Ethyl Carbamate Formation in Wine: Use of Radioactively Labeled Precursors to Demonstrate the Involvement of Urea

FRANCISCO F. MONTEIRO[1], EUGENE K. TROUSDALE[2], and LINDA F. BISSON[3*]

The formation of ethyl carbamate was investigated in model solutions and in wines using radioactively labeled urea and monitoring the appearance of that radioactivity in ethyl carbamate. There was a striking temperature dependence for the rate of the reaction as well as a dependence upon urea and ethanol concentrations. The source of urea during fermentation was investigated using radioactively labeled arginine (*guanido*-^{14}C). Under the conditions used here, arginine was degraded to urea, and some urea was released into the fermentation medium. More urea could be released from the cells when ethanol concentration was elevated, as a consequnce of fortification of the must. There was significant turnover and degradation of arginine in cells incubated in the presence of ethanol, as would occur during commercial fortification of musts. Thus, urea can be formed during vinification and, if released into the medium, will yield ethyl carbamate through reaction with ethanol.

KEY WORDS: ethyl carbamate, urea

Ethyl carbamate is a suspected carcinogen (7) found in a variety of fermented foods and beverages as a natural consequence of the metabolic activity of microorganisms (8). One such beverage containing low levels of ethyl carbamate (μg/L) is wine (8,9,10). In order to design the fermentation process to minimize ethyl carbamate production, it is first necessary to identify the precursor molecules and to investigate the physiological conditions leading to their synthesis and release from the cell. Ethyl carbamate can be formed in a spontaneous chemical reaction involving ethanol and a compound containing a carbamyl group (9,14). The rate of formation and final yield of ethyl carbamate is dependent upon the ease of transfer of the carbamyl moiety and, in complex solutions, upon the presence or absence of competing acceptor molecules. Compounds containing carbamyl groups abound in natural systems and are fundamental components of anabolic and catabolic pathways in living organisms. In yeast, such compounds can be found in the metabolism or biosynthesis of arginine, purines and pyrimidines. Arginine, along with proline, is generally the major amino acid found in grape juice. Arginase catalyzes the cleavage of arginine to ornithine and urea, and all known yeasts possess this enzymatic activity. It is well known that urea can react with ethanol to form ethyl carbamate, although the reaction mechanism and reactive intermediates have not yet been elucidated. Another potential source of urea is the degradation of purines. However, purine and pyrimidine levels are relatively low in grape juices (see below), and it is, therefore, unlikely that these compounds are utilized as nitrogen sources during vinification. Urea can be further metabolized by the wine yeast *Saccharomyces cerevisiae* via urea carboxylase and allophanate hydrolase to yield ammonia and carbon dioxide or it can be excreted from the cell (2). The purpose of this research was to use radioactively labeled substrates to investigate the contribution of urea to the production of ethyl carbamate in wine and to trace the source of that urea during fermentation.

Materials and Methods

Reagents: All chemicals were reagent grade unless otherwise noted. Kinetic studies on model systems were carried out as described in the Experimental section. Radio-labeled ^{14}C-urea (57.0 mCi/mmol, Dupont New England Nuclear, Wilmington, DE) was evaporated to dryness in a vacuum dessicator and made up with 0.05 *M* citrate buffer, pH 3.19, to give the appropriate concentration of labeled reactant upon addition of H_2O and EtOH. L-Arginine (*guanido*-^{14}C, 54.4 mCi/mmol) and (2,3-^{3}H, 50.4 Ci/mmol) were from Dupont New England Nuclear, Wilmington, Delaware.

Yeast strains: All studies were conducted using UCD 522 (Montrachet) unless otherwise noted. For analysis of adenine and uracil content of juices MCY 501 (*MATα ade2-101*) and MCY 638 (*MATα his4-539 lys2-801 ura3-52 GAL SUC2*) were used, respectively, and were originally obtained from Dr. Marian Carlson, Columbia University, New York, New York.

Analysis of adenine and uracil levels in juice: Adenine levels were analyzed by determining cell yield of MCY 501 in a minimal medium 6.7 g/L [Yeast Nitrogen Base containing $(NH_4)_2SO_4$ (from Difco Laboratories via Fisher Scientific)] containing 20% glucose (w/v). Up to 1.0 mL of juice was added to 3.9 mL of medium to provide a source of adenine, inoculated with 0.1 mL of a

[1]Graduate Student, [2]Research Associate, and [3]Assistant Professor, Department of Viticulture and Enology, University of California, Davis, CA 95616-5270.

*Author to whom correspondence should be addressed.

This research was conducted at the University of California, Davis, CA.

The authors would like to acknowledge the following individuals for helpful discussions of the research and sharing of unpublished data: Dr. C. S. Ough of the Department of Viticulture and Enology, University of California. Davis, CA; A. J. Caputi and R. Morenzoni of E. & J. Gallo Winery; and Drs. R. Boulton, V. L. Singleton and D. Adams, also of the Department of Viticulture and Enology, University of California, Davis, CA.

The research was supported by funds from the Wine Institute and, in part, by a grant from the Winegrowers of California. F.F. Monteiro was the recipient of Fulbright-Hays and Wine Spectator scholarships and a Leon Adams research scholarship. We thank these generous donors for these funds.

Manuscript submitted for publication 10 August 1988.

cell suspension, and growth was monitored for a period of two to three days, until culture absorbance attained a constant value. A standard curve was generated by the addition of known amounts of adenine. Inhibition of cell growth caused by addition of juice was investigated by adding an excess of adenine to control tubes receiving 0.5 mL of juice. Such inhibition was generally not observed. Samples were incubated at 30°C in 18 × 150 mm test tubes on a roller drum (New Brunswick) set at 40 rpm. Absorbance was monitored using a Spectronic 21 (Bausch and Lomb) spectrophotometer, and dilutions were made where appropriate for accurate determination of absorbance at 580 nm. For an inoculum, MCY 501 was grown on plates YPD (Yeast extract, 10%; peptone, 20%; and glucose, 2%), and colonies were resuspended in sterile water to prevent transfer of any residual nutrients.

Assay of uracil content was performed similarly, except the yeast strain used was MCY 638.

Amino acid analysis: The amino acid analysis of the pink juice concentrate was performed by the Health Sciences Research Laboratory of the University of California, Davis, using a Beckman 6300 Amino Acid Analyzer.

Analysis of ethyl carbamate in model systems: Thin layer chromatography (TLC) was performed using pre-coated analytical Silica Gel G Uniplates (250 μ, 10 × 20 cm, Analtech, Inc., Newark, DE) developed in the short direction using $CHCl_3$:CCl_4:HOAc:H_2O (90:60:100:50) as mobile phase (3). Sample origins were pre-spotted with 1-μL samples of unlabeled ethyl carbamate and unlabeled urea (10 μg/μL) to facilitate visualization. After drying the pre-spotted samples, 10-μL samples of the ^{14}C-labeled reaction mixture were applied and air dried to maintain minimum spotting area. The samples were run in duplicate with the mobile phase until the solvent front had migrated to the top of the plate. The plate was removed, air dried, sprayed with modified Ehrlich's reagent (4) and heated at 100°C until bright yellow spots appeared. The outline of the spots were marked with a needle and the plates coated according to the method of Redgwell, *et al.* (11). After coating, the plates were air dried, the spots cut out and placed in scintillation vials, 0.5 mL H_2O added (and allowed to stand 5 to 10 min), and 5-mL scintillation cocktail (Optifluor, Fisher Scientific, Pittsburgh, PA) added. The samples were immediately counted in a Packard Tri-Carb 2000 Liquid Scintillation Counter (Packard Instrument Co., Downers Grove, IL). A count time of five minutes with a count window adjusted for ^{14}C was used. Labeled ^{14}C-urea was used as a standard for both quench determinations and in all experiments. Counting efficiency was determined to be 89.5% for sprayed/coated samples. R_f data for ethyl carbamate and urea in this system was 0.66 and 0.21, respectively.

Procedure for analysis of urea in high-sugar musts: TLC was performed as described for ethyl carbamate except as noted. Pre-coated, analytical Silica Gel G Redi/Plates (250 μ, 20 × 20 cm, Fisher Scientific, Pittsburgh, PA) were used and developed with nBuOH:HOAc:H_2O (60:20:20) (13). Samples (100 μL) of radio-labeled ^{14}C-arginine (54.5 mCi/mmol, Dupont NEN, Wilmington, DE) must were placed in 1.5-mL microfuge tubes and 5 μL of a standard mixture of unlabeled arginine and urea (100 μg/μL) added to improve visualization. These samples were then each applied to the plate in a 1.5-inch sample streak and air dried. The plate was then developed with the mobile phase until the solvent front had migrated 10 to 12 cm. The plate was removed from the TLC chamber, dried, visualized, coated, and counted as previously described. R_f data for arginine and urea in this system was 0.06 and 0.60, respectively.

Tracer studies using radioactively labeled arginine: Tracer studies were performed using reconstituted pink juice concentrate obtained from the E. & J. Gallo winery. Deionized distilled water was added to adjust the juice to a final °Brix of 23. UCD 522 was pregrown in YPD (see above) for approximately 24 hours at 30°C with aeration, until the culture reached stationary phase. A volume equivalent to 1% of the final volume of juice was added as inoculum. ^{14}C- or ^{3}H-arginine was added to obtain a concentration of 0.2 μCi/mL. The juice was continuously mixed using a magnetic stirring bar and stirrer at the lowest speed possible. The fermentation vessel was a modified one liter glass-stoppered Pyrex bottle. A sampling port was constructed above the juice line in the vessel by the Glass Blowing Shop, Department of Chemistry, University of California, Davis. There was no aeration of the juice as samples were removed using a syringe through the sampling port. The fermentations were run using 150 or 200 mL of juice. Since degradation of *guanido*-^{14}C-arginine will lead to the formation of radioactively labeled CO_2, a series of traps were set up to allow capture and quantitation of labeled CO_2. Two vessels were set up, each containing 200 mL of 2 *N* potassium hydroxide. The exhaust from the fermentation was connected to a gas sparger in the first trap, and the first trap was connected to a gas sparger in the second trap. Greater than 99% of the labeled CO_2 was recovered in the first trap. In the first experiment, a third trap was included but found to be unnecessary as no label was detected in this trap. Samples from the traps were removed during fermentation by use of a syringe through a sampling port constructed in the plug for the bottles. The progress of the fermentation was monitored by hydrometry using a parallel fermentation that had not received any label, but was otherwise identical to the first fermentation.

Permeabilization studies: At several time points during the fermentation, juice samples were removed for analysis of free and cell-associated radioactivity and to determine the effect of addition of ethanol (fortification) upon release of label. Thin layer chromatography was used to assess the amount of label appearing as urea, as previously described. Samples were filtered through 0.45-μm pore filters and the filtrate analyzed directly for radioactivity. Total radioactivity was determined using a sample of unfiltered juice. Cells collected

on the filter were either washed rapidly with 2 volumes of water, followed by an equal volume of buffer (20 m*M* sodium citrate, pH 3.2), and placed in scintillation vials for determination of radioactivity. In the case of the extraction experiments, cells were washed with water, then buffer and resuspended in buffer alone or in buffer with 17.5% ethanol, incubated at a given temperature for a given period of time, refiltered, and filtrate and collected cell material analyzed for radioactivity. All samples received 10 mL of Optifluor (Packard) as scintillation cocktail prior to being counted.

Results and Discussion

Kinetics of ethyl carbamate formation from urea and ethanol in model solution: Using radioactively labeled urea (^{14}C), the kinetics of formation of ethyl carbamate were investigated in a model solution (50 m*M* Na-citrate, pH 3.2). A thin layer chromatographic method was utilized for separation of the reactant, urea, from product, ethyl carbamate, and potential precursors of urea such as arginine. The relative amounts of each compound were then quantitated by scintillation counting. Thus, for a variety of conditions the rate of formation of ethyl carbamate as well as the rate of disappearance of urea can be readily determined. Replicate samples were run and the variation in the method was less than 10%. As expected from published work (9), the rate of ethyl carbamate formation was dependent upon both urea (Fig. 1) and ethanol (Fig. 2) concentration. The effect of temperature on the rates of reaction of urea with ethanol and with water revealed a striking temperature dependence in both cases (Fig. 3) with a 6- to 7-fold increase in reaction rate for ethyl carbamate formation with a 10° rise in temperature and a 15- to 16-fold increase in the rate of reaction with water over an equivalent temperature change. Thus, at higher temperatures hydrolysis of urea proceeds at a faster rate than ethanolysis, while at low temperatures the reverse is the case. At 40°C, the rate of ethyl carbamate formation was approximately 0.75 n*M*/h at 0.175 m*M* urea, and there was no detectable hydrolysis at this temperature (Fig. 4). At higher temperature (80°C), the hydrolysis of urea proceeded at such a rapid rate (40% conversion in 48 h) that accurate estimates of the rate of ethyl carbamate formation under these conditions could not be made (Fig. 5). In contrast, in a 48 hour time period at 60°C less than 5% of the starting urea had been hydrolyzed, and the rate of ethyl carbamate formation remained linear for an extended period of time (Fig. 4). Standard assay conditions were chosen to be 48 hours at 60°C. A value of 1.56 μ*M*/h has been reported for the rate of ethyl carbamate formation in a solution containing 20% ethanol and 100 mg/L

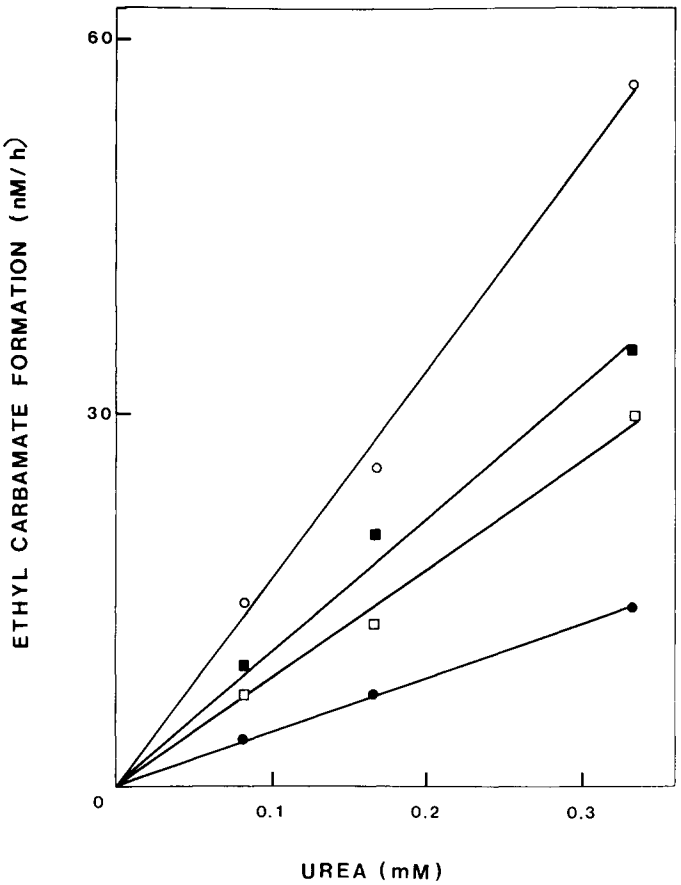

Fig. 1. Dependence of rate of ethyl carbamate formation upon urea concentration at four different concentrations of ethanol. Ethanol concentration in moles/L: 3.4 (○); 2.6 (■); 1.7 (□); 0.86 (●).

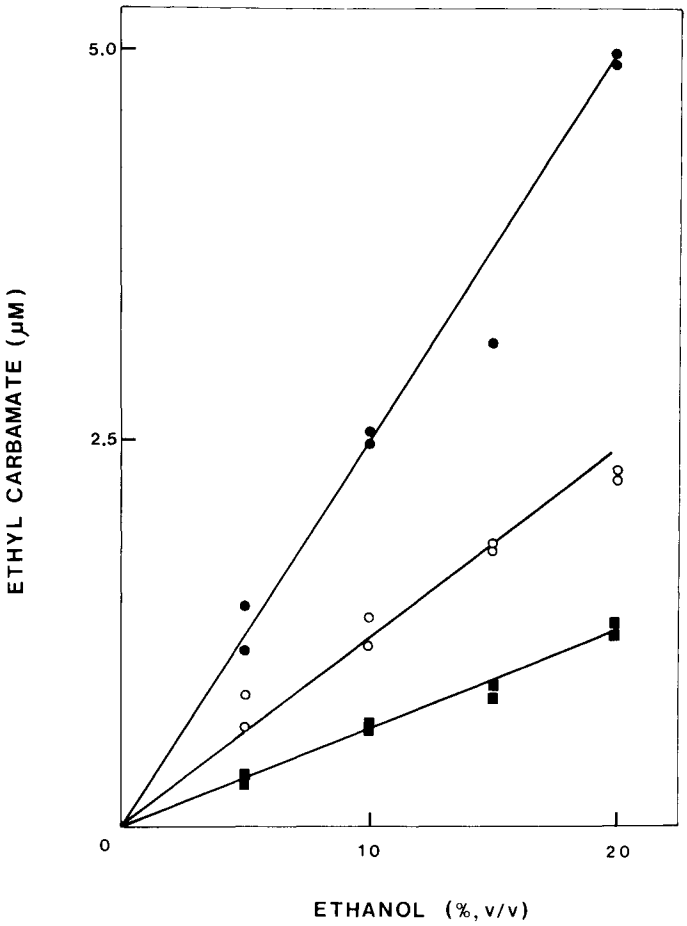

Fig. 2. Dependence of rate of ethyl carbamate formation upon ethanol concentration at three different concentrations of urea. Urea concentration, m*M*: 0.33 (●); 0.167 (○); 0.093 (■).

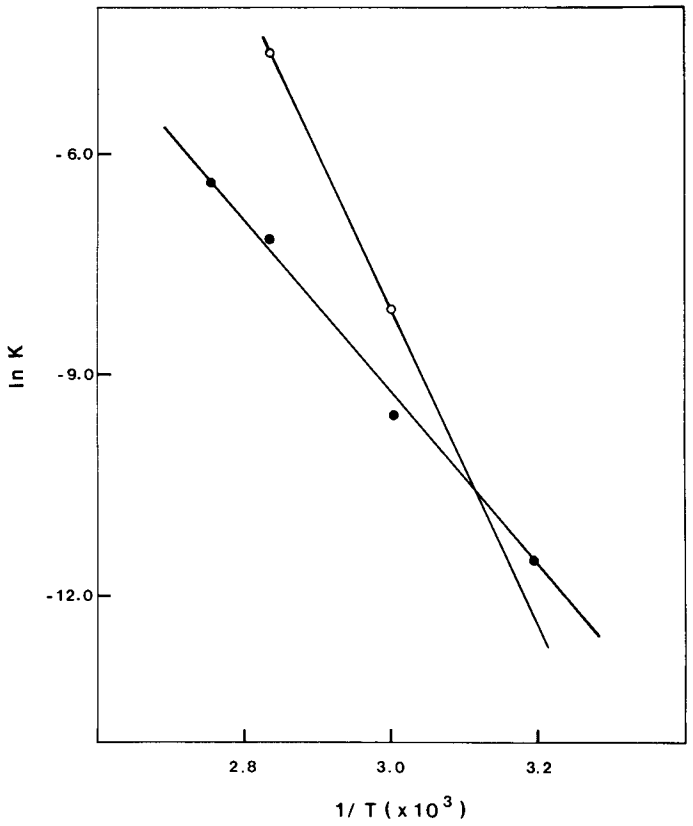

Fig. 3. Arrhenius Plot demonstrating temperature dependence of rate of hydrolysis of urea (○) and rate of formation of ethyl carbamate (●).

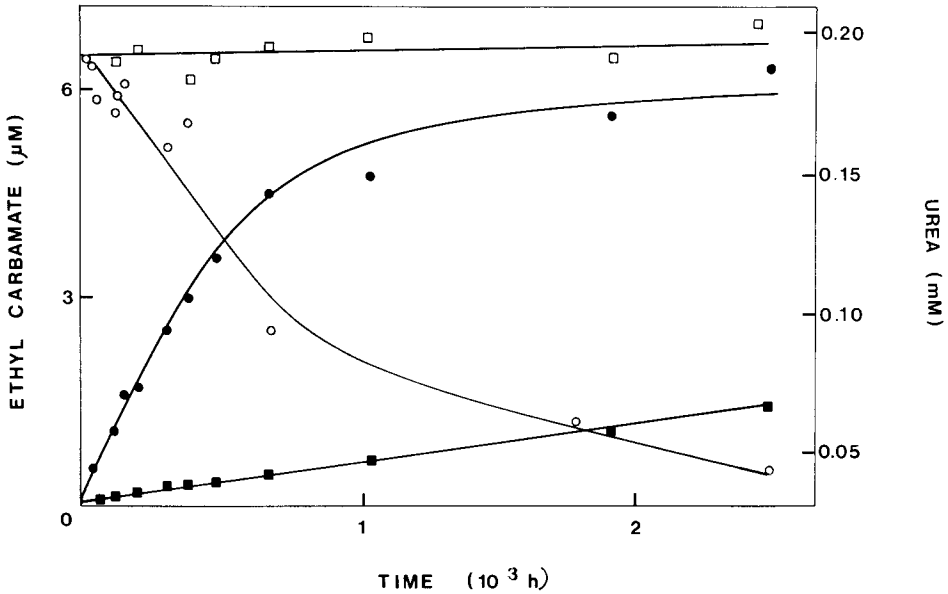

Fig. 4.Rates of ethyl carbamate formation and loss of urea at 40°C and 60°C.
40°C: ethyl carbamate formation (■), loss of urea (□).
60°C: ethyl carbamate formation (●), loss of urea (○).

(1.67 m*M*) of urea at 80°C estimated over a 48 hour time period (9, C.S. Ough, personal communication). Using our assay conditions and correcting for concentration differences between the two systems, we obtain a value of 3.83 m*M*/h at 80°C, in good agreement considering the sharp temperature dependence and relative effects

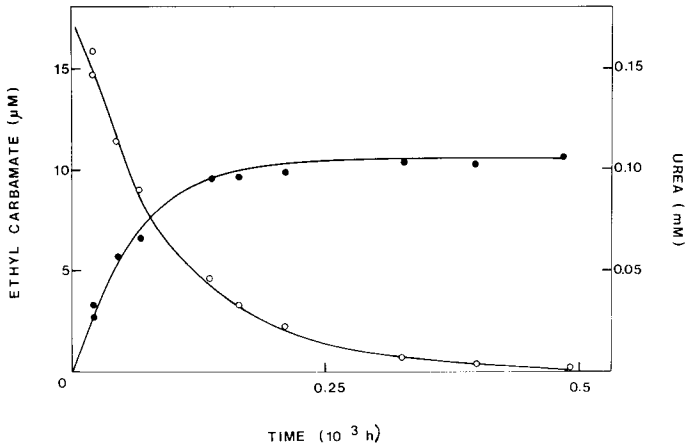

Fig. 5. Rates of ethyl carbamate formation and loss of urea at 80°C, ethyl carbamate formation (●); loss of urea (○). (Note scale differences with Fig. 4.)

of the hydrolysis reaction.

Ethyl carbamate formation from labeled urea in wine: To determine if urea is indeed a precursor of ethyl carbamate in wines, radioactively labeled urea was added to two wines and the incorporation of radioactivity into ethyl carbamate was investigated. Two wines produced from grapes of the University of California, Davis, vineyard were chosen, a French Colombard and a Cabernet Sauvignon, for which ethyl carbamate levels following treatment at 80°C for 48 hours were known from a gas chromatographic/mass spectrometric analysis (performed by E. Crowell and C. S. Ough). Samples of the two wines were dried down and resuspended in 20% ethanol, labeled urea was added, and the rate of formation of labeled ethyl carbamate was determined. Both wines initially contained some urea and given the dependence of rate of ethyl carbamate formation upon initial urea concentration, the actual total urea concentration in the wines could be determined (Table 1). Knowing the time and temperature dependence on rate of ethyl carbamate formation combined with actual total urea concentration, an ethyl carbamate value could be predicted from the tracer analyses (Table 1) and compared to the value obtained experimentally using the GC/MS analysis. There was surprisingly excellent agreement between the values. In the case of these two wines, it appears that the urea level initially present in the wine is probably sufficient to account for the ethyl carbamate produced during the heating regime.

The effect of alcohol content of the wine on the rate of formation of ethyl carbamate at a given concentration of urea was also investigated using the French

Table 1. Ethyl carbamate formation in wines from radioactively labeled urea.

Wine	Added urea (mg/L)	Rate of ethyl carbamate formation (nmol/L/h)	Total urea (mg/L)	Urea originating in wine (mg/L)	Predicted ethyl carbamate level*	Ethyl carbamate level (μg/L) 80°C, 48 h
French Colombard	10	32.2	11.9	1.9 ± 0.4	132	108
Cabernet Sauvignon	10	29.8	11.0	1.0 ± 0.2	63	66

*Calculated based upon urea originating in wine, assuming 4.5% conversion at 80° after 48 hours incubation (9).

Table 2. Percent conversion of initial urea to ethyl carbamate at different ethanol concentrations.

Urea (mg/L)	Solution* (%)	Ethanol (%)	Percent conversion of initial urea
20	Buffer	20	1.5
		15	0.9
		10	0.8
		5	0.4
10	Buffer	20	1.4
		15	1.1
		10	0.8
		5	0.4
5	Buffer	20	1.6
		15	1.0
		10	0.8
		5	0.4
2	French Colombard	20	1.9
		10	1.0
		5	0.7

*Reaction conducted in either French Colombard wine or citrate buffer. All samples were incubated at 60°C, data is from the 88 hour time point.

Colombard wine as a base. Ethanol concentration had the same effect on rate of ethyl carbamate formation (expressed as % of total initial urea) in the wine as in citrate buffer (Table 2). In addition, a value of an increase in reaction rate of 2.56 ± 0.5 nmol/L/h ethyl carbamate/mg/L urea was calculated for the reaction in buffer. Values of 3.22 ± 0.64 for the French Colombard and 2.98 ± 0.6 for the Cabernet Sauvignon were calculated from the difference in reaction rates observed with added urea, again in excellent agreement. Thus, while wine clearly represents a much more complex medium than citrate buffer, the kinetics of ethyl carbamate formation using labeled urea seems highly reproducible in these systems. However, when the rate of loss of urea was investigated in buffer solutions and in wines, there was a greater conversion of urea to some compound other than ethyl carbamate, particularly in the case of the Cabernet Sauvignon wine. Obviously, wine contains many other species capable of reacting with urea. The influence of these reactions on ultimate ethyl carbamate yields in wine cannot be discounted.

Investigation of the source of urea in wine: The previous results had demonstrated that urea, if present in wine, could react with ethanol to form ethyl carbamate. Urea can be found in wines and is an intermediate in the degradation of arginine and of purines. Other compounds, also potential precursors of ethyl carbamate, can be obtained from the biosynthesis or degradation of pyrimidines (9). The concentration of purines and pyrimidines in grape juice appears to be too low to be utilized as a source of nitrogen. The high phenolic content of grape juices interferes with conventional methods to estimate purine and pyrimidine concentrations. Therefore, a biological

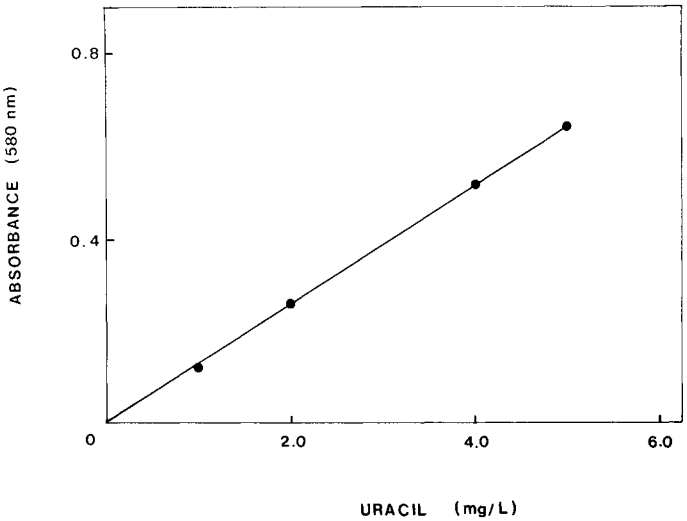

Fig. 6. Dependence of cell yield (absorbance at 580 nm) of strain MCY 638 upon uracil concentration.

Table 3. Amino acid composition of pink concentrate.

Compound	Concentration (mg/L)
Aspartic acid	65.8
Threonine	66.7
Serine	79.7
Glutamic acid	84.7
Glutamine	36.2
Proline	2150.3
Glycine	3.9
Alanine	141.5
Valine	30.4
Cystine	6.8
Methionine	5.1
Isoleucine	13.4
Leucine	25.2
Tyrosine	23.2
Phenylalanine	13
α-amino butyric acid	152.6
NH_3	66
Ornithine	13.2
Lysine	11.1
Histidine	32.3
Arginine	944
Total	3970

assay was developed to determine adenine and uracil content of juices. Yeast strains MCY 638, carrying a mutation in the *URA3* gene, and MCY 501 carrying a mutation in the *ADE2* gene were grown under conditions of limiting uracil or adenine concentration, with all other nutrients in excess. Under these growth conditions, cell yield is directly proportional to the amount of added uracil in the case of MCY 638 and of adenine in the case of MCY 501 (Fig. 6, shown for uracil only). Using a sample of the concentrate juice, values of 7.7 ± 1.0 mg/L for uracil and 7.8 ± 0.8 mg/L for adenine were obtained. To support good growth of adenine and uracil requiring mutants in laboratory media, approximately 10 mg/L of each compound was found to be required. Several other juices were analyzed for adenine and uracil content. The concentration range observed for adenine was from 4 to 15 mg/L and that of uracil from 4 to 8 mg/L. Thus, in a variety of juices there may be sufficient adenine and uracil to provide cellular needs for biosynthesis of these compounds, but in all cases insufficient concentrations to serve as a primary source of nitrogen. At these low concentrations, these compounds would not be used as nitrogen sources.

In contrast, arginine is present in much higher concentration in grape juice and is one of the major nitrogen sources used by yeast during vinification (1). To determine if arginine could serve as a source of urea in wine, a series of juice fermentations were conducted using radioactively labeled arginine. Arginine labeled in the *guanido* carbon (^{14}C) was used to trace the fate of the urea moiety, while arginine tritiated on carbons 2 and 3 was used to trace the ornithine group. The juice used was a pink concentrate obtained from E. & J. Gallo Winery that was reconstituted to 23°Brix. The amino acid composition of this juice is given in Table 3. In the first experiment, the concentration of labeled arginine was varied [total arginine concentration (940 mg/L) was not significantly altered by the addition of labeled material], and samples removed at 15°Brix, and the harvested cells were subjected to various extraction procedures. Very little of the ^{14}C-arg label was extractable by buffer (Na-citrate) or water alone. In 17.5% ethanol there was an immediate release of about 0.026 μmol/mL of label. Approximately 0.0052 μmole comigrated with urea, none comigrated with ethyl carbamate, with the bulk of the label remaining at the origin (as would arginine). The pattern of release in the case of tritiated arginine was very different. Approximately 50% of the accumulated label was released from the cells quickly, whether in buffer, water, or ethanol suspension, compared to about 3 to 4% release of ^{14}C-arg in the same time period. This observation gives an important clue to the nature of the material derived from tritiated arginine as well as one potential metabolic reason for the degradation of arginine during active growth in juice. The active excretion of tritium upon growth arrest of the culture is most consistent with the label appearing in polyamines for which ornithine is the direct precursor. In the presence of arginine, other biosynthetic routes to ornithine are repressed, thus arginine serves as the major precursor of polyamines. While little is understood about polyamine metabolism and function in yeast, actively growing yeast have very high cellular concentrations of these compounds (6) which are not found in cells arrested in growth. Thus, arginine can be degraded to provide cellular nitrogen as well as to provide precursors of polyamine biosynthesis. Essentially, nothing is known about polyamine levels in grape juices nor about any potential control of arginase activity mediated by the need for polyamines as opposed to the need for nitrogen. Clearly, both areas merit further investigation.

In a second experiment using the same juice and temperature of incubation, samples of cells were removed at 19°Brix and 8°Brix and at dryness. Disappearance of *guanido*-carbon-labeled arginine from the juice was investigated as well as the effect of length of time of incubation of the cells in the presence of ethanol (17.5%) on extraction of label. By the time the culture reached 19°Brix (starting at 23°Brix), the bulk of the labeled arginine had been consumed by the cells (Fig. 7). Of that label, 80% was trapped as CO_2, the rest being associated with the cells. Similar results were obtained for a replicate of the same experiment. Therefore, the urea moiety of the arginine molecule was being degraded to NH_3 and CO_2. Quantitation of the amount of label stably associated with cells during the course of the fermentation demonstrated a rapid increase in incorporation correlating with period of most active utilization of arginine, as expected (Fig. 7). The cells were harvested from the juice by filtration, washed with water and citrate buffer, and resuspended in citrate buffer (50 m*M*, pH 3.2) with ethanol (17.5%). Samples were removed at various time points, cells removed by filtration, and the amount of radioactivity appearing in the filtrate determined. At 19°Brix, a graph of counts extracted versus time (Fig. 8) was biphasic with a rapid (over a time period of one hour) initial extraction of about 12% of the label followed by a slow release of label, linear over

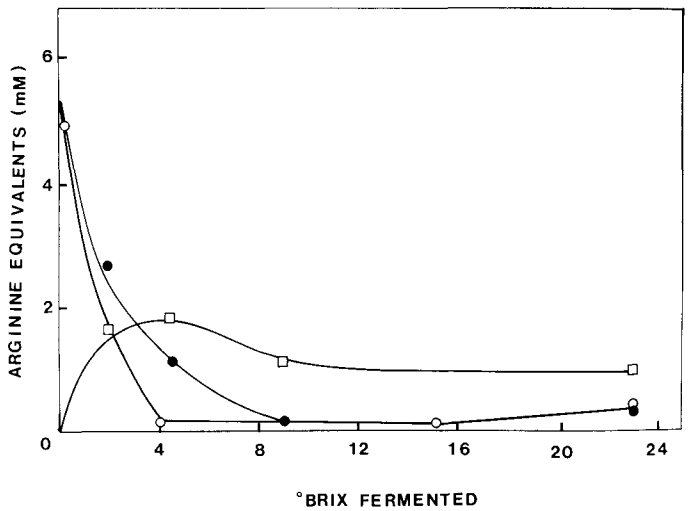

Fig. 7. Pattern of disappearance of radioactively labeled arginine during fermentation of rehydrated juice concentrate and accumulation of label in cell material. Replicate experiments are shown for loss of radioactive material from juice (○, ●). Label accumulating in cells (□) is shown for one of the replicates only and corresponds to the fermentation depicted by closed circles.

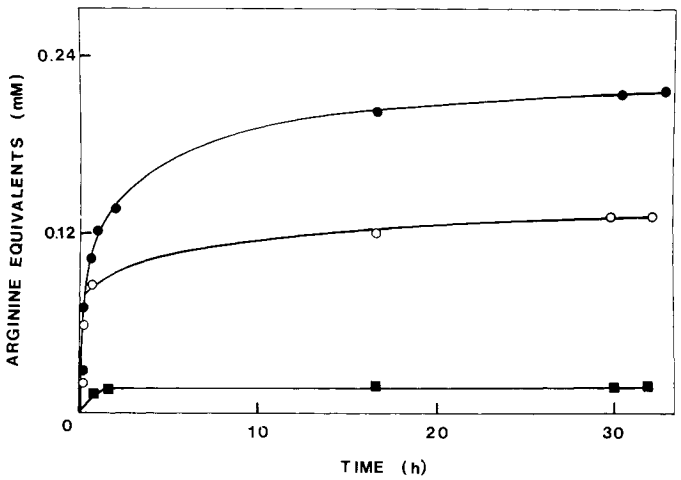

Figure 8. Ethanol-induced extraction of accumulated radioactively labeled material from cells grown in rehydrated juice concentrate in the presence of labeled arginine, harvested at differing substrate concentrations (°Brix): 19°Brix(●)); 8°Brix (○); 0°Brix (■). Radioactivity is expressed as molar equivalents of arginine.

the entire time course of seven days. By seven days, 90% of the label initially associated with the cells was extracted. A similar pattern was obtained for the 8°Brix sample, but overall yields decreased somewhat (Fig. 8). Extraction of such a high percentage of label from the cells is indicative of massive protein turnover combined with plasma membrane damage and permeabilization caused by the ethanol (12). To determine the fraction of extractable label appearing as urea, samples were run on TLC plates to determine the amount of radioactivity comigrating with the urea spot. Samples taken from the initial rapid phase of extraction gave about 90% of the label migrating as urea, while later time points gave only 50% migrating as urea. Other factors such as low temperature also affect integrity of the plasma membrane. Leakage of label from the cells incubated at low temperature (4°C) was examined. Instead of a rapid release of only 12% of the label, about 33% of the label was extractable in the first 10 minutes of incubation with ethanol. Whether the effects of ethanol and low temperature are additive or synergistic in certain conditions remains to be determined and is being investigated, as this has important implications for the appropriate temperature of fermentation.

A different picture of extraction emerged from examination of the cells at dryness. In this case, 50% of the label was stably associated with the cells—not extractable by ethanol—and more of the label appeared in the juice, most likely due to the ethanol already present in the wine (Fig. 8). However, as cells approach dryness, significantly less label is extractable by fortification (Fig. 8). This finding implies that if the cells are allowed to go to dryness prior to ethanol supplementation, less label will be released from the cells, suggesting a greater resistance to ethanol disruption of the plasma membrane. Therefore, it may be possible to modify plasma membrane composition by appropriate nutrient additions or use of ethanol resistant strains.

To determine the nature of label in the juice during fermentation, samples were filtered at a given °Brix to remove cellular material and analyzed by thin layer chromatography (Table 4). At 18.5°Brix, radioactively labeled material comigrating as urea could be detected in the juice, the level present decreased slightly during the course of the fermentation. The levels of urea found in the juice are too low to be detectable in a standard enzymatic method for determination of urea, so the identity of the compound could not be readily confirmed by an independent procedure; values presented in Table 4 should be viewed as rough estimates of the amount of the compound present.

Table 4. Concentration of material in juice comigrating as arginine and urea.

	Concentration of radioactively labeled material (μM)	
°Brix	Comigrating as argine	Comigrating as urea
23	5240	—
18.5	210	23
13.8	33.5	13.5
0	60.3	13.5

The standard error replicates of the thin layer chomatographic method used is ± 20%.

Conclusions

Delineation of the pathway or pathways responsible for the formation of ethyl carbamate in fermented foods and beverages such as wine is essential in order to limit or eliminate production of this compound. That yeast metabolic activities are involved is well known (5,8,10). One potential precursor is urea which can be formed from the degradation of arginine or of the purines adenine and guanine. Analysis of purine content of juices indicated that adenine is not present in sufficient quantity to be used as a nitrogen source during fermentation; however, the contribution of purine catabolism and nucleic acid turnover during stationary phase requires investigation. We have shown that radioactively labeled ethyl carbamate is formed when radioactively labeled urea is added to wines. The kinetics of formation of ethyl carbamate from labeled urea in wine were in good agreement with the results obtained in model solution and with previously published reports (9).

Radioactively labeled arginine (*guanido*-^{14}-C) was shown to yield labeled material comigrating as urea which could be further extracted from the cells during fermentation by ethanol fortification. Thus, arginine can be a precursor of urea (which is itself a precursor of ethyl carbamate) via the catabolic activity of arginase, confirming one potential pathway for the formation of ethyl carbamate in wine. However, other pathways may also exist, as there is a tremendous variation in composition of juices.

Literature Cited

1. Castor, J. G . B. The free amino acids of musts and wines. II. The fate of amino acids of must during aerobic fermentation. Food Res.

10:146-151 (1953).

2. Cooper, T. B. Nitrogen metabolism in *Saccharomyces cerevisiae. In:* The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression. J. N. Strathern, E. W. Jones and J. R. Broach (Eds.). pp 39-99. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

3. Davis, T. W. M. Thin-layer chromatographic identification of thirteen medicinally important carbamates. J. Chromatogr. 29:283-287 (1967).

4. Hais, I. M., and K. Macek (Eds.). Paper Chromatography: A Comprehensive Treatise. p 804. Academic Press, New York (1963).

5. Ingledew, W. M., C. A . Magnus, and J. R. Patterson. Yeast foods and ethyl carbamate formation in wine. Am. J. Enol. Vitic. 38:332-5 (1987).

6. Jones, E. W., and G. R. Fink. Regulation of amino acid and nucleotide biosynthesis in yeast. *In:* The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression. J. N. Strathern, E.W . Jones and J.R. Broach (Eds.) pp 181-299. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

7. Mirvish, S. S. The carcinogenic action and metabolism of urethan and N-hydroxyurethan. Adv. Cancer Res. 11:1-42 (1968).

8. Ough, C. S. Ethyl carbamate in fermented beverages and foods. I. Naturally occurring ethyl carbamate. J. Agric. Food Chem. 24:323-7 (1976).

9. Ough, C. S., E. A. Crowell, and B. R. Gutlove. Carbamyl compound reactions with ethanol. Am. J. Enol. Vitic., 39:139-42 (1988).

10. Ough, C. S., E. A. Crowell, and L. A. Mooney. Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acids, urea and ammonia; effects of fortification on intercellular and extracellular precursors. Am. J. Enol. Vitic. 39:243-9 (1988).

11. Redgwell, R. J., N. A. Turner, and R. L. Bieleski. Stripping thin layers from chromatographic plates for radio tracer measurements. J. Chromatogr. 88:25-31 (1974).

12. Salgueiro, S. P., I. Sa-Correia, and J. M. Novais. Ethanol-induced leakage in *Saccharomyces cerevisiae*: kinetics and relationship to yeast ethanol tolerance and alcohol fermentation productivity. Appl. Env. Micro. 54:903-9 (1988).

13. Stahl, E. Thin Layer Chromatography: A Laboratory Handbook. p 740. Springer-Verlag, New York (1969).

14. Werner, E. A. The Chemistry of Urea. The Theory of its Constitution, and of the Origin and Mode of its Formation in Living Organisms. 212 pp. Longmans, Green and Co., New York (1923).