-^{14}*C* is transformed into isoamyl alcohol.

Another enzyme that acts upon threonine is threonine aldolase. In Pathway D, this enzyme transforms threonine into glycine and acetaldehyde (Dainty, 1967) and the acetaldehyde can be reduced to ethyl alcohol. The existence of this system could explain the transformation of threonine-*U*-^{14}*C* into ethyl alcohol.

The data obtained using isoleucine-*U*-^{14}*C* showed that *d*-amyl alcohol was the primary radioactive product. This was expected since this amino acid, as shown in Figure 3, is transformed into *d*-amyl alcohol *via* AKBMVA. Table IV shows that some radioactive isoamyl alcohol was formed when isoleucine-*U*-^{14}*C* was added to the mash. Isoleucine-*U*-^{14}*C* may contain small amounts of leucine-*U*-^{14}*C*, which could be responsible for the formation of radioactive isoamyl alcohol.

The existence of Pathways A and B could explain the observation of Guymon *et al.* (1961). In the two threonine-deficient mutants that produced more *n*-propyl alcohol than *d*-amyl alcohol, Pathway A would be the most active. The mutant that did not produce either alcohol lacked both systems.

Effect of Different Types of Mash. A smaller amount of fusel oil is produced in malt than in corn mashes. This is explained by a depression of fusel oil production due to a shorter fermentation period, *i.e.*, depletion of carbohydrates and/or to a high amino nitrogen content.

Malt mash contains more protein and proteolytic enzymes than the corn mash, resulting in a larger amino acid pool in the malt fermentations. This inhibits the metabolic systems producing higher alcohols from carbohydrate and threonine. In corn mash fermentation the amino acid pool is exhausted in the early stages of fermentation (Reazin *et al.*, 1970) and most of the fusel oil is produced in the absence of an amino acid pool. This is in agreement with Ayrapaa (1971), who observed that the longer a fermentation proceeds in the absence of nitrogen, the higher the yield of fusel oil.

The amount of fusel oil produced during a fermentation is reported to be governed by a keto acid overflow mechanism (Ayrapaa, 1971; Guymon *et al.*, 1961; Lewis, 1964). That is, the amount of keto acid produced by the yeast varies with the nitrogen concentration of the mash, and changes in this ratio govern the amount of fusel oil produced. Thus, in corn mashes low in nitrogen, an excess of keto acids is present and fusel oil production is high, while in malt mashes the high nitrogen level not only lowers the amount of keto acid produced from carbohydrates and certain amino acids, but more of the keto acid will probably be transformed into amino acids and cell protein. The result will be a lowering in the amount of fusel oil produced.

The changes in higher alcohol specific activity caused by the fermentation of different types of mash can also be explained by the keto acid overflow theory. If, in the production of *d*-amyl alcohol, the conversion rate of isoleucine into the yeast metabolic intermediate AKBMVA is assumed to remain constant, any increase in the amount of this intermediate produced will be due to its formation from substrates other than isoleucine. Therefore, in malt mash fermentations, the high level of nitrogen will cause AKBMVA to be produced mostly from isoleucine, and the specific activity of the *d*-amyl alcohol will be higher than if corn mash with a lower nitrogen level had been used. The data indicate that this occurred, which is consistent with the overflow theory.

Although the relationship of specific activity to mash type observed for isoleucine differed from that observed when threonine was used, the data obtained using radioactive threonine are also consistent with the overflow theory. Threonine is produced from pyruvic acid and not its keto acid isomer, as is isoleucine. Therefore, the high nitrogen level of the malt mash will cause some of the pyruvic acid normally transformed into AKBMVA or AKICA to be

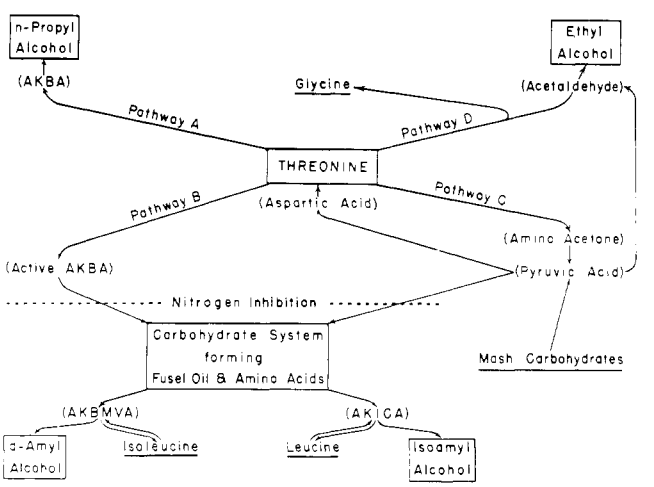

Figure 3. Biochemical relationships of threonine and isoleucine in higher alcohol production.

shunted into aspartic acid and subsequently into threonine. As a result, the threonine-U-^{14}C added in the malt fermentations will be diluted with nonradioactive threonine, causing a lower specific activity. The lower threonine specific activity will result in lower higher alcohol specific activity. This relationship of higher alcohol specific activity was demonstrated.

When threonine-U-^{14}C is added, the specific activity of *n*-propyl and *d*-amyl alcohol changed, using different types of mash. This may be due to rate differences in the transformation of threonine into these higher alcohols. Since most of the threonine, due to blockage of Pathway B by high nitrogen levels of the malt mash, is transformed *via* Pathway A into AKBA, the concentration of AKBA should be higher than in the corn fermentations, where both systems are active. This would result in a higher level of *n*-propyl alcohol in malt than in corn fermentations. Although more *n*-propyl alcohol is produced in the malt fermentations, had the malt fermentations lasted as long as the corn fermentations, this difference probably would have been greater.

Although the overflow concept accounts for much of the variation in fusel oil production in different mashes, there are some observations it does not explain. For example, in the malt fermentations, a large amino acid pool existed throughout the fermentation period, but fusel oil was formed only during the time of active ethanol production. It may well be that the level of reduced diphosphopyridine nucleotide, a necessary cofactor for the transformation of keto acids into higher alcohols (SentheShanmuganathan, 1960), becomes limiting after the cessation of ethanol production from sugar. Although large amounts of amino acid are present, there may not be enough energy for the Ehrlich mechanism to function.

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Chemistry of Thiamine Degradation in Food Products and Model Systems: A Review

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The instability of thiamine to heat in neutral or alkaline systems has prompted extensive study of the chemistry of thiamine degradation. Literature dealing with the effect of pH and heat on the thiamine molecule is reviewed. Thermal degradation products which have been reported, such as hydrogen sulfide, elemental sulfur, 4-methyl-5-(β-hydroxyethyl)thiazole, and numerous minor products, are discussed. The extent of thermal degradation and the nature of the products formed appear to be determined by which of two proposed reaction mechanisms predominates, which is controlled by pH. Literature dealing with the effects of other factors, including oxidation–reduction systems, inorganic bases (sulfites, bisulfites), thiaminase enzymes, metal complexes, radiation, and ultrasonic waves, is also reviewed. Reactions of thiamine in model systems with proteins, amino acids, carbohydrates, other organic compounds, and certain inorganic compounds are presented. Chemical structures of thiamine degradation products reported in the literature are shown.

The sensitivity of thiamine to heat and alkali was recognized almost immediately after thiamine was discovered. Considerable information about the destruction of thiamine during cooking, processing, and storage of foods has appeared since the isolation of thiamine by Jansen and Donath (1926). However, most of this information is concerned with the loss of biological activity of thiamine during the treatment of a particular food under specified conditions. Only recently have the reaction products of thiamine in foods or in model systems been identified.

Temperature, pH, and time of heating, processing, or storage are the most important factors contributing to the loss of thiamine in food products. Rice and Beuk (1945) studied thiamine decomposition in pork at different temperatures. Farrer and Morrison (1949) studied thermal destruction of thiamine in buffered solutions and showed that the rate of destruction follows the Arrhenius equation, $\ln k = I - E/RT$, where I = constant, R = gas constant, E = energy of activation, k = rate constant, and T = temperature in degrees Absolute.

Subsequent studies showed that this equation can be successfully used in predicting thiamine retention in foods

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