Swelling and Gelatinization of Cereal Starches. IV. Some Effects of Lipid-Complexed Amylose and Free Amylose in Waxy and Normal Barley Starches[1]

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ABSTRACT

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Amylose (AM) and lysophospholipid (LPL) contents were directly correlated in barley starches, but the linear regressions that described the relationships in waxy and nonwaxy starches were quite different. The data indicated that AM exists partially as lipid-complexed amylose (L·AM), with an LPL-to-L·AM ratio of 1:7 and partially as lipid-free amylose (F·AM). The ^{13}C-cross polarization/magic angle spinning-nuclear magnetic resonance (CP/MAS-NMR) spectra of the nonwaxy starches had a broad resonance with a chemical shift of 31.2 ± 0.4 ppm, which is characteristic of midchain methylene carbons of fatty acids in the solid state or in V-amylose (V-AM) complexes.

The extracted lipid was a viscous liquid that, when mixed with seven parts AM, did not give a discernible peak under the conditions used to acquire the solid-state spectra. However, when the lipid was complexed with AM, it gave a typical V-AM spectrum and a broad resonance at 31.8 ppm. This proves lipid complexed with L·AM existed in the native starches and was not an artifact formed subsequently from free LPL and F·AM. The intensity of the resonance was consistent with the LPL content of the starches. Independent supporting evidence was obtained by differential scanning calorimetry that showed a constant enthalpy for disordering of amylopectin, ΔH(AP), for all waxy and nonwaxy starches, regardless of L·AM content and, hence, no exothermic formation of L·AM during starch gelatinization. Twelve waxy barley starches used in this study contained 0.8–4.0% L·AM and 0.9–6.4% F·AM; six nonwaxy starches contained 6.1–7.2% L·AM and 23.1–25.0% F·AM. All starches had essentially identical AP structures, as shown by the chain lengths of debranched starches fractionated by gel-permeation chromatography and high-performance liquid chromatography. L·AM and F·AM appeared to have quite different effects on starch gelatinization behavior. Peak gelatinization temperature (T_p) of the waxy starches was positively correlated with L·AM content; because the T_p of the nonwaxy starches was much lower than predicted from the regression equation for the waxy starches, we concluded that F·AM lowered T_p. Swelling of starches at 80°C is essentially a property of AP content that is inhibited by LPL, but the relationship is not strictly linear. An improved equation to describe swelling properties assumed that 80% of the F·AM swelled with the AP fraction, although swelling was inhibited by L·AM$^{0.485}$.

A relationship was reported between amylose (AM) and lysophospholipid (LPL) contents in waxy barley starches that differed from the relationship describing normal (nonwaxy) barley starches (Tester and Morrison 1992). This led to the conclusion that AM may exist in two forms in these starches: as lipid-complexed amylose (L·AM) and as lipid-free amylose (F·AM). In this paper, we prove that L·AM does exist in native barley starches (i.e., it is not an artifact), and we show that L·AM and F·AM have different effects on the swelling and gelatinization properties of the granules.

MATERIALS AND METHODS

Starches

Twelve waxy and six nonwaxy starches from barleys grown in adjacent field plots, in the same year, at the Scottish Crop Research Institute (SCRI), Dundee, Scotland (Tester and Morrison 1992), were used for most of this work. Starch was isolated, as before, from Waxy Hector and Hector barleys taken at 20, 30, 40, and 50 days after anthesis.

Methods

Analytical methods were as described before (Morrison et al 1980; Tester and Morrison 1990a,b; Tester et al 1991) and included determination of total AM (on lipid-free starch) and apparent AM (on starch with native lipids) using a colorimetric method (Morrison and Laignelet 1983) with a revised calibration for barley AP (Tester and Morrison 1992). Differential scanning calorimetry (DSC) was used to obtain the onset, peak, and conclusion temperatures (T_o, T_p, and T_c) and the enthalpy (ΔH) of the endotherm for gelatinization, or disordering, of amylopectin (AP), as well as the T_p and ΔH of the endotherm for the dissociation of L·AM. Triplicate samples (3–4 mg of starch, 15 μl of water) were heated from 5 to 120°C at 10°C/min, using a Mettler DSC 30 with TC 10A processor. All results are given on a dry weight basis.

Cross polarization/magic-angle spinning (CP/MAS) ^{13}C-nuclear magnetic resonance (NMR) spectra of air-dried native starches were obtained at 25 and 75 MHz, using Bruker MSL 100 and 300 instruments, respectively. Over 20,000 scans were accumulated, enabling peaks from any complexed lipid to be identified. MAS speeds of ~4 kHz, a contact time of 1 ms, and a line broadening of 5 MHz were used with both field strengths. The low-field (25 MHz) spectra of physical mixtures and inclusion complexes of amylose with extracted-starch lipids and oleic acid were also obtained.

RESULTS AND DISCUSSION

AM and Lipid Relationships

Table I data describes the composition of the starches. AM and LPL contents were closely correlated for the waxy starches (Table II). Inclusion of data for six waxy starches from a previous study (Morrison et al 1986) gave similar regressions (n = 18). Extrapolation of regression *ii* (Table II) to 922 mg of LPL per 100 g (mean value for the six nonwaxy starches) gave a predicted value of 11.9% AM, which was 18.7% less than the actual mean value. There were insufficient samples to obtain a satisfactory regression for the nonwaxy starches. Inclusion of values for four Hector starches and 11 comparable nonwaxy starches from a previous study (Morrison et al 1986) gave a set of data that could be described by a regression line with a similar slope predicting 19.0% AM at 0 LPL (Fig. 1). This intercept value is nearly the same as the discrepancy in AM content obtained by the extrapolation described above; the results presented here show that it is caused by F·AM content.

These observations confirm that the range of AM contents in the waxy starches was not caused by starch from nonwaxy kernels; if it were, the nonwaxy starches would have fitted the waxy regression *i* (Table II) and the slope would have been 26.4 instead of 75.8. The results agree with McDonald et al (1991), although their regressions were not the same, partly because they determined AM using the original colorimetric calibration (Tester and Morrison 1992).

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Lipid-saturated complexes of V_6 AM with free fatty acids (FFA), averaging six parts helical AM with six glucosyl residues per turn to one part nonhelical AM, contain ~8% lipid (Karkalas and Raphaelides 1986; Raphaelides and Karkalas 1988). When the guest molecule is lysophosphatidylcholine (LPC), the complex contains from 10.2 (Acker and Becker 1971) to 12.5% lipid (Kugimiya and Donovan 1981). The difference (ΔAM) between total AM and apparent AM, determined colorimetrically, is a measure of lipid complexed with AM under assay conditions (Morrison and Laignelet 1983). Regression equation *iii* (Table II), predicting LPL from ΔAM for the waxy starches, gives a ratio of 149.9 mg of LPL per gram of AM, which corresponds to an AM complex containing 13.0% LPL. Using data for all waxy and nonwaxy starches described in this article and also from unpublished work, a very similar regression equation was obtained ($n = 42$, $r = 0.982$). Comparable regressions describe the data of Tester et al (1991). From all these regressions, a mean value of 12.5% LPL in the complex formed under colorimetric assay conditions was obtained.

The LPL in cereal starches contains ~70% LPC (Morrison 1988). Kugimiya and Donovan (1981) measured ΔH for the decomposition of L·AM, formed by adding egg yolk LPC to various starches in DSC experiments, and obtained a saturating ratio of one part LPC to seven parts AM, which also gives 12.5% LPC in the complex. This is not necessarily identical to the stoichiometry of the complex that may exist in native starch granules or to that of complexes prepared in the laboratory, but it does give acceptable conversion factors (LPL × 7.0 or fatty acid methyl esters [FAME] × 11.4) to obtain the amount of AM that could be complexed with lipid. This fraction is termed *lipid-complexed amylose* (L·AM), and the remainder is consequently termed *lipid-free amylose* (F·AM). It should be emphasized that the weights of L·AM given in this article refer to the polysaccharide only; LPL + L·AM is referred to as amylose-lipid complex.

There are several reports in the literature that some lipids and surfactants can interact with AP (e.g., causing altered rates of retrogradation). There is no evidence that lipids can form a true inclusion complex comparable to the L·AM complex (Kugimiya and Donovan 1981, Evans 1986) unless subjected to very unusual treatment (Slade and Levine 1987). We isolated pure barley AP by preparative GPC for colorimetric measurements using I_2/KI reagent, as for the determination of AM (Morrison and Laignelet 1983), and then added LPC at various levels, but it did not alter iodine binding and color development as it does with AM. The exceptionally low λ_{max} of barley AP (Tester and Morrison 1992) indicates that there are no long internal or external chain segments, which are characteristic of anomalous types of AP found in some other cereal starches (Morrison and Karkalas 1990). Thus, we consider that complexing of LPL with the short chains of barley AP, in solution or in the native starch granules, is most unlikely. This is consistent with the regression equation for the waxy starches that shows that lipid is negatively correlated with AP content, reaching a negligible value at 100% AP.

Calculated values for L·AM and F·AM are given in Table III together with ΔAM values for comparison with L·AM values. For the 12 waxy starches, 54 ± 5% of the total AM was L·AM (51% calculated from Table II, regressions *i* and *iii*). In the six nonwaxy starches, 21 ± 2% was L·AM. In nine comparable nonwaxy starches analyzed previously (Morrison et al 1986), there were 4.1–6.6% L·AM and 22.2–25.4% F·AM, giving 18 ± 3% L·AM in the total AM. The discrepancy of 18.7% in the extrapolated total AM content of the nonwaxy starches (Table II,

TABLE I
Composition and Properties of Starches from 12 Waxy and Six Nonwaxy Barley Varieties[a]

Barley Variety	Total Amylose (%)	Lipids as LPL[b] (mg/100 g)
Waxy		
Summire Mochi	1.7	120
Dango Mugi	2.1	169
Masan Naked	2.4	209
Tokushima Mochimugi (a)	3.1	246
Chalbori	3.4	233
Tokushima Mochimugi (b)	3.6	300
Iyatomi Mochi	3.9	296
Waxy Oderbrucker	5.2	397
Bozu Mochi	5.4	460
Wapana	6.5	480
Wanupana	6.5	493
Washonupana	7.4	569
Nonwaxy		
Chalky Glen	29.2	873
Midas	30.2	881
Hector	30.4	774
Shopana	30.5	1,000
Compana	30.5	1,032
Glen	32.7	969

[a] Taken from Tester and Morrison (1992).
[b] LPL = Lysophospholipid. LPL (mg) = (total starch phosphorus −2) × 16.16, where 2 is the correction for nonlipid Phosphorous.

TABLE II
Linear Regression Equations (form $y = A + Bx$) for Pairs of Variables Describing 12 Waxy Barley Starches

Line	Equation[a]	r	P
i	LPL (mg) = 7.7 + 75.8 total AM(g)	+0.990	<0.001
ii	Total AM = −0.02 + 12.9 LPL (g)	+0.990	<0.001
iii	LPL (mg) = 15.1 + 149.9 AM(g)	+0.982	<0.001
iv	$T_p = 54.60 + 0.780$ AM	+0.966	<0.001
v	$T_p = 54.67 + 9.851 \times 10^{-3}$ LPL	+0.935	<0.001

[a] Units are lysophospholipid (LPL) = mg or g/100 of g starch (as shown), AM = %, T_p = °C.

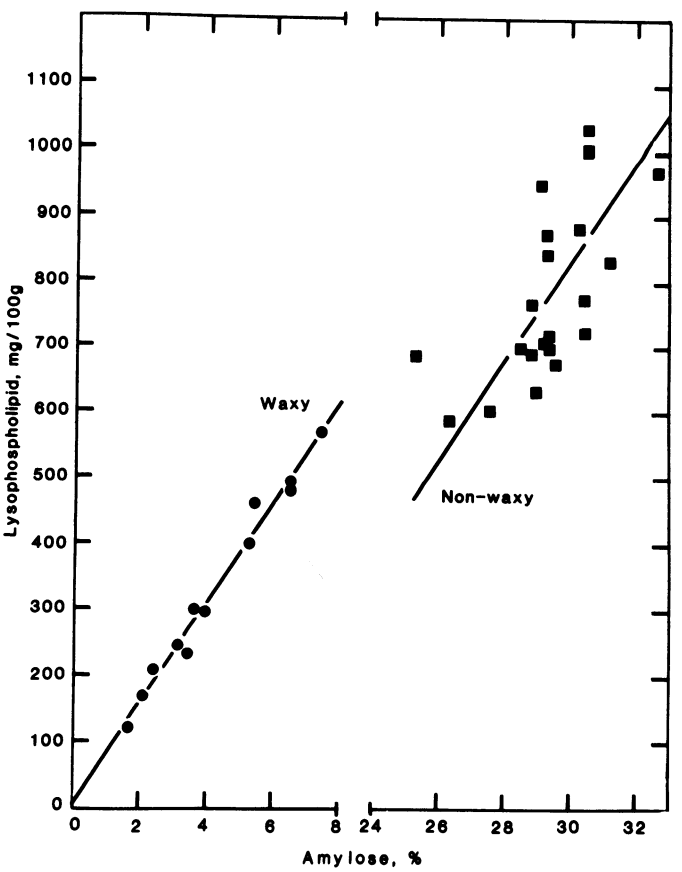

Fig. 1. The amylose-lysophospholipid (AM-LPL) relationship in starches from 12 waxy barleys (left) and 21 nonwaxy barleys (right). Regression equations for the waxy barleys are given in Table II (*i* and *ii*). The regression equation for the nonwaxy starches, including six starches in this study, is AM (g) = 19.0 + 13.3 LPL (g).

regression *ii*; LPL = 922 mg/100 g, mean value) is, in effect, the difference between the average F·AM content of the nonwaxy starches (24.1 ± 1.1%) and the extrapolated F·AM contents of the waxy starches (46% of 11.9% = 5.5%).

Evidence for L·AM and F·AM in Native Starch Granules

It is often assumed, because AM can complex with monoacyl lipids, that L·AM must exist in the native cereal starch granules, but available evidence has been ambiguous, and the point has been neither proved nor disproved (Morrison and Milligan 1982, Morrison 1988). Any experiment in which starch granules swell could cause L·AM to form (if it did not already exist) from free LPL and F·AM; this must be borne in mind when appraising evidence.

Figure 2 shows the low-field (25 MHz) ^{13}C-CP/MAS NMR spectrum of Chalky Glen starch. The spectrum contained a very weak resonance with a chemical shift of 31.2 ± 0.4 ppm that is characteristic of midchain methylene carbons of solid-state fatty acids or of V-AM complexes. This peak was also observed in the high-field (75 MHz) spectrum, but other regions of the aliphatic carbon chemical shift range were obscured by spinning sidebands from the dominant polysaccharide peaks. The concentration of the midchain methylene carbons, deduced from the intensity of the peak at 31.2 ppm, was 0.3 ± 0.05 mol % of carbon, compared with a value of 0.4 mol % calculated from the LPL content of the starch and the FA composition of the LPL. Similar results were obtained with other nonwaxy barley starches. When 60% of the starch (mostly AP) was removed by lintnerization, the spectrum of the insoluble residue showed essentially the same polysaccharide features and noise level, with considerable enhancement of the peak at 31 ppm assigned to methylene carbons of fatty acids in segments of acid-resistant L·AM (Morrison et al, *in press*).

The starch LPL have 50% *cis*-unsaturated FA (Tester and Morrison 1992), and the extracted lipid was a viscous liquid. Seven parts AM mixed with one part extracted-starch lipid (both essentially anhydrous) gave a typical spectrum (Fig. 3, top) for amorphous AM with a low content of helical order (Gidley and Bociek 1985, Veregin et al 1986, Horii et al 1987). Minor features between 20 and 40 ppm were not reproducible in their chemical shifts or intensity and were attributed to impurities. These minor features were invariably narrow peaks (4 ppm width) and could not be confused with the broad resonance from 21 to 38 ppm, peaking at 31–33 ppm, given by FFA in the solid state. Oleic acid and other *cis*-unsaturated, liquid FA mixed with seven parts AM gave no detectable lipid signals under the CP/MAS conditions.

When an inclusion complex of starch lipid with AM was prepared, it gave a typical spectrum for single-helix V-AM (Fig. 3, bottom) with narrowing of all polysaccharide carbon resonances (peaks B–E) and partial resolution of the C-3 resonance (peak C′), as reported previously (Horii et al 1987). There was also a broad resonance (21–38 ppm) peaking at 31.8 ppm with a characteristic profile downfield (peak A) assigned to midchain methylene carbons of fatty acids, showing that complexation had changed the acyl chains of the LPL from a liquid state to a near-solid state due to steric restraints within the V-AM helix. Resonances assigned to double-bond carbons at 130 ppm (peak F) and carboxylic ester carbons at 175 ppm (peak G) were likewise revealed. The spectrum of oleic acid complexed with AM showed the same general behavior.

In a nonwaxy starch granule containing only 6–7% L·AM (Table III), the characteristic V-AM features would not be evident in the spectrum of AP and F·AM, but the strongest lipid feature (Fig. 3, bottom, peak A) would be detectable as a weak resonance with the same strength and characteristic chemical-shift dispersion that we observed (Fig. 2). The minor features between 20 and 40 ppm attributed to impurities or artifacts (Fig. 3, top) would have been undetectable against background noise. From this, we concluded that most, if not all, of the LPL in Chalky Glen starch was complexed with L·AM and did not occur as free lipid.

In a supplementary experiment, native starch granules were ball-milled for 48 hr to destroy all crystalline order detectable by DSC, X-ray diffraction, and ^{13}C-NMR analysis (Cooke and Gidley 1992) and then extracted with methylene chloride, which is a good solvent for LPL. Methylene chloride does not extract lipid from amorphous (or crystalline) AM-lipid compexes at am-

TABLE III
Lipid-Complexed Amylose (L·AM) and Lipid-Free Amylose (F·AM) in Waxy and Nonwaxy Barley Starches

Variety	L·AM[a] (%)	ΔAM[b] (%)	F·AM[c] (%)
Waxy			
Summire Mochi	0.8	0.9	0.9
Dango Mugi	1.2	1.2	0.9
Masan Naked	1.5	1.3	0.9
Tokushima Mochimugi (a)	1.7	1.3	1.4
Chalbori	1.6	1.5	1.8
Tokushima Mochimugi (b)	2.1	1.5	1.5
Iyatomi Mochi	2.1	2.1	1.8
Waxy Oderbrucker	2.8	2.5	2.4
Bozu Mochi	3.2	3.0	2.2
Wapana	3.4	3.1	3.1
Wanupana	3.5	3.3	3.0
Washonupana	4.0	3.6	3.4
Nonwaxy			
Chalky Glen	6.1	6.4	23.1
Midas	6.2	6.4	24.0
Hector	5.4	6.1	25.0
Shopana	7.0	7.4	23.5
Compana	7.2	8.3	23.3
Glen	6.8	7.2	25.9

[a] Lypophospholipid. (LPL) × 7 × 10^{-3} = (total P-2) × 0.1129, where LPL (mg).
[b] Total AM − apparent AM (Tester and Morrison 1992).
[c] Total AM − L·AM.

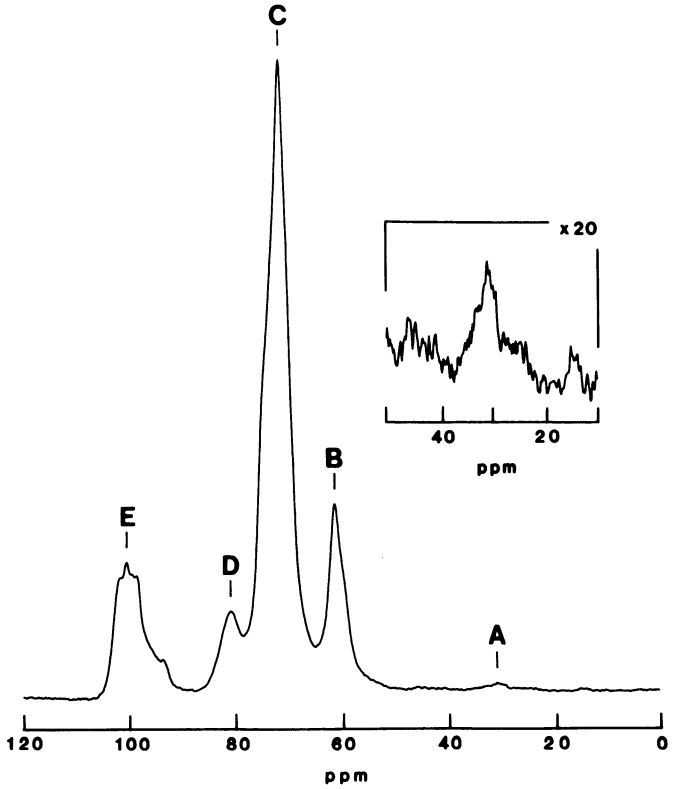

Fig. 2. The ^{13}C-cross polarization/magic angle spinning-nuclear magnetic resonance spectra (25 MHz) of Chalky Glen starch, with inset of the 10–50 ppm region at ×20 scale expansion. The marked resonance is for midchain methylene carbons of fatty acids in lysophospholipid with a chemical shift of 31.2 ppm (**A**). Resonances from polysaccharide carbons (**B–E**) are **B** = C-6 (61.3 ppm), **C** = unresolved C-2, C-3, C-5 (71.4 ppm), **D** = C-4 (80.9 ppm), **E** = C-1 (100.5 ppm) with some helical amylose (103 ppm).

bient temperature, but it should extract lipid that is not complexed. No trace of LPL was detected in extracts from the ball-milled starch, showing that it was still firmly retained in some amorphous structure, such as L·AM helices. Further evidence for the existence of L·AM in native starches was obtained from gelatinization enthalpy measurements.

AP in all the starches had essentially identical structures, as shown by the GPC elution profiles of the debranched structures

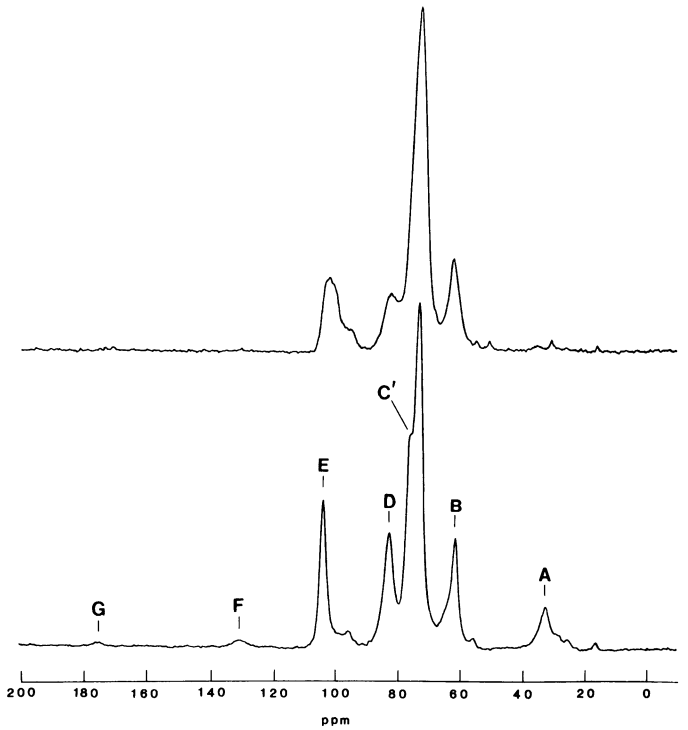

Fig. 3. The ^{13}C-cross polarization/magic angle spinning-nuclear magnetic resonance spectra (25 MHz) of seven parts amylose mixed with one part solvent-extracted starch lipid (top) and an inclusion complex of amylose with starch lipid (bottom). **A–E** are as in Fig. 2. **C'** = C-3 (~75 ppm), **F** = double-bonded carbons (130 ppm), **G** = carboxylic ester carbon (175 ppm).

(Tester and Morrison 1992). Because the parent barleys were all grown together and they were all exposed to the same temperature and growing conditions, their AP crystallinity should have been very similar; therefore, it was not likely to cause any variation in gelatinization enthalpy or in gelatinization temperature and swelling behavior.

Table IV shows that the gelatinization enthalpies of the waxy starches (13.0 ± 0.7 J/g) were appreciably greater than those of the nonwaxy starches (9.3 ± 1.1 J/g). However, when calculated on the basis of AP content (the source of ordered structures), the enthalpies, ΔH(AP), were nearly identical (13.55 ± 0.71 and 13.35 ± 1.50 J/g, respectively). Similar results were obtained before with four barley genotypes grown at 10, 15, and 20°C (Tester et al 1991) and with Riso mutants (Tester et al 1993). Because there were no significant effects of AM or lipids on enthalpy, other than by dilution of AP, AM and lipids probably did not affect the degree of ordering in AP but merely the ease of disordering, as shown by T_p.

A second implication arises from the constant ΔH(AP) values. If L·AM did not exist in native granules, the exothermic energy of complexation between AM and lipids during gelatinization would be masked by the greater endothermic disordering of AP over the same temperature range (Kugimiya et al 1980; Morrison and Milligan 1982; Biliaderis et al 1986 a,b; Morrison 1988; Biliaderis 1991), and the net enthalpy would be less. If this were correct, ΔH(AP) should have been inversely correlated with L·AM content. In practice, there was no correlation in the waxy starches ($r = -0.252$), in the nonwaxy starches ($r = -0.011$), or overall ($r = -0.134$), so the assumption appears to be wrong.

A comparison can also be made between experimental values for ΔH(AP) and calculated values for ΔH(AP) − ΔH(L·AM), assuming an enthalpy of 25 J/g of AM for the dissociation (endo) or formation (exo) of L·AM (Kugimiya and Donovan 1981, Raphaelides and Karkalas 1988, Biliaderis and Seneviratne 1990). The measured enthalpy is primarily a measure of disordering of single L·AM helices rather than crystalline structures; hence, ΔH for type I complexes is similar to ΔH for type II complexes (Biliaderis and Galloway 1989). Consequently, the polymorphic form of L·AM in the native starch granules is not important in this discussion.

In practice, ΔH(L·AM) for the six nonwaxy starches was difficult to measure accurately because of the uncertainty about the endotherm baseline, but it was ~21 J/g of L·AM. Similar

TABLE IV
Gelatinization or Disordering of Amylopectin and Dissociation of Lipid-Complexed Amylose (L·AM) Measured by Differential Scanning Calorimetry

	AP Endotherm					Predicted[a]	L·AM Endotherm	
Variety	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	ΔH(AP) (J/g)	Δ(AP) (J/g)	T_p (°C)	ΔH (J/g)
Waxy								
Summire Mochi	43.3	55.6	74.0	12.9	12.9	13.3	...	...
Dango Mugi	43.0	56.4	74.7	13.5	13.8	13.2	...	...
Masan Naked	40.7	56.1	74.0	13.3	13.6	13.2	...	...
Tokushima Mochimugi (a)	43.7	57.5	74.7	13.5	13.9	13.1	...	...
Chalbori	44.0	57.8	76.0	13.8	14.3	13.1	...	...
Tokushima Mochimugi (b)	47.2	57.5	74.0	12.9	13.4	13.0	...	...
Iyatomi Mochi	42.0	57.4	74.7	14.0	14.6	13.0	...	...
Waxy Oderbrucker	44.0	55.6	74.0	11.9	12.6	12.8	...	...
Bozu Mochi	44.5	58.2	76.7	12.8	13.5	12.7	...	...
Wapana	48.0	60.1	78.0	13.6	14.5	12.6	...	...
Wanupana	50.7	60.0	76.3	11.7	12.5	12.6	...	...
Washonupana	46.3	60.0	75.0	12.0	13.0	12.5	...	...
Nonwaxy								
Chalky Glen	43.3	53.9	69.3	9.1	12.9	11.4	96.1	1.2
Midas	42.2	53.1	70.0	8.4	12.0	11.3	94.4	1.0
Hector	42.3	53.8	70.0	10.4	14.9	11.6	95.8	1.5
Shopana	45.3	56.2	71.0	9.3	13.4	11.0	96.1	3.0
Compana	44.8	57.3	73.2	10.6	15.3	11.0	98.5	1.7
Glen	42.3	53.8	70.7	7.8	11.6	11.0	95.8	1.3

[a] Calculated as 13.55 − 25 L·AM/(100−T·AM), where L·AM is as in Table III, T·AM is total AM (Table I). 13.55(J/g) is an arbitrary value for the endothermic enthalpy of disordering AP in waxy barley starch (zero AM), and 25 (J/g AM) is the exothermic enthalpy for the formation of L·AM, assuming it equals the dissociation enthalpy as reported.

low values for dissociation of L·AM on first heating of native starches were reported previously (Kugimiya and Donovan 1981). Using 25 J/g of AM, we obtained the predicted values given for ΔH(AP) in Table IV.

For the 12 waxy starches, the calculated effect of L·AM was less than the error in measuring ΔH (0.6 J/g). Experimental values of ΔH(AP) were reasonably constant (13.55 ± 0.71 J/g), whereas predicted values decreased with increasing L·AM content, so that the two sets of data were not correlated ($r = 0.233$, $n = 12$), but the predicted values (mean 12.93 ± 0.27 J/g) were generally not significantly different from the experimental values.

For the six nonwaxy starches, experimental values of ΔH(AP) were more variable (13.35 ± 1.51 J/g) and very similar to the values for the waxy starches. The predicted values were significantly lower (mean 11.22 ± 0.26 J/g) and not correlated with the experimental values ($r = 0.179$). From this we conclude that there was no measurable exothermic effect from formation of L·AM during the endothermic disordering of AP; this is independent evidence that L·AM probably did exist in the native starch granules.

When similar previous studies were reexamined, the results no longer supported the original interpretation that L·AM is formed during starch gelatinization. Biliaderis et al (1986b) compared ΔH of waxy and nonwaxy rice starches without taking into account the lower AP content of the latter. When their data were recalculated as ΔH(AP), the low-GT waxy and nonwaxy starches had identical enthalpies, as did the high-GT starches, in agreement with the present work.

Numerous studies have shown exothermic formation of L·AM when lipids were added to any nonwaxy starch, but attempts to demonstrate the effects of removing native lipids by solvent extraction have given conflicting results. In some cases, partial annealing of the starch may have occurred, while in others there was a partial gelatinization of AP (shown by a large decrease in ΔH). Biliaderis (1991) concluded that such experiments are unreliable, and we agree. It is improbable that lipids can be removed quantitatively from barley or wheat starches without disturbing their crystallinity, and there is no guarantee that lipids added back would return to the sites occupied by the original native lipids, which are asymmetrically distributed within the native starch granules (Morrison and Gadan 1987, McDonald et al 1991).

Are L·AM and F·AM Separate Entities (Molecules)?

Lipid could be uniformly distributed throughout the amylose fraction so that each molecule in a nonwaxy starch would be about 21% L·AM and 79% F·AM, or it could be complexed with lipid-saturated L·AM molecules, leaving 79% of the total AM as lipid-free F·AM molecules. (Intermediate states are also possible.) To test these possibilities, leaching experiments were carried out with nonwaxy starches in the expectation that F·AM would be water-soluble and leachable, while L·AM would be stable until 94–98°C and insoluble (Karkalas and Raphaelides 1986, Raphaelides and Karkalas 1988). In practice, more material was lost by partial disintegration of starch granules than by selective leaching.

At temperatures up to 80°C, the starch granules lost 4–5% material with a composition almost identical to the original starches (69–71% AP, 6–7% L·AM, 23–25% F·AM), and there was little evidence of selective leaching of F·AM. From 85 to 95°C, there were progressively larger losses of starch (16–44% of the original weight, at 95°C, depending on the starch used) plus additional losses of F·AM (21–41% of the original F·AM content), presumably by selective leaching. When starches were incubated at 100°C, some L·AM dissociated into F·AM and LPL, leaving a residue (86–93% AP, 0–2.5% L·AM, 7–12% F·AM) that represented 42–67% of the original starch weight. Although the material lost into the supernatant at 90°C did not form a gel that excluded blue dextran dye (Tester and Morrison 1990a), it did not retain a structure like gelatinized starch or damaged starch gel. From this we conclude that the AP and F·AM in the supernatant were in true solution, and that the L·AM was probably in colloidal dispersion. This would explain why we found that the swelling of Oderbrucker barley starch decreased above 80°C, unlike starches from wheat and maize (Tester and Morrison 1990a), which were evidently less susceptible to disintegration.

We could find no way to fractionate starch to obtain a quantitative recovery of L·AM free from F·AM, or vice versa; therefore, we could not prove directly that AM exists exclusively in these two distinct forms rather than some mixed state(s). However, the very different ratios of L·AM/F·AM in the residues (0.44–0.70) and supernatants (0.14–0.19) at 90–95°C and in the original starches (0.26–0.28), show that AM does not exist as uniform molecules with constant proportions of L·AM and F·AM. That evidence, coupled with the fact that there was a constant ratio of L·AM-AP in all fractions at all temperatures up to 95°C, is good evidence that AM exists either as L·AM or as F·AM. There was no evidence for transfer of free LPL between fractions, except at 100°C when nearly all L·AM dissociated into F·AM and free LPL, which appeared in the water-soluble fraction.

Results presented in this article show that L·AM and F·AM have opposite effects on starch gelatinization temperature and on swelling at 80°C. This behavior can be explained in terms of separate L·AM and F·AM molecules but not by molecules with mixed L·AM-F·AM character. Also, uniform distribution of lipid throughout AM cannot be explained in simple biochemical terms (South et al 1991), but separate L·AM and F·AM molecules are compatible with established biochemical pathways when an additional stage to introduce lipid is invoked (Morrison, *in press*).

Amorphous Nature of L·AM and F·AM

In the nonwaxy starches, 79% of the total AM was F·AM, which is generally considered to be part of the amorphous polysaccharide in the granule (Banks and Greenwood 1975, French 1984, Blanshard 1987). However, the crystallinity of L·AM has not been discussed explicitly, although some evidence is available.

Models for L·AM show the AM molecule with single-chain V_6 helices enclosing single acyl chains of lipid, interspersed with short lipid-free regions with a more random conformation (Jane and Robyt 1984, Biliaderis and Galloway 1989) that are about one-seventh of the total AM (Karkalas and Raphaelides 1986). Crystallinity arises when the helices are packed in antiparallel arrays. Crystalline (type II) complexes give a V-type wide-angle X-ray diffraction pattern characteristic of single (V-helix) segments of AM enclosing monoacyl lipids or other guest molecules, but the spectrum of the amorphous (type I) complex does not have any special features (Seneviratne and Biliaderis 1991). Waxy and normal cereal starches give a simple A-type X-ray pattern from ordered regions of AP, with no trace of a V-pattern until the starches have been gelatinized (Eberstein et al 1980, Stute and Konieczy-Janda 1983, Blanshard 1987, Biliaderis 1991, Zobel 1992), although a mixed V + B pattern is given by some high-amylose mutants of maize (Zobel 1992). Thus, L·AM is either amorphous in waxy and normal native starch granules and only becomes crystalline during swelling and gelatinization, or crystalline L·AM is formed from free lipid and F·AM during gelatinization. The NMR and other results given in this article require the former explanation.

The dissociation temperature of type I (amorphous) L·AM, containing FFA or monoglyceride (MG) in excess water is 94–100°C; for type II (crystalline) complexes, it is 110–125°C. (Raphaelides and Karkalas 1988, Biliaderis and Galloway 1989, Biliaderis 1991, Seneviratne and Biladeris 1991). The dissociation endotherm of L·AM is normally <105°C in barley (Table IV) and in most other cereal starches (Eberstein et al 1980; Kugimiya et al 1980; Kugimiya and Donovan 1981; Stute and Konieczy-Janda 1983; Biliaderis et al 1986 a,b; Eliasson 1986). The DSC evidence thus complements the X-ray evidence and is compatible with the existence of amorphous type-I L·AM complexes in the native starch granules.

Gelatinization Temperature

The gelatinization endotherm is one measure of the progressive disordering of AP crystallites as starch granules swell when heated

in excess water (Biliaderis 1991, Cooke and Gidley 1992). However, although the enthalpy of gelatinization is closely associated with the level of AP double-helical order, the DSC endotherm is observed at temperatures significantly higher than that required for loss of granular birefringence (Cooke and Gidley 1992). Nondouble-helical regions within the granule are thus implicated in the control of gelatinization temperatures; therefore, it was of interest to examine the contributions of amorphous L·AM and F·AM to gelatinization temperatures. It should be remembered that it is most unlikely that there were any differences in AP structure and crystallinity of the samples that could have significantly altered gelatinization temperature or enthalpy (see discussion of evidence for L·AM based on enthalpy measurements).

For the waxy starches, T_p was positively correlated with AM content and with the closely correlated L·AM or LPL values (Table II, regressions *iv, v*). Predicted values for T_p of nonwaxy starches, taking mean values, were 78.5°C from AM content and 63.9°C from LPL content. As the actual values were only 53.1–57.3°C, these regression equations apply only to the waxy starches. Similar differences in T_p between waxy and normal Oderbrucker barley starches and between waxy and normal W64A maize starches were noted previously (Tester and Morrison 1990a). This means amorphous F·AM, which is potentially soluble, has a different effect on T_p than L·AM, which should be stable and insoluble over the whole AP gelatinization temperature range.

In the waxy starches, ~54% of the AM was in L·AM and ~46% in F·AM, but, in the nonwaxy starches, only ~21% of the AM was in L·AM and ~79% in F·AM. If the action of insoluble L·AM increased T_p, then F·AM must have decreased T_p by an almost equal amount in the nonwaxy starches; in the waxy starches, there was not enough F·AM to counteract the effect of L·AM. From regression equations for the waxy starches (Table II, *v*; others not shown), T_p would be increased by 0.985°C per 100 mg of LPL, by 0.999°C per 100 mg of lipid (from FAME), or by 1.407°C per gram of L·AM in 100 g of starch. Using the L·AM value, it follows that there is a decrease in T_p of 0.39°C (SD 0.05) for each gram of F·AM. The full equation describing peak gelatinization temperature is:

$$T_p = 54.67 + 1.407\ \text{L·AM} - 0.375\ \text{F·AM} \quad (1)$$

Confirmation of the effect of L·AM on T_p, independent of other variables, was obtained from a separate experiment (Tester and Morrison 1993). Starches from Waxy Hector, taken at 20, 30, 40, and 50 days after anthesis, had ranges of AM (3.1–6.7%) and LPL (310–560mg/100g) contents that were closely correlated (r = 0.954 and 0.990, respectively) with T_p (55.0–57.7°C). Four corresponding starches from normal Hector had parallel variation in AM (26.4–29.4%) and LPL (636–755mg/100g) contents, as well as T_p (54.0–55.9°C), from which it was calculated that:

$$T_p = 51.97 + 1.558\ \text{L·AM} - 0.193\ \text{F·AM} \quad (2)$$

Equation (2) resembles equation (1), but neither gave accurate predictive values of T_p for the other set of starches. The different temperature constants (54.67 and 51.97°C) indicate substantial effects of ambient temperature on the ordering of AP. (Waxy Hector and Hector were field-grown one year previous to the barleys grown for the present study.) We have also demonstrated the effects of ambient temperature on the ordering of AP, lipid content, and T_p using barleys grown in controlled environment chambers (Tester et al 1991).

When equations (1) and (2) were used with data for other barley starches, the results were unsatisfactory. We conclude that there was too much variation in the ordering of AP, coupled with independent variation in L·AM and F·AM, to be able to predict the effects of L·AM and F·AM on T_p in starches other than those grown and isolated under very closely controlled conditions (as for the waxy-nonwaxy sets discussed above). However, this does not invalidate the basic premise that L·AM increases T_p of AP and F·AM decreases it. Recent evidence (Cameron and Donald, *in press*) shows that the degree of hydration of the amorphous material (where F·AM is probably located) between crystalline lamellae of AP relates to the cooperative melting temperature.

Starch Granule Swelling

Swelling above T_p and T_c is considered a property of the intact AP molecule, with AM acting as a diluent of AP and lipid acting as an inhibitor of swelling (Tester and Morrison 1990a,b; Tester et al 1991; Tester and Morrison 1992). Consequently, the swelling factor of the AP fraction, SF(AP), is negatively correlated with lipid content. However, experimental data are not described adequately by a simple linear regression (despite very significant correlation coefficients); predicted values for waxy starches and high-amylose starches are invariably low. Data for the swelling factor at 80°C and for the composition of 38 starches from several studies (Tester et al 1991, Tester and Morrison 1992; Tester et al 1993), including the 18 starches used in the present study, were analyzed further. Regressions of SF(AP) on lipid content were not strictly linear, and the waxy and nonwaxy starches sometimes behaved as separate populations.

An adequate description was obtained when it was realized that F·AM might contribute to swelling, despite some F·AM leaching from the granules. If amorphous AM (i.e., F·AM) can initiate swelling below T_p (French 1984), it seems reasonable that the fraction that does not leach into solution would also contribute to swelling above T_p. We assumed that AP and F·AM retained within the granule would both swell, and we have shown that the fraction of F·AM leached at 80°C (Morrison et al 1986; Tester and Morrison 1990a), plus any starch released by granule disintegration, would not be measured. Swelling was then calculated for AP + f F·AM (where f = the effective fraction of F·AM). The inhibition caused by L·AM was determined by regression analysis. The data were described best (r^2 = 80%) by the equation:

$$\text{SF} = 41.1\ (\text{AP} + 0.8 \times \text{F·AM})\ (1 - 2.7\ \text{L·AM}^{0.485}) \quad (3)$$

where AP, F·AM, and L·AM are fractions of unity instead of percentages. This equation describes the very low AM and very high AM starches well, and it gives a more realistic value of 41.1 for the swelling factor of a barley starch comprised of pure AP (zero L·AM and F·AM) than do simple linear regressions (Tester et al 1991, Tester and Morrison 1992). The term (1–2.7 $\text{L·AM}^{0.485}$) means that the active part of the L·AM inhibits the swelling of 2.7 times its weight of (AP + 0.8 × F·AM). We have not measured the effects of added lipids on the swelling factor, so we cannot say whether they inhibit swelling in a linear or a nonlinear manner.

CONCLUSIONS

^{13}C-CP/MAS-NMR experiments prove that LPL in nonwaxy barley starch occurs as an inclusion complex with AM. This conclusion was supported by other independent evidence. It appears that AM in barley starches occurs as two fractions, L·AM and F·AM, that are probably separate types of molecules rather than molecules with mixed character. L·AM and F·AM have quite different effects when starch granules gelatinize and swell. In practice, the granules should be regarded as consisting of three types of polysaccharide: AP, L·AM, and F·AM. Similar considerations almost certainly apply to the other nonwaxy cereal starches, although definitive evidence for L·AM in them has yet to be obtained.

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