30 °C (O'Leary and Fox, 1974), probably due to an electrostatic expansion of the negatively charged polypeptide chains (Lowenstein, 1974). The decrease in net negative charge on the carboxyl-modified pepsin molecule (Figure 2) may lower the extent of electrostatic expansion and subsequent denaturation. An improvement of enzyme stability was also reported in pepsin covalently bound to a soluble polyanionic carrier, ethylene maleic anhydride, and was attributed to different environmental states of the native and modified enzymes (Lowenstein, 1974).

The present results show that native pepsin was inactivated rapidly under simulated cheese-making conditions. This is consistent with the findings of Green (1972) that crude pepsin was almost completely denatured in milk dialysate under simulated cheese-making conditions, while rennet was fairly stable, retaining about 60% of its activity. In another report, active enzyme was not recovered from Cheddar curd made by porcine pepsin, whereas 5% of the added calf rennet was recovered (Holmes and Ernstrom, 1977).

The poor quality of cheese made with pepsin has been attributed to the instability of pepsin near neutral pH. It was suggested that in cheese made with calf rennet, the active enzyme retained in the curd would aid the starter enzymes in the ripening of cheese, while in pepsin cheese, proteolysis would be almost entirely dependent on starter activity (Green, 1972; Lawrence et al., 1972). Hence, cheese made with pepsin alone requires a longer aging period and develops flavor slowly (Melachouris and Tuckey, 1964; Emmons et al., 1971; Green, 1972). An increase in stability under simulated cheese-making conditions would make pepsin a more effective milk coagulant in cheese manufacture. A larger amount of active enzyme would be retained in the curd to aid ripening, thus reducing the production cost. For practical application, carboxyl-modified crude pepsin (1:10 000), with improved stability without losing the milk clotting activity more than 50% (data will be published elsewhere), would be a better coagulant than the modified crystalline enzyme.

The results presented in this paper show that the carboxyl groups modified by EDC and glycine methyl ester are not essential for pepsin activity. However, changes in the charge distribution on the enzyme may affect some pepsin functions such as milk clotting and the hydrolysis of dipeptide substrates, possibly through an interference with the enzyme–substrate binding process as shown by an increase in the K_m values upon carboxyl modification. The changes in the charge distribution may also alter some physicochemical properties of pepsin, such as enzyme stability and pH optimum for proteolysis.

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Fatty Acid Biogenesis in Ripening Mango (*Mangifera indica* L. var. Alphonso)

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The biogenesis of fatty acids in the ripening mango (var. Alphonso) was investigated with the aid of [2-^{14}C]acetate and [1-^{14}C]palmitic acid. It was observed that [2-^{14}C]acetate incorporation was maximal into palmitic acid and to a lesser extent in palmitoleic acid, while the radioactivity of [1-^{14}C]palmitic acid could be recovered essentially in the hydroxy fatty acids. The contribution of C-16 fatty acids to the formation of lactones in ripe Alphonso mango was discussed.

Lipid components in fruits, though occurring in minor amounts, are presumed to contribute to characteristic aroma and flavor during ripening. These are essentially considered as precursors for various volatile odorous principles of fruits. Several lactones identified in peaches and apricots (Jennings and Sevenants, 1964), as well as esters in bananas (Tressl and Drawert, 1973), are believed to be of lipid origin. Ripening of mango fruit (var. Alphonso) has been shown to be accompanied by an increase in glyceride content of the pulp, followed by changes in component fatty acids, particularly with respect to the ratio of palmitic to palmitoleic acids, and fruit aroma

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Table I. Incorporation of Sodium [2-^{14}C]Acetate into Different Fractions of Fatty Acids[a]

substrate	% distribution of ^{14}C in different fractions	% distribution of ^{14}C in component fatty acids						
		$C_{12:0}$	$C_{14:0}$	hydroxy fatty acid (?)	$C_{16:0}$	$C_{18:0}$	$C_{16:1}$	$C_{18:1}$
sodium [2-^{14}C]acetate	88.6 ± 2.60^{A}	0.86 ± 0.08	3.2 ± 0.12	27.5 ± 2.32	57.1 ± 3.14	10.8 ± 2.02		
	9.3 ± 1.06^{B}						40.8 ± 3.25	49.7 ± 2.83
	1.9 ± 0.09^{C}							

[a] Values are the average of five independent determinations ± standard deviation. (A) Saturated fatty acids, (B) monoenoic fatty acids, (C) polyenoic fatty acids. See details of the fractionation of fatty acid methyl esters on TLC in the Experimental Section.

(Bandyopadhyay and Gholap, 1973). Fatty acids, particularly palmitic acid, present in mango pulp have been reported to stimulate the activity of the citrate cleavage enzyme (Mattoo and Modi, 1970) and also of mango peroxidase (Mattoo and Modi, 1975) during fruit ripening. The mitochondrial fatty acids with 16 carbon atoms have recently been shown to undergo a significant change in correlation with the development of chilling injury of the mango fruit (Kane et al., 1978). Thus, the fatty acid components of mango pulp are presumed to play a significant metabolic role in the ripening fruit. The present paper reports the biogenesis of fatty acids in ripening mango (var. Alphonso) and the possible involvement of fatty acids in the development of characteristic aroma in the ripe fruit.

EXPERIMENTAL SECTION

Fully matured, unripe Alphonso mangoes were purchased from a local market and kept at ambient temperature (25–30 °C) for ripening. Half-ripe mangoes were selected according to color, flavor, and texture by five expert judges and were used for subsequent studies.

The sodium [2-^{14}C]acetate (sp act., 22.8 mCi/mmol) and chromatographically pure [1-^{14}C]palmitic acid (sp act., 15.25 mCi/mmol) were obtained from the Isotope Division, Bhabba Atomic Research Centre, (Bombay, India).

Incorporation of Label Substrate. At the stem end of half-ripe mango, 4 μmol (ca. 100 μCi) of an aqueous solution of sodium [2-^{14}C]acetate was carefully injected, and the fruit was ripened for up to 4 days at 25–30 °C. The procedures for extraction of pulp lipid, recovery of fatty acids after removal of unsaponifiables, and preparation of methyl esters were essentially followed as described earlier (Bandyopadhyay and Gholap, 1973). The methyl esters of fatty acids were then fractionated according to the degree of unsaturation on a silver nitrate impregnated preparative silica gel G plate, using petroleum ether (bp 40–60 °C)–benzene (65:35, v/v) as developing solvent (Morris, 1966). The bands were located under ultraviolet light after spraying the plate with 1% aqueous solution of sodium fluorescin, and each fraction after recovery from the plate with diethyl ether was assayed for radioactivity. These fractions were further separated into methyl esters of component fatty acids by reversed-phase, thin-layer chromatography (TLC) on a 10% paraffin impregnated kieselguhr G plate, using acetone–water (90:10, v/v) as developing solvent (Bandyopadhyay and Chakrabarty, 1968). The radioactivity of each fraction was determined after recovery from the plate with diethyl ether. A portion of the total fatty acid methyl esters was resolved into three fractions (R_f values of 0.94, 0.32, and 0.24, respectively) on a preparative silica gel G plate, using petroleum ether–diethyl ether (80:20 v/v) as developing solvent (Bandyopadhyay and Gholap, 1973). The radioactivity of each fraction after recovery was measured as above. A portion of the near-base fraction (R_f 0.24) thus obtained was acetylated with acetic anhydride in anhydrous pyridine. The reaction mixture after concentrating under high vacuum was rechromatographed along with the unacetylated fraction on a silica gel G plate, using petroleum ether–diethyl ether (60:40, v/v) as developing solvent, according to the method described by Jurriens and Oele (1965). The change in R_f value was noted. Both the acetylated and unacetylated fractions after TLC separation, followed by recovery from the plate, were subjected to IR analysis as well as radioactive assay. An authentic sample of 12-hydroxystearic acid methyl ester (Sigma Chemicals, USA) was used for identification of hydroxy fatty acid on the TLC plate.

In another set of experiments, 0.7 μmol (ca. 10 μCi) of an alcoholic solution of [1-^{14}C]palmitic acid was similarly injected in the half-ripe fruit. The fruit was incubated at ambient temperature for 48 h before the pulp was taken for analysis. According to the procedures detailed above, the fatty acids were recovered from the pulp and methylated. The methyl esters of fatty acids were subjected to preparative argentation TLC, and each fraction was assayed for radioactivity. A portion of each fraction was decarboxylated by the Schmidt reaction and the radioactivity of the liberated CO_2 was measured (Phares, 1951). Further, the near-base fraction representing polyenoic acid and possibly hydroxy fatty acid methyl esters obtained from argentation TLC were again separated on a preparative silica gel G plate, using petroleum ether–diethyl ether–acetic acid (70:30:3, v/v) as developing solvent. The three bands with R_f values of 0.84, 0.49, and 0.40, respectively, were located by exposing the plate to iodine vapors, and each fraction was recovered from the plate and assayed for radioactivity. Each of the subfractions was also subjected to IR analysis.

Radioactive Assay. The respective samples obtained either directly or from the TLC plate were counted for radioactivity in a Beckman scintillation counter, using 2,5-bis(5-*tert*-butyl-2-benzoxazolyl)thiophen in toluene (Stern et al., 1969).

IR Analysis. IR spectra were recorded on a Perkin-Elmer Infra Cord Model 237 and the sample was used as a thin film between sodium chloride windows.

RESULTS AND DISCUSSIONS

The distribution of ^{14}C in fatty acid fractions and component fatty acids of pulp lipid after argentation and reverse-phase TLC, using [2-^{14}C]acetate as a substrate, is depicted in Table I. The saturated fatty acid fraction gave a significantly higher count than the other fractions, and the maximum ^{14}C was detected in palmitic acid, indicating its preferential synthesis. The counts obtained in the monoene fraction were equally distributed in palmitoleic and oleic acids. In earlier studies on the changes in fatty acid composition of the pulp of ripening mango (var. Alphonso) (Bandyopadhyay and Gholap, 1973; Gholap and Bandyopadhyay, 1975), palmitoleic acid content was found

Table II. Distribution of ^{14}C in Different Fractions of Fatty Acids with [1-^{14}C]Palmitic Acid Incorporation[a]

substrate	% distribution of ^{14}C in different fraction[b]	% $^{14}CO_2$ recov	% distribution of ^{14}C in fraction C[c]		
			polyenoic fatty acids	hydroxy fatty acids	unidentified
[1-^{14}C]palmitic acid	67.9 ± 2.36[A]	89.2 ± 3.52			
	8.8 ± 1.02[B]	47.1 ± 2.67			
	23.3 ± 7.94[C]	89.7 ± 2.05	2.2 ± 0.42	83.5 ± 4.65	14.3 ± 1.68

[a] Values are the average of five independent determinations ± standard deviation. (A) Saturated fatty acids, (B) monoenoic fatty acids, (C) polyenoic fatty acids. [b] Fractionation of fatty acid methyl esters on argentation TLC. [c] Fractionation of fraction C on silica gel TLC. See details of the fractionation of fatty acid methyl esters on TLC in the Experimental Section.

to increase during ripening, while the content of palmitic acid remained practically constant. Hence, the increasing content of palmitoleic acid without apparent change in palmitic acid content in the ripening fruit could possibly be due to desaturation of the endogenous palmitic acid (Hitchcock and Nichols, 1971). Further, methyl esters of total fatty acids on TLC separation resolved into three fractions on the silica gel plate, where the maximum ^{14}C incorporation (88.6%) was accounted for in the major fraction (R_f 0.94), representing methyl esters of normal fatty acids. The near-base fraction (R_f 0.24) exhibited 10% of the total count, which on acetylation followed by rechromatography on a silica gel plate changed its R_f value to 0.87 without altering the level of its radioactivity. Thus, this fraction could be identified as hydroxy fatty acid(s) since it was also evidenced by IR analysis, which showed the appearance and disappearance of the characteristic absorption of a hydroxyl group at 2.8 μm before and after acetylation.

Table II represents the distribution of ^{14}C in different fatty acid fractions of pulp lipid obtained from [1-^{14}C]-palmitic acid incorporated Alphonso mango. On decarboxylation of individual fractions obtained from argentation TLC, about 90% of the label was recovered as $^{14}CO_2$ from both the saturated and polyene fractions, whereas only 50% recovery was obtained in the monoenoic fraction. The fact that more than 6% activity was recovered as $^{14}CO_2$ on decarboxylation of the monoenoic acid fraction suggested that monoenoic acids were not solely produced by de novo synthesis, but some degree of direct desaturation seemed to have taken place. Direct desaturation of exogenous palmitate and stearate in plant systems such as soybean cotyledons has been demonstrated (Inkpen and Quackenbush, 1969). If only desaturation was operative in the present studies, then at least 90% incorportation would have been obtained as $^{14}CO_2$ on decarboxylation of the monoenoic fraction. However, the 50% recovery of $^{14}CO_2$ for the monoenoic fraction suggested that the exogenous substrate ([1-^{14}C]palmitic acid) was perhaps degraded to acetyl-CoA, followed by resynthesis, allowing for the observed degree of randomization of the ^{14}C in the monoenoic fractions. Further, IR analysis of polyene fractions with the characteristic absorption at 2.8 μm indicated the presence of hydroxy fatty acids. This fraction on further fractionation on a silica gel G plate resolved into three major fractions. The maximum ^{14}C was recovered in the fraction (Table II) with a R_f value of 0.40; the fraction was identified as hydroxy fatty acids by comparing its R_f value to the R_f value (0.42) of authentic 12-hydroxystearic acid methyl ester and by IR analysis. Thus it appeared that hydroxylation of exogenous fatty acids might be operative.

Recent evidence (Angelini et al., 1973; Hunter et al., 1974) on the occurrence of C_6–C_{10} γ- and δ-lactones in the aromatic principles of ripe Alphonso mango suggested the contribution of fatty acids in mango aroma. Hydroxy fatty acids are believed to be immediate precursors of lactones in various foods (Maga, 1976), and, therefore, incorporation of ^{14}C in hydroxy fatty acid fractions seemed to be of further interest.

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