A Mechanistic Study of Alkylpyrazine Formation in Model Systems

Several α-dicarbonyl compounds were reacted with α-amino acids in an attempt to validate the hypothesis that alkylpyrazines are formed from similar precursors during the roasting of foodstuffs. α-Diketones yielded the expected pyrazines, but pyruvaldehyde produced trimethylpyrazine in addition to isomeric dimethylpyrazines. Aminoacetone, a proposed reaction intermediate in the later reaction, spontaneously condensed with itself to form 2,5-dimethyl-, trimethyl-, and 2,5-dimethyl-3-ethylpyrazine.

Recent years have witnessed the discovery of numerous alkyl-substituted pyrazines in a wide variety of foodstuffs (Maier, 1970). In view of the apparent organoleptic importance of pyrazines in foods, information pertaining to their origin was deemed to be of significant value for future food process or development work. A consideration of data obtained in this and other laboratories indicated that pyrazines probably arise *via* complex interactions between α-amino acids and carbohydrate (or small oxycarbon fragments derived therefrom). Such pathways have been suggested by others (Hodge, 1967; Koehler and Odell, 1970; Wang *et al.*, 1969) and seem most reasonable at this time. Alkylpyrazines have also been obtained from carbohydrate and aqueous ammonia in model systems (van Praag *et al.*, 1968) and by direct pyrolysis of oxy-α-amino acids (Kato *et al.*, 1970); however, the latter conditions do not reasonably approximate actual food roasting circumstances under which pyrazines are formed, *e.g.*, the roasting of cacao beans (Rohan and Stewart, 1967).

In order to learn more about the interactions of potential pyrazine precursors we studied some reactions of α-dicarbonyl compounds with α-amino acids. α-Dicarbonyl compounds were chosen because they are widespread in foods and in heated fats (Cobb and Day, 1965) and because they are known to participate in the Strecker degradation of α-amino acids (Schönberg and Moubacher, 1952). In this study, representatives of three types of α-dicarbonyls including glyoxal, α-ketoaldehydes (pyruvaldehyde), and several symmetrical and unsymmetrical α-diketones were allowed to react with glycine, alanine, or β-phenylalanine. Volatile reaction products were separated by steam distillation and analyzed by gas chromatography (gc). Subsequently, the pyrazines were identified by spectral means or by direct comparison with authentic samples.

EXPERIMENTAL SECTION

Materials. Diethyleneglycol dimethylether (diglyme) was freshly distilled from calcium hydride before use. α-Amino acids were high purity commercial samples and were used as received. 2,3-Butanedione (diacetyl) and 2,3-pentanedione were obtained commercially and redistilled prior to use. 2-Hydroxy-2-cyclohexenone, 3-methyl-1,2-cyclopentanedione, 30% aqueous pyruvaldehyde, and benzil were reagent grade commercial products and were used directly without purification. 2,5- and 2,6-Dimethyl- and tetramethylpyrazine were commercial samples which were used without purification. 2,5-Dimethyl-3-ethylpyrazine and trimethylpyrazine were prepared according to Klein and Spoerri (1951). 3,4-Hexanedione (dipropionyl) was prepared by Bi_2O_3 oxidation (Rigby, 1951) of propionoin. Aminoacetone hydrochloride was prepared by the method of Gabriel and Pinkus (1893).

Methods of Analysis. Volatile pyrazines were isolated from reaction mixtures by simple steam distillation at atmospheric pressure. Aqueous distillates were generally saturated with sodium chloride and extracted with ether. The ether extracts were dried ($MgSO_4$) and concentrated to small volumes prior to gas chromatography. Gas chromatographic conditions for analysis and small scale isolation have been previously described (Rizzi, 1967, 1968). Quantitative analyses of volatile products were obtained by planimeter integration of gc curves. All melting points were taken in open capillaries and were uncorrected. In cases where solid products were obtained (see below), the materials were recrystallized and melting points were compared with literature values. Infrared spectra were obtained on either liquid film samples or in CS_2 solution with a Perkin-Elmer Model 321 instrument. Nmr spectra were taken in CCl_4 solution using a Varian Associates Model HA-100 instrument employing tetramethylsilane (TMS) as internal reference standard. Chemical shifts were recorded in parts per million (ppm expressed in δ units) downfield from the TMS signal. Mass spectra were obtained with an Atlas Model CH-4 instrument operating with an ionizing energy of 70 eV.

Reaction of DL-β-Phenylalanine and 3,4-Hexanedione. A mixture containing DL-β-phenylalanine (3.30 g, 0.02 mol) 3,4-hexanedione (2.28 g, 0.02 mol), and diglyme (25 ml) was stirred and refluxed under nitrogen for 3 hr. The clear orange-red reaction mixture was transferred to a 1-l. flask and steam distilled until 400 ml of aqueous distillate was obtained. The distillate was saturated with salt and subjected to continuous ether extraction for 16 hr. The ether extract was concentrated under reduced pressure using a rotatory evaporator until a diglyme solution of the product remained. After adding 10 ml of 4 *N* HCl excess HCl, water and diglyme were evaporated to leave a crude hydrochloride salt. The residue was treated with 10 ml of 6 *N* NaOH and extracted with ether (3 × 50 ml) to remove the product. Concentration of the dried ($MgSO_4$) ether solution gave 1.27 g of an oil. Gc analysis of this material revealed a single major component which was trapped from the effluent He stream in a cooled glass capillary. The compound was a colorless oil at 25°C with a strong spice-like aroma. It was identified as tetraethylpyrazine on the basis of its ir and nmr spectra. The seven most intense ir bands in order of decreasing intensity appear at: 7.07, 3.29, 6.81, 8.53, 7.61, 3.40, and 9.57 μ (liquid film sample); nmr (CCl_4) δ 1.24 (t, J = 7 Hz), 2.68 (q, J = 7 Hz). The ratio of triplet to quartet protons was 3:2.

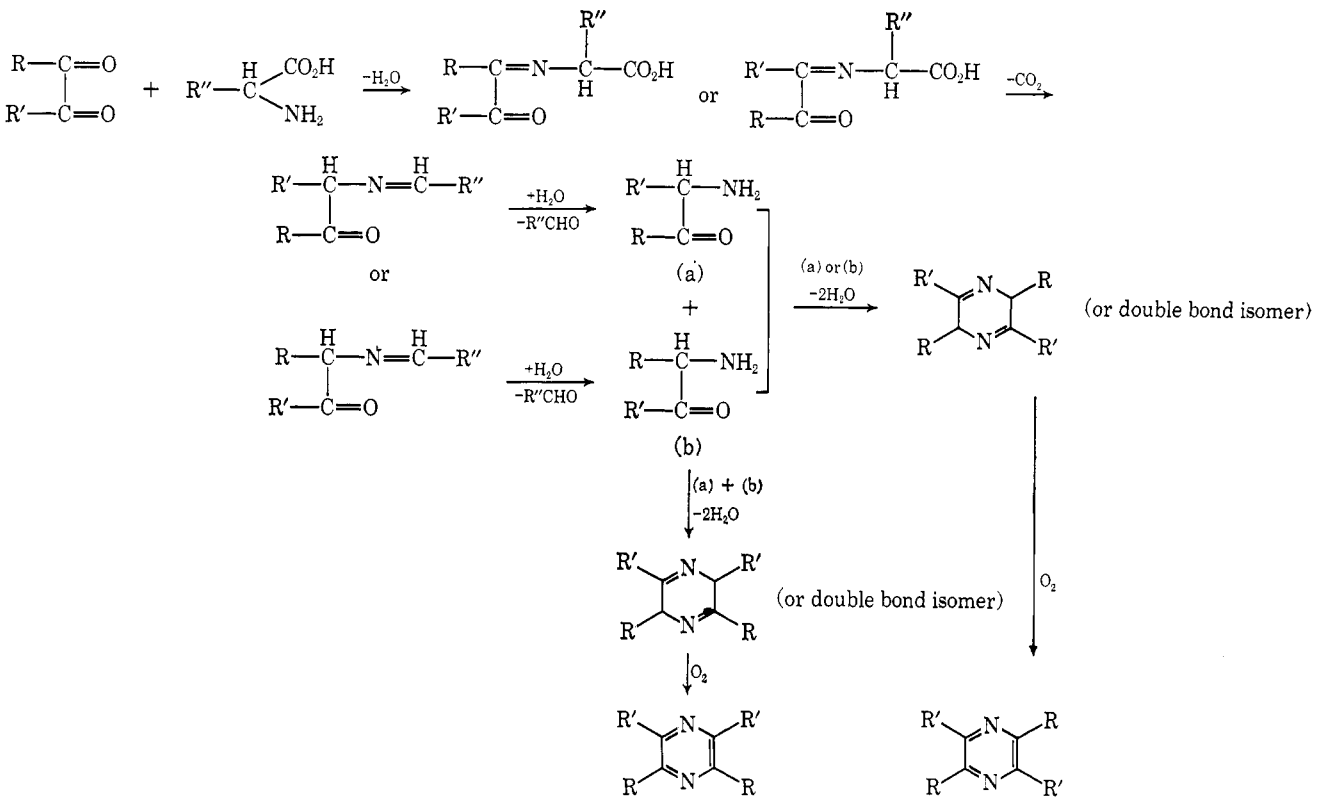


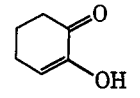
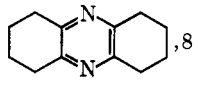
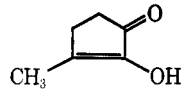
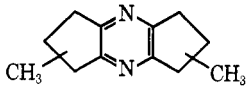
Figure 1. Reaction scheme for the formation of pyrazines from α-amino acids and α-dicarbonyl compounds

Reaction of 2-Hydroxy-2-cyclohexenone with DL-Alanine. A mixture of 2-hydroxy-2-cyclohexenone (2.80 g, 0.025 mol), DL-alanine (2.22 g, 0.025 mol), and 30 ml of diglyme was refluxed under N_2 for 2 hr. The reddish-brown reaction mixture was steam distilled and the steam distillate, after saturation with NaCl, was extracted with ether. The ether solution was washed with water to remove diglyme, dried over $MgSO_4$, and finally concentrated to a small volume under reduced pressure. Treatment of the residue with ethanolic picric acid solution produced a solid picrate which was recrystallized from ethanol to yield 0.669 g of yellow crystals, mp 162–163°C (8% yield). Subsequent decomposition of the purified picrate with aqueous NH_3 afforded 0.192 g of crude, 1,2,3,4,6,7,8,9-octahydrophenazine, mp 92–102°C. Two recrystallizations from hexane yielded the pure octahydrophenazine as thin colorless plates, mp 109–111°C. Smith (1948) reported mp 108–109°C, picrate mp 162–163°C.

Reaction of Pyruvaldehyde with Glycine. A mixture consisting of 7.5 g (0.10 mol) of glycine and 50 ml of diglyme was stirred and refluxed under N_2. While these conditions were maintained, a mixture of 30% aqueous pyruvaldehyde (23.0 g, 0.10 mol) and 20 ml of diglyme was added dropwise over the course of 40 min. Under these conditions a vigorous reaction took place which yielded copious amounts of dark resinous tar. After an additional 30 min reflux the reaction mixture was worked up in a manner similar to that described above. Gc analysis of the volatiles indicated the presence of a single major volatile reaction product. This product was isolated by preparative gc and identified as trimethylpyrazine by means of nmr and mass spectral data and by the melting point of its picrate derivative (found 135–137°C; Klein and Spoerri (1951) reported 138–138.5°C). The yield of trimethylpyrazine was about 1%.

Autocondensation of Aminoacetone. Aminoacetone hydrochloride (28.21 g, 0.258 mol) was dissolved in 50 ml of water, and after dissolved oxygen was removed under vacuum, the mixture was stirred in an atmosphere of N_2 at room temperature (*ca.* 25°C). The hydrochloride solution was treated with a similarly degassed solution of sodium hydroxide (11.84 g, 0.303 mol) in 30 ml of water and the resulting dark reaction mixture was stirred for 0.75 hr at 25°C. Reaction products were isolated by extracting the reaction mixture with CH_2Cl_2 under an atmosphere of N_2. Removal of the solvent under reduced pressure afforded a clear, reddish-brown oil which was distilled. In this way there was obtained 2.44 g of mobile pale yellow oil, bp 67–100°C (21 mm). The pot residue amounted to about 10 g of material which was a dark viscous oil at 160°C, and which solidified to a tenacious resin on cooling to 25°C. A center fraction (1.16 g) of the distillate, bp 86–98°C (21 mm), was used for subsequent experiments. This fraction exhibited properties like those reported by Cornforth (1958) for a 2,5-dimethyldihydropyrazine in that it had ir absorption bands at 2.95 μ. The oil showed no visible signs of decomposition when stored at 4°C under N_2. Gc analysis of the distillate indicated three products which were identified as 2,5-dimethylpyrazine (2,5-DMP), trimethylpyrazine, and 2,5-dimethyl-3-ethylpyrazine. Identification of products was established by comparing spectral and retention time data with like data of known materials. Structure assignments were unequivocal in each case. The dihydropyrazine present in the original distillate was not detected by gc analysis and was presumed to have decomposed to 2,5-DMP plus relatively nonvolatile products during chromatography. A more efficient thermal conversion to 2,5-DMP was realized when a sample of the distillate was heated to 200°C for 16 hr in a sealed glass tube prior to analysis. No new volatile compounds were formed during heating.

Table I. Pyrazines Formed in Reactions of α-Diketones and DL-Alanine

Diketone	Reaction conditions,[a] temperature °C, time, hr	Pyrazine obtained, % yield
2,3-Butanedione (diacetyl)	160, 2	Tetramethylpyrazine, 0.5
3,4-Hexanedione (dipropionyl)	160, 2	Tetraethylpyrazine
2,3-Pentanedione	160, 2	2,5-Diethyl-3,6-dimethylpyrazine[b] or 2,6-diethyl-3,5-dimethylpyrazine
Benzil	160, 7 No solvent	Tetraphenylpyrazine, 14
	150–160, 2	 ,8
	150–160,[c] 2	

[a] Equimolar amounts of reactants were refluxed in diglyme for the specified times. [b] Nmr did not allow unequivocal structure assignment to the single gc homogeneous product isolated. [c] A complex mixture of geometric isomers was obtained.

RESULTS AND DISCUSSION

Reactions of DL-Alanine with α-Diketones. Mixtures of α-diketones and DL-alanine were heated to 150–160°C in diethyleneglycol dimethylether (diglyme). Diglyme was chosen as a reaction medium to mimic the available partial solubilization and low water content that might be present in the interior of a roasting cacao or coffee bean (Rohan and Stewart, 1967). It was assumed for the present that all reactions leading to pyrazines were completed during the diglyme reflux period and that no further changes took place during steam distillation. In accord with the mechanism originally proposed by Schönberg and Moubacher (1952) and later elaborated by Rizzi (1969) (Figure 1), a series of symmetrically substituted pyrazines was obtained from structurally related α-diketones (Table I). The reaction of 2,3-butanedione (diacetyl) and DL-alanine yielded tetramethylpyrazine as the sole pyrazinic product. It was apparent from the diacetyl reaction and others involving symmetrical α-diketones that all the pyrazine carbon atoms were derived from the carbonyl moiety, whereas the heterocyclic nitrogen atoms came from the α-amino acid. A similar conclusion was reached earlier by Koehler *et al.* (1969) in a study of pyrazine formation in another model system using radiotracer technique. The unsymmetrical α-diketone, 2,3-pentanedione, gave a single, apparently homogeneous product; however, nmr analysis could not distinguish whether the material obtained was 2,5-diethyl-3,6-dimethylpyrazine or 2,6-diethyl-3,5-dimethylpyrazine. It is, in fact, possible that a mixture of both pyrazines was obtained. Highly enolic cyclic diketones also gave pyrazines. This result is particularly interesting since it suggests that cyclic diketones which have been isolated from a coffee aroma concentrate (Gianturco *et al.*, 1963) may also be participating in heterocycle formation during the roasting of coffee beans. To date, however, no tricyclic pyrazines such as those shown in the table have been reported among coffee volatiles.

In accord with the proposed scheme (Figure 1) it should be possible that mixtures of two or more α-diketones and a single α-amino acid might yield several pyrazines containing carbon skeletons derived from the participating diketones. Such a possibility that a single α-amino acid interacts with more than one diketone to form a variety of alkylpyrazines was suggested earlier by Wang *et al.* (1969). We found this to be true in the case of phenylalanine and mixtures of 2,3-butanedione (diacetyl) and 3,4-hexanedione (dipropionyl). Reactions involving various mixtures of the two diketones and phenylalanine led to varying amounts of tetramethylpyrazine, 2,3-diethyl-5,6-dimethyl-, and tetraethylpyrazines (Table II). Under competitive reaction conditions (Run 2) tetraethylpyrazine was the major product, although more methylpyrazines were observed when a higher proportion of diacetyl was employed (Run 3). It is significant that a relatively small change in the α-diketone ratio (4:1) caused a large change in product ratio (36:1 in the case of tetramethylpyrazine).

Reactions of Glycine with Glyoxal and Pyruvaldehyde. If the reaction scheme (Figure 1) were completely general, the formation of mono-, di-, tri-, and tetraalkylpyrazines might be expected in reactions of α-amino acids with mixtures of α-diketones, α-ketoaldehydes, and glyoxal. According to our proposed mechanism reductive amination would be expected to occur to yield α-aminocarbonyl compounds which could then dimerize to give dihydropyrazines. The simplest dicarbonyl, glyoxal, reacted rapidly with glycine in hot diglyme but the only volatile product observed was carbon dioxide. Much dark tarry material was formed which presumably resulted from linear condensation polymerization of the initial reductive amination product, aminoacetaldehyde. No trace of pyrazine was observed among the steam volatile reaction products.

Reductive amination of pyruvaldehyde can theoretically proceed at either carbonyl site; however, reaction at the aldehyde group is preferred *a priori* since aldehydes are generally more reactive than ketones toward α-amino acids in the Strecker degradation. Reaction at the aldehydic site of pyruvaldehyde would lead to aminoacetone as a penultimate precursor to pyrazines. Aminoacetone has already been implicated as a possible pyrazine precursor in carbohydrate/amino acid reactions by Newell *et al.* (1967). Alternatively, if amination did occur at the keto position on pyruvaldehyde, the initial product would be α-aminopropanal. Dimerization of either aminoacetone or α-aminopropanal will eventually lead to 2,5-dimethylpyrazine (Figure 1, where R = H, R′ =

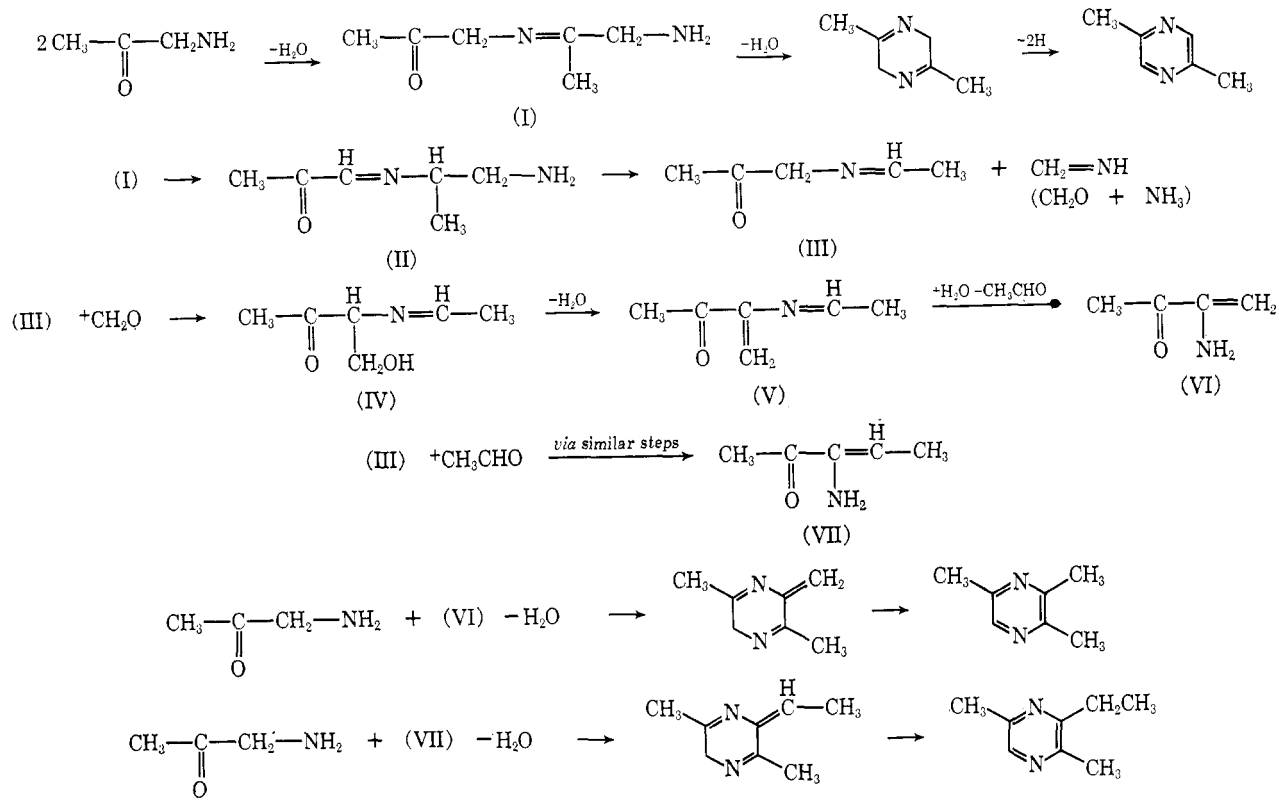


Figure 2. Proposed condensation reactions of aminoacetone

Table II. Formation of Tetraalkylpyrazines in the Strecker Degradation of DL-Phenylalanine with Mixtures of Diacetyl and Dipropionyl[a]

Run	Mole ratio of diacetyl to dipropionyl[b]	Pyrazines[c] (relative % yields) Tetramethyl	Diethyl-dimethyl	Tetraethyl
1	0.54	1.27	7.33	91.3
2	1.00	7.93	7.93	84.2
3	2.00	45.8	13.35	40.8

[a] Reactions were run by refluxing mixtures of the diketones and DL-phenylalanine in diglyme for 2 hr. [b] One mole of DL-phenylalanine was used per mole of ketone mixture, and initial total diketone concentration was 0.145 *M*. [c] Pyrazines were identified *via* their nmr spectra.

$-CH_3$). Two different amino carbonyl compounds also can react, in which case 2,6-dimethylpyrazine will be the product. In fact, when pyruvaldehyde was reacted with glycine in hot diglyme, trace amounts of 2,5- and 2,6-dimethylpyrazine and trimethylpyrazine were detected among the steam volatile reaction products. The formation of trimethylpyrazine from pyruvaldehyde is not explained by the mechanism outlined in Figure 1. According to Figure 1, trimethylpyrazine would only be predicted as a product in a reaction involving simultaneous or sequential amination of 2,3-butanedione and pyruvaldehyde, followed by combination of the aminated intermediates and subsequent oxidation of the dihydropyrazine. Since trimethylpyrazine was formed in very low yield it is possible that its formation was due to a trace of diacetyl originally present in our sample of commercial 30% aqueous pyruvaldehyde. It is also possible that pyruvaldehyde underwent decomposition to yield diacetyl during the overall reaction.

Reactions of Aminoacetone. In view of the apparently anomalous formation of trimethylpyrazine from pyruvaldehyde, we decided to examine our mechanism more thoroughly in terms of proposed reaction intermediates. Because the Strecker degradation was expected to yield aminoacetone, we elected to prepare aminoacetone by an independent route and determined the products which formed during its subsequent reactions. Aminoacetone hydrochloride was converted to aminoacetone with sodium hydroxide in the absence of air. The condensation products which formed spontaneously at about 25°C were solvent extracted, distilled, and analyzed by gas chromatography. Gc separation gave three products in relative yields of 50, 33, and 17% which were identified as 2,5-dimethylpyrazine, trimethylpyrazine, and 2,5-dimethyl-3-ethylpyrazine, respectively. The unexpected formation of trimethyl- and 2,5-dimethyl-3-ethylpyrazines from aminoacetone immediately suggested a more complex mechanism than the one shown in Figure 1. The observed formation of polyalkylpyrazines from aminoacetone can be explained by the hypothetical series of reactions shown in Figure 2. We propose that the reaction of aminoacetone to form a dimethyldihydropyrazine proceeds *via* a two-step sequence. In the first step, two molecules of aminoacetone condense with loss of water to form the acyclic imino ketone (I), followed by a second step in which intramolecular condensation takes place with additional loss of water to yield the expected dihydropyrazine product. Isomerization of I to its more stable conjugated isomer II and retro-Mannich condensation of II could take place under our experimental conditions, in which case *N*-ethylideneaminoacetone (III), formaldehyde, and ammonia would be the likely products. A similar retrograde process has been reported for β-dimethylaminopivalophenone by Snyder and Brewster (1949); also, the elimination bears close resemblance to the loss of CO_2 in

the Strecker degradation, *cf.* Baddar (1959). Realdolization of III with formaldehyde followed by dehydration of the intermediate carbinol IV would lead to a new imino ketone V which now contains a chain of four carbon atoms (*cf.* compound I with a chain of three carbon atoms). Hydrolysis of V could yield acetaldehyde plus a four-carbon aminoketone VI; and condensation of VI with aminoacetone with loss of water could ultimately yield trimethylpyrazine. Similarly, compound III may undergo realdolization with acetaldehyde (instead of formaldehyde), in which case a homologous aminoketone VII would be the product. Further condensation of VII with aminoacetone could ultimately lead to 2,5-dimethyl-3-ethylpyrazine as shown. Thus, the formation of trimethyl- and 2,5-dimethyl-3-ethylpyrazines can be explained in terms of a nonoxidative pathway involving a series of potentially reversible aldol condensations followed by thermal rearrangement of hydropyrazine intermediates to form aromatic products. Although highly speculative, the mechanism of Figure 2 does explain the formation of the wide variety of polyalkylpyrazines which have been reported to date. Extensions of the mechanism could involve participation of the various aldehydes formed during Strecker degradation of α-amino acids. Using this type of reasoning it is possible to predict the formation of many complex branched chain pyrazines, only a few of which have already been isolated from browned foodstuffs.

LITERATURE CITED

Baddar, F. G., *J. Chem. Soc.* S163 (1949).
Cobb, W. Y., Day, E. A., *J. Amer. Oil Chem. Soc.* **42,** 1110 (1965).
Cornforth, J. W., *J. Chem. Soc.* 1174 (1958).
Gabriel, S., Pinkus, G., *Chem. Ber.* **26,** 2197 (1893).
Gianturco, M. A., Giammarino, A. B., Pitcher, R. G., *Tetrahedron* **19,** 2051 (1963).
Hodge, J. E., "Chemistry and Physiology of Flavors," Schultz, H. W., Day, E. A., Libby, L. M., Eds., Avi Publishing Co., Westport, Conn., 1967, pp 465–491.
Kato, S., Kurata, T., Ishitruka, R., Fujimaki, M., *Agr. Biol. Chem. (Tokyo)* **34,** 1826 (1970).
Klein, B., Spoerri, P. E., *J. Amer. Chem. Soc.* **73,** 2949 (1951).
Koehler, P. E., Odell, G. V., J. AGR. FOOD CHEM. **18,** 895 (1970).
Koehler, P. E., Mason, M. E., Newell, J. A., J. AGR. FOOD CHEM. **17,** 393 (1969).
Maier, H. G., *Angew. Chem. Int. Ed. Engl.* **9,** 917 (1970).
Newell, J. A., Mason, M. E., Matlock, R. S., J. AGR. FOOD CHEM. **15,** 767 (1967).
Rigby, W., *J. Chem. Soc.* 793 (1951).
Rizzi, G. P., J. AGR. FOOD CHEM. **15,** 549 (1967).
Rizzi, G. P., *J. Org. Chem.* **33,** 1333 (1968).
Rizzi, G. P., *J. Org. Chem.* **34,** 2002 (1969).
Rohan, T. A., Stewart, T., *J. Food Sci.* **32,** 625 (1967).
Schönberg, A., Moubacher, R., *Chem. Rev.* **50,** 261 (1952).
Smith, P. A. S., *J. Amer. Chem. Soc.* **70,** 323 (1948).
Snyder, H. R., Brewster, J. H., *J. Amer. Chem. Soc.* **71,** 1061 (1949).
van Praag, M., Stein, H. S., Tibbetts, M. S., J. AGR. FOOD CHEM. **16,** 1005 (1968).
Wang, P., Kato, H., Fujimaki, M., *Agr. Biol. Chem.* **33,** 1775. (1969).

George P. Rizzi

Miami Valley Laboratories
The Procter & Gamble Co.
Cincinnati, Ohio 45239

Received for review December 6, 1971. Accepted January 28, 1972.

Extraction of Oil Samples from Seeds with Little Impairment of Viability

Seeds immersed in lipid solvents for several days release small amounts of their lipids without losing their viability. Thus, it is possible to determine the fatty acid composition of a seed sample and still be able to plant it and produce offspring.

The ability to determine the quantity and quality of oil in seeds prior to germination is of paramount importance both in basic genetic research involving parent–offspring comparisons and in plant breeding experiments. The traditional method of oil extraction for analytical purposes has consisted of expelling the oil by crushing the seed, followed by solvent extraction. Comparison of seed weight before and after oil removal determines the oil content of the seed, and a chemical analysis of the oil sample gives its composition. The problem with this technique, however, lies in the destruction of the seed, which the researcher may need for planting as parental material because of a desirable genetic constitution.

In recent years, wideline nmr spectroscopy has provided a quantitative means of rapid and accurate measurement of the lipid content of whole seeds without subjecting them to treatment which impairs their viability (Alexander *et al.*, 1967). In the absence of a better technique for oil quality determination, however, some investigators have cut off portions of a seed or removed one of its cotyledons for oil quality analysis and then planted the remainder of the seed to obtain progeny (Downey and Harvey, 1963).

The authors have now found an alternative, nondestructive method for oil extraction without impairment of seed viability. The technique consists of immersing seed samples for 4 to 6 days in lipid solvents at room temperature. The immersion process extracts amounts of oil from the seed which are sufficient for chemical analysis after evaporation of the solvent.

The technique has been employed with seed of safflower, flax, sunflower, soybean, and sesame and has proven satisfactory with samples as large as 50 g of seed in 500 ml of solvent and as small as a single seed in a 15 × 125 mm test tube with 5 ml of solvent. Since several drops of oil can be obtained from large seed samples, any of the conventional analytical methods may be used. Sufficient oil can be extracted from single seeds for glc analysis. Samples of 50 to 100 seeds from those plants mentioned above provide 15 to 25 mg of oil, sufficient for obtaining a reading on a refractometer. After termination of the immersion, seeds are air dried under a hood, and they may then be planted. Plants