- Dodgson, K. S. & Spencer, B. (1956). *Rep. Progr. Chem.* **53**, 318.
- Gorini, L. & Maas, W. K. (1957). *Biochim. biophys. Acta*, **25**, 208.
- Harada, T. (1952). *J. agric. chem. Soc., Japan*, **26**, 95.
- Harada, T. (1959). *Bull. agric. chem. Soc., Japan*, **23**, 222.
- Harada, T., Kono, K. & Yagi, K. (1954). *J. agric. chem. Soc., Japan*, **28**, 337.
- Harada, T. & Spencer, B. (1960). *J. gen. Microbiol.* **22**, 520.
- Hilz, H. & Kittler, K. (1958). *Biochim. biophys. Acta*, **30**, 650.
- Hilz, H., Kittler, K. & Knape, G. (1959). *Biochem. Z.* **332**, 151.
- Hockenhull, D. J. D. (1949). *Biochim. biophys. Acta*, **3**, 326.
- Horiuchi, T., Horiuchi, J. & Mizuni, D. (1959). *Nature, Lond.*, **183**, 1529.
- Magasanik, B. (1955). *J. biol. chem.* **213**, 557.
- Monod, J. (1941). Thesis: University of Paris.
- Monod, J. & Cohen-Bazire, G. (1953). *C.R. Acad. Sci., Paris*, **236**, 530.
- Nakamura, T. & Sato, R. (1960). *Nature, Lond.*, **185**, 163.
- Neuberg, C. & Korono, K. (1923). *Biochem. Z.* **140**, 295.
- Peck, H. (1960). *J. biol. Chem.* **235**, 2734.
- Schlossman, K. & Lynen, F. (1957). *Biochem. Z.* **328**, 591.
- Shepherd, C. J. (1956). *J. gen. Microbiol.* **15**, 29.
- Spencer, B. & Harada, T. (1960). *Biochem. J.* **77**, 305.
- Torriani, A. (1960). *Biochim. biophys. Acta*, **38**, 460.
- Wainwright, T. (1961). *Biochem. J.* **80**, 27f.
- Whitehead, J. E. M., Morrison, A. R. & Young, L. (1952). *Biochem. J.* **51**, 585.
- Wilson, L. G. & Bandurski, R. S. (1958). *J. Amer. chem. Soc.* **80**, 5576.

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Studies on the Biosynthesis of Starch Granules

2. THE PROPERTIES OF THE COMPONENTS OF STARCHES FROM SMOOTH- AND WRINKLED-SEEDED PEAS DURING GROWTH*

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Mature pea starches have been reported to contain more amylose than the 20–25 % usually present in root or cereal starches, there being about 35 % in smooth-seeded and about 70 % in wrinkled-seeded varieties (McCready, Guggolz, Silveira & Owens, 1950). Work on these starches by Potter, Silveira, McCready & Owens (1953) indicated that, although the amylose components were similar, the amylopectin component from a wrinkled-seeded variety had an abnormal length of unit-chain of 36 glucose units and a smaller molecular size than that from a smooth-seeded pea. These hitherto unconfirmed results suggest that either some radical alteration has occurred in the balance of the synthesizing enzymes, or a new type of branch-synthesizing enzyme exists.

In the work described here the components from mature smooth- and wrinkled-seeded pea starches have been characterized by measurements of extents of conversion by β -amylase, iodine absorption, periodate oxidation and molecular size. These properties have been compared with those of typical root and cereal starches. Further, in an attempt to gain some insight into the method of biosynthesis of the starch granules, the properties of pea starch and its component amylose and amylopectin during growth have been studied.

* Banks & Greenwood (1959) is regarded as Part 1.

EXPERIMENTAL

Isolation of starches. Freshly gathered peas were exhaustively extracted with 0.01M-HgCl₂ and then deproteinized as described by Banks & Greenwood (1959). (It has been found that the starch in dried peas can be similarly extracted and purified if the peas are initially soaked overnight in 0.01M-HgCl₂ at room temperature.) The purified starches were then defatted with boiling aqueous 80 % methanol. The varieties of pea investigated and the properties of the isolated starches are given in Table 1.

Development of granules. Peas were harvested at various stages of growth and graded in size by sieves before the starch was extracted as above.

Table 2 shows the properties of the resultant materials. Percentages of starch were estimated by the method described by MacWilliam, Hall & Harris (1956). Gelatinization temperatures were measured as described by Banks & Greenwood (1959).

Fractionation of starches. Fractionation was achieved by both conventional dispersion and aqueous-leaching experiments (Banks & Greenwood, 1959; Banks, Greenwood & Thomson, 1959). In agreement with the work of Potter *et al.* (1953), pea starches would not disperse directly into water and pretreatment with liquid NH₃ was necessary. This technique causes no modification of the molecular size or β -amylolysis limit of amylose (Banks *et al.* 1959).

The purity of the separated components was measured as described by Banks & Greenwood (1959). β -Amylolytic limits were measured with purified soya-bean β -amylase

and also with mixtures of β -amylase and Z-enzyme (Banks *et al.* 1959). Extinction measurements on polysaccharide-iodine complexes were made with a Unicam SP. 600 spectrophotometer under the conditions described by Hassid & McCready (1943). The average length of unit-chain for the amylopectin fractions was determined by periodate oxidation with NaIO_4 at 2° as described by Banks & Greenwood (1959).

Measurement of molecular size. The methods used to measure limiting viscosity numbers ($[\eta]$) and sedimentation coefficients (S_{20}) of the components in alkali have been described (Banks & Greenwood, 1959). In collaboration with Dr W. Banks, weight-average molecular weights ($\bar{M}_w$) of some amylopectin fractions were obtained from light-scattering measurements on the polymers dissolved in water. Measurements were made with a Brice-Phoenix-

Table 1. *Varieties of pea and properties of mature starches*

Iodine affinities are expressed as mg. of I_2 bound/100 mg. of starch; amylose is calculated from (iodine affinity/19.0) $\times$ 100.

Variety	Pea		Starch		
	Type	Starch (%)	Nitrogen (%)	Iodine affinity	Amylose (%)
British Lion	Smooth-seeded	43	0.04	6.6	35
Kelvedon Monarch	Smooth-seeded	42	0.04	6.5	34
Alaska*	Smooth-seeded	—	0.06*	7.1*	34.5*
Gradus	Wrinkled-seeded	32	0.03	12.5	66
Gladstone	Wrinkled-seeded	31	0.02	12.4	65
Onward	Wrinkled-seeded	32	0.02	12.4	65
Perfection*	Wrinkled-seeded	—	0.24*	13.3*	66*

* Results quoted by Potter, Silveira, McCready & Owens (1953).

Table 2. *Properties of amylose fractions obtained by successive aqueous leaching of the granule followed by dispersion*

Procedures I–IV were as follows: granules were successively leached with water for 10 min. at 70° (I), 80° (II) and 90° (III). The residue was then treated with liquid NH_3 (Banks, Greenwood & Thomson, 1959) and dispersed in water for 1 hr. at 100° (IV). All experiments were carried out in N_2 . Amount of amylose extracted is calculated from iodine-titration experiments. S, Smooth-seeded pea; W, wrinkled-seeded pea.

	Procedure	Amylose extracted (%)	$[\eta]$	β -Limit*
British Lion (S)	I	2	110	100
	II	3	125	88
	III	4	170	83
	IV	91	190	80
Kelvedon Monarch (S)	I	2	95	100
	II	3	120	90
	III	3	170	81
	IV	92	195	80
Potato (var. Redskin)†	I	35‡	250	99
	II	—	—	—
	III	—	—	—
	IV	50	570	75
Gradus (W)	I	2	105	100
	II	2	115	89
	III	3	130	83
	IV	93	150	79
Gladstone (W)	I	2	95	100
	II	3	110	91
	III	4	125	83
	IV	91	145	79
Barley (var. Ymer)§	I	26	89	96
	II	15	146	74
	III	20	246	65
	IV	39	272	62

* Expressed as percentage conversion into maltose.

† Results from Banks *et al.* (1959).

‡ This leaching was at 60° . A further aqueous leach at $63\text{--}56^\circ$ extracted another 15% of the amylose ($[\eta] = 320$; β -limit = 82%).

§ Results from Greenwood & Thomson (1959).

photometer (model 1000D) with cylindrical cells and the narrow diaphragm system. Solvent was filtered through sintered-glass (G5) directly into the light-scattering cell. Amylopectin solutions (approx. 0.6%) were centrifuged for 1 hr. at 15 000 rev./min., and successive samples of the clarified solution were added to a weighed amount of solvent in the cell to form a concentration series in the range $(1-20) \times 10^{-5}$ g./ml. The angular distribution of scattered light was measured over the range $20-45^\circ$ at $546 \text{ m}\mu$. The light-scattering data were treated by the method of Zimm (1948). The results obtained confirmed the work of Jones (1959), who showed that, for the above experimental conditions, the concentration dependence of (c/R_θ) as a function of $\sin^2(\theta/2)$ was negligible. (Here, c is the concentration; R_θ the Rayleigh ratio measured at the angle θ .)

RESULTS AND DISCUSSION

Properties of the mature granular starches

Preliminary studies on several varieties of peas (see Table 1) confirmed the results of earlier workers (McCready *et al.* 1950; Potter *et al.* 1953) that smooth-seeded varieties contained more starch (42–43%) than wrinkled-seeded varieties (31–32%). Although it has been suggested that pea starches are extremely difficult to deproteinize (Senti & Dimler, 1959), no difficulty has been found with the method of Banks & Greenwood (1959).

Fig. 1. Typical potentiometric iodine-titration curves for pea-starch products. Conditions: $[I^-]$, 0.01M; pH, 5.85; temperature, 20° . Δ , British Lion amylose; $\bullet$, Gradus starch; $\circ$, British Lion starch; $\blacksquare$, British Lion amylopectin.

For all starch samples, the nitrogen content was reduced to 0.04% or less without treating the granules with any chemical reagents that might have modified the structure of the starch. This purification is comparable with that achieved for other laboratory-extracted starches (cf. Clendenning & Wright, 1945; Greenwood, 1960). Potentiometric iodine-titration experiments showed that the amylose content of the starches from smooth-seeded varieties was 34–35%, whereas that in the wrinkled-seeded varieties was as high as 65–66%. As shown in Table 1, these results have confirmed the earlier observations in the literature. Typical potentiometric iodine-titration curves are shown in Fig. 1. For all the pea-starch samples, although the amylose content varied so much between the two types, the average gelatinization temperature (i.e. the temperature when 50% of the granules were gelatinized) was greater than 98° . As a result the starches would not disperse in boiling water and their behaviour on aqueous leaching was abnormal. This gelatinization behaviour is uncommon. We have examined a large number of starches from a wide variety of botanical sources (C. T. Greenwood & J. Thomson, unpublished work), but have found nothing comparable.

The granules of the starches from smooth-seeded varieties on microscopic examination were found to be 'simple', whereas those from wrinkled-seeded varieties appeared to be essentially 'compound'. The morphology of the pea-starch granule is obscure, however, and it is not certain whether true 'compound' granules exist (see Badenhuizen, 1959). This problem is discussed further below.

Aqueous leaching and dispersion of mature granules

Starches from two varieties of smooth-seeded pea (British Lion and Kelvedon Monarch) and two varieties of wrinkled-seeded pea (Gradus and Gladstone) were selected for more detailed study. The properties of the starch subfractions obtained by successive aqueous leaching and complete dispersion of the granules were examined.

Leaching of typical cereal and tuber starches with water at $60-70^\circ$ has been shown to result in the preferential extraction of some 30–40% of short-chain, linear amylose (Banks *et al.* 1959; Greenwood & Thomson, 1959). With all the pea starches, however, the amount of extracted amylose was only 2–3% of the total. Indeed, even after leaching successively at 70° , 80° and 90° , more than 90% of the total amylose remains inaccessible. The remarkable contrast in behaviour of the pea starches compared with that of potato and barley starch is shown in Table 2. This suggests a profound difference in granular structure, which is independent of the percentage of amylose. The

Table 3. *Properties of components from dispersion experiments on mature starches*

Starches after treatment with liquid NH₃ (Banks *et al.* 1959) were dispersed in boiling water at 100° for 1 hr. under N₂. β-Amylolysis limits were measured as the percentage conversion into maltose on treatment with (i) β-amylase, and (ii) β-amylase + Z-enzyme. S, Smooth-seeded pea; W, wrinkled-seeded pea.

Starch ...	...	...	...	Pea						Potato	Barley
Variety	...	...	...	British Lion	Kelvedon Monarch	Alaska*	Gradus	Gladstone	Perfection*	Home Guard†	Ymer‡
Type	...	...	...	S	S	S	W	W	W	—	—
Amylose											
Iodine affinity (%)				19.0	19.0	19.0	19.0	18.9	18.7	19.5	19.0
β-Amylolysis limit (i)				81	80	—	82	81	—	83	72
β-Amylolysis limit (ii)				100	100	—	100	101	—	—	98
[η] (ml./g.)				180	170	176	160	130	134	450	240
Degree of polymerization (7.4 [η]; Cowie, 1958)				1300	1300	770	1200	1000	650	3300	1800
Amylopectin											
Iodine affinity (%)				1.0	1.0	1.6	2.7§	2.7§	—	0.1	0.38
Purity (%)				95	95	92	86	86	—	99.5	98
β-Amylolysis limit (i)				58	57	—	65	66	—	56	58
[η] (ml./g.)				150	140	157	120	120	122	160	188
Apparent chain-length				26	27	26.6	36	35	36	24	25.5
Apparent internal chain-length¶				8	9	—	10	9	—	8	8

* Results from Potter *et al.* (1953). † Results from Banks & Greenwood (1959).
‡ Results from Greenwood & Thomson (1960). § See text.
|| Calculated from iodine titrations. Typical percentages of nitrogen were: British Lion, 0.06; Gradus, 0.04. This percentage will not interfere with periodate oxidation (Anderson & Greenwood, 1955).
¶ Calculated from [chain-length - {(chain-length × β-limit) + 2.5}]; results corrected to nearest whole number.

structural difference precludes the easy preferential extraction of material, which is linear as shown by its complete β-amylolysis.

As the pea starches would not disperse directly into water, pretreatment of the granules was necessary. This was successfully accomplished with liquid ammonia (Potter *et al.* 1953; Banks *et al.* 1959). The properties of the resultant components are shown in Table 3, together with comparative results for potato and barley starch.

The iodine affinity and the β-amylolysis limits for the amyloses from both the smooth- and wrinkled-seeded pea starches were similar. The incomplete conversion into maltose under the action of β-amylase was not due to contaminating amylopectin, as, under the concurrent action of β-amylase and Z-enzyme, conversion was complete. The latter provides a sensitive test for the purity of amylose (Banks, 1960). The values for β-amylolysis limits were similar to that found for potato starch, but higher than that for barley (see Table 3). There was a tendency for the limiting viscosity number for the amyloses from smooth-seeded pea starches to be larger than that for the wrinkled-seeded samples. This is discussed further below. Our values for viscosity are similar to those of Potter *et al.* (1953).

Potentiometric iodine titration showed that the amylopectin components were more impure than is usual (see Table 3 for potato and barley amylo-

Fig. 2. Potentiometric iodine-titration curves for pea amylopectins. Conditions were as for Fig. 1. ●, Original Gradus amylopectin (see text); ▲, British Lion amylopectin; ○, Gradus amylopectin after treatment with methanol (see text).

pectin). The titration curves for the British Lion and Kelvedon Monarch amylopectins were normal, but the corresponding ones for the Gradus and Gladstone samples possessed an abnormally large slope for the linear portion of the curve (see Fig. 2). This made estimation of the amount of contaminating amylose difficult; the value quoted is minimal. As a result, the corrections applied for the amylose content in both β-amylolysis experiments and chain-length determinations are not

known with any high accuracy for the latter samples (see below).

The values for β -amylolysis limit (57–58%) and chain-length (26–27 anhydroglucose units) for the amylopectins from the smooth-seeded peas are comparable with those reported earlier for other amylopectins (e.g. barley in Table 3). The corresponding values of 65–66% conversion into maltose and an apparent chain-length of 35–36 anhydroglucose units for the wrinkled-seeded samples are abnormal, but agree with the earlier unconfirmed report (Potter *et al.* 1953). Calculation showed that, within experimental error, the internal chain-lengths of all samples were the same. This suggests, in conjunction with the extra apparent amylose, the presence of a new or modified branching enzyme in wrinkled-seeded varieties of pea. These samples were examined therefore in detail.

*Properties of amylopectins from
mature pea starches*

The absorption spectra of the amylopectin-iodine complexes were measured under the conditions described by Hassid & McCready (1943). The curves for the amylopectins from wrinkled-seeded peas were abnormal. The result for the Gradus sample in Fig. 3 shows that the maximum of the curve was at 5750 Å compared with 5500 Å for the British Lion amylopectin and 6800 Å for amylose. A mixture of amylopectin of normal chain-length and the same amount of amylose as appeared to contaminate the Gradus amylopectin gave a completely different type of absorption curve (see Fig. 3). The characteristics of the absorption curve did not appear therefore to be accounted for by the presence of contaminating amylose. A comparable result has been shown for the abnormal amylopectin reported to be present in maize of high amylose content (Wolff, Hofreiter, Watson, Deatherage & MacMasters, 1955).

However, when the amylopectins of apparently abnormal chain-length were studied in alkaline solution in the ultracentrifuge, they were found to be unhomogeneous. [A preliminary account of this result has been given elsewhere (Greenwood & Thomson, 1960)]. The Gradus and Gladstone amylopectins both showed a rapidly moving component (S_{20} approx. 100×10^{-13}) together with material with a very low sedimentation coefficient ($S_{20} < 1 \times 10^{-13}$). In contrast, the British Lion and Kelvedon Monarch amylopectins were homogeneous.

Differential ultracentrifuging of the Gradus and Gladstone samples separated the two components. Table 4 shows the results together with a control experiment on British Lion amylopectin. The sedimented material from both '36-unit' amylopectins had a chain-length and β -amylolysis limit comparable with that for the British Lion amylo-

Fig. 3. Absorption curves for pea amylopectins. Conditions were as in the Experimental section. ○, Original Gradus amylopectin; ●, mixture of British Lion amylose and amylopectin containing 20% of the linear component (see text); △, Gradus amylopectin after differential ultracentrifuging (see text); ■, British Lion amylopectin.

Table 4. *Some properties of the amylopectins and subfractions of the amylopectins from pea starches*

Aqueous solutions (0.2%) of the amylopectins (obtained from a dispersion) were spun for two periods of 2 hr. at 44 000 rev./min. in the preparative rotor of the Spinco model E ultracentrifuge. Material was isolated from the supernatant liquor by freeze-drying. Apparent purity of the amylopectin fraction was obtained by iodine titration. W, Wrinkled-seeded pea; S, smooth-seeded pea.

Pea	Type	Component	Amount (%)	Apparent purity (%)	Chain-length calc. from apparent purity	β -Limit	$[\eta]$
Gradus	W	Original	100	86	36	65	120
		Sediment	80	88	27	57	150
		Supernatant	20	—	—	100	30
Gladstone	W	Original	100	86	35	66	120
		Sediment	—	91	28	58	140
		Supernatant	—	—	—	100	—
British Lion	S	Original	100	95	26	58	150
		Sediment	—	97	27	57	160
		Supernatant	—	—	—	—	—

pectin when corrections were made for the apparent amylose impurity. Further, the iodine-absorption curve was normal (cf. Fig. 3). The properties of the British Lion amylopectin were effectively unaltered on differential ultracentrifuging, although some amylose was removed. The materials isolated from the supernatant liquors of the Gradus and Gladstone samples were each completely degraded into maltose by β -amylase and had a very low limiting viscosity number; they appeared to be degraded, short-chain, linear amyloses. No comparable material was isolated from the supernatant liquors from the British Lion sample.

The above results suggested that the observed low iodine affinities for the amylopectins from wrinkled-seeded peas may have been due to residual fatty acids (cf. Schoch & Williams, 1944; Mikus, Hixon & Rundle, 1946); the fissured and irregular shape of the granules might well prevent complete defatting by our normal procedure. When samples of the Gradus pea starch and the component amylopectin were re-extracted with boiling methanol for 12 hr., material was extracted which in carbon tetrachloride solution yielded a spectrum, in a Perkin-Elmer Infracord spectrophotometer, which was very similar to that for authentic samples of stearic and arachidic acids. Further, the methanol-extracted amylopectin on potentiometric iodine titration yielded a curve with less of a slope and a corresponding higher iodine affinity than that for the original sample (see Fig. 2); the apparent chain-length was then 32 glucose units, compared with 36.

It seems therefore that the apparently abnormal amylopectins occurring in wrinkled-seeded varieties of peas are artifacts arising from the presence of residual fatty acids and small amounts of short-chain amylose in amylopectins of normal chain-length. It is unnecessary to suggest that a radical upset has occurred in the balance of the normal starch-synthesizing enzymes; nor has another 'chain-lengthening' enzyme to be postulated.

Molecular weights of the components from mature starches

Molecular weights (M) for the amylose samples from British Lion and Gradus starches were obtained by use of the relation:

$$M = \left[\frac{N\eta_0}{\beta(1-\bar{v}_\rho)} \right]^{\frac{1}{3}} (S^0)^{\frac{1}{3}} \left(\frac{[\eta]}{100} \right)^{\frac{1}{3}}$$

(Scheraga & Mandelkern, 1953). Here, η_0 is solvent viscosity; N , Avogadro's number; $\bar{v}$, partial specific volume of solute; ρ , solvent density; S^0 , sedimentation coefficient at infinite dilution; $[\eta]$, limiting viscosity number; β , a constant. The value of β is related to the molecular shape: for rigid

spheres, $\beta = 2.12 \times 10^6$; for rigid ellipsoids of axial ratio 1:6, $\beta = 2.28 \times 10^6$; for random coils in good solvents, $\beta = 2.5 \times 10^6$. Hence if a value for β of 2.25×10^6 is used, the value of $M^{\frac{1}{3}}$ will have about 10% accuracy, and the actual value of M will be accurate to 15% without making any assumptions regarding molecular shape.

The results of measurements made on aqueous solutions of the amyloses were: British Lion amylose: $[\eta]$, 150; S_{20} , 6.6×10^{-13} ; M , 3.9×10^6 ; degree of polymerization, 2400.

Gradus amylose: $[\eta]$, 120; S_{20} , 5.8×10^{-13} ; M , 2.9×10^6 ; degree of polymerization, 1800. [A value for $\bar{v}$ of 0.62 was used (Cowie, 1958).]

These values for the degree of polymerization are much larger than earlier values in the literature (see Table 3), because the latter were number-average values and our calculated ones are more nearly weight-average values.

As has been stressed in the first paper in this series (Banks & Greenwood, 1959), the determination of the molecular weight of amylopectin is difficult. The earlier values reported in the literature (Potter *et al.* 1953) are not likely to be very accurate as osmometry experiments on such high-molecular-weight materials are difficult. Further, the probable presence of contaminating short-chain amylose in the original sample of amylopectin from wrinkled-seeded peas would make molecular-weight determinations inaccurate. In this work, viscosity measurements (Table 3) indicated that the amylopectins from all varieties were comparable. This conclusion was confirmed by light-scattering measurements made by Dr W. Banks. Molecular weights of 1.5×10^9 and 1.4×10^9 for the Gradus and British Lion amylopectins respectively were obtained. These values are comparable with those of our measurements on other amylopectins (W. Banks & C. T. Greenwood, unpublished work), and will be discussed in detail elsewhere.

Growth experiments

Table 5 shows that, with increase in maturity, there is an increase in the amount of starch present in both British Lion and Gradus peas. The properties of the isolated granular starches also shown in this Table indicate that with increase in maturity there is an increase in (i) granule size, (ii) average gelatinization temperature, and (iii) the percentage of amylose. As mentioned earlier (Banks & Greenwood, 1959), there is no simple relation between these three values. On microscopic examination, there were obvious differences between the granules isolated at different stages of growth. The granules in the smooth-seeded British Lion starches were oval, but somewhat irregular structures, which simply increased in average size

with maturity. However, in the earlier stages of growth the granules of the wrinkled-seeded *Gradus* starches were small and round (there was no evidence of compound granules) but these appeared during the final stages. In the mature *Gradus* starch, as well as 'compound' granules, there were a large number of small spherical granules. Further, some granules showed evidence of enzymic attack and the larger ones were deeply fissured. Evidence for the existence of true 'compound' granules in wrinkled-seeded varieties of pea is, in fact, conflicting (see Badenhuizen, 1959).

Properties of starch components during growth

The starch samples isolated during growth were fractionated. The properties of the separated components are shown in Table 6.

The complete conversion of the amylose fractions into maltose under the concurrent action of β -amylase and Z-enzyme shows that they are not contaminated with amylopectin. Consequently, the results show that as the pea starches mature, the β -amylolysis limit of the amylose decreases, i.e. there is an increase in the extent of the natural barrier to β -amylase in amylose with maturity. The

apparent β -amylolysis limit for any sample of amylose probably depends therefore on the maturity of the botanical source of the starch. The nature of this natural barrier has been discussed elsewhere (Greenwood, 1960; Banks & Greenwood, 1961). Since we established that Z-enzyme (the enzyme capable of effectively removing the barrier) is α -amylolytic and therefore non-specific in nature (Banks, Greenwood & Jones, 1960), the problem of establishing the identity of the barrier is made more difficult.

Also, during growth, the size of the amylose increases as shown by the increase in limiting viscosity number. The apparent molecular size of any sample of amylose is dependent, therefore, on the sample-maturity, and it is unlikely that amylose samples from different varieties of the same botanical source will be found to have the same molecular weight.

The amylopectin components isolated from the smooth-seeded peas show small differences in chain-length and β -amylolysis limit with increase in maturity (see Table 6). The significance of this is not known. The samples from the wrinkled-seeded pea show a gradual increase in both β -amylolysis limit and chain-length. Calculation shows that the

Table 5. *Properties of the granular starches obtained during growth*

Average granular size is estimated from photomicrographs; average gelatinization temperature is the temperature at which 50% of the granules are gelatinized; amylose is calculated from iodine affinity as in Table 1. S, 'Simple'; C, 'compound'; S/C, mixture of 'Simple' and 'Compound'.

	Pea		Starch				
	Diameter (mm.)	Starch (%)	Sample	Average granule size (μ)	Granule type	Average gelatinization temp.	Amylose (%)
British Lion	2-6	22	S1	10	S	62°	15
	6-9	26	S2	15	S	81	19
	9-11	32	S3	30	S	92	29
	11-12	40	S4	40	S	> 98	37
Gradus	2-6	12	W1	7.5	S	64	23
	6-9	16	W2	12	S	83	36
	9-11	22	W3	20	S/C	97	59
	11-12	31	W4	30	S/C	> 98	69

Table 6. *Variation in properties of starch components during growth of peas*

Sample numbers refer to Table 5.

Sample	...	...	...	...	S1	S2	S3	S4	W1	W2	W3	W4
Amylose												
β -Limit (i)*					90	86	79	74	90	82	80	74
β -Limit (ii)*					100	101	100	100	100	100	100	101
$[\eta]$ (g./ml.)					145	160	195	210	120	130	135	150
Amylopectin												
Purity (%)					98	96	95	92	90	89	86	83
Apparent chain-length					24	26	26	27	29	31	33	37
Apparent β -limit (i)*					54	56	56	58	58	60	64	66
Apparent internal chain-length†					9	9	9	9	10	10	10	10

* Percentage conversion into maltose on treatment with (i) β -amylase and (ii) β -amylase and Z-enzyme.

† Calculated as in Table 3.

apparent internal chain-lengths remain constant and this could imply the action of an enzyme capable of forming longer branches on normal amylopectin. However, the experiments described above show that the presence of the short-chain amylose accounts for these results. It is therefore likely that the amount of this short-chain amylose increases with maturity. How far this is related to the microscopic appearance of apparent enzymic attack on the granules is not known. The presence of degraded amylose may be due to limited α -amylolytic activity, or a partial inhibitor for amylose synthesis may be present. In the first case, preferential α -amylolysis of amylose might well occur as it does during the malting of barley (Greenwood & Thomson, 1959).

Biosynthesis of the starch granule

Little is yet known of the biosynthesis of starch granules. Electron-microscope studies (Whistler & Thornberg, 1957) have shown that granules originate in the amyloplasts. Wolf, MacMasters, Hubbard & Rist (1948) studied the general properties of maize-starch granules during growth, and observed an increase in average granule size, and an apparent increase in amylose content with maturity. Harris & MacWilliam (1958) have shown that, during the growth of barley, the starch content increased from 2 to 66 %, whereas the apparent amylose content of the starch increased from 14 to 26 %. A similar increase in amylose-content during the growth of maize has been reported by Erlander (1960). Tracer techniques have been used to study the formation of wheat starch (McConnell, Mitra & Perlin, 1958) and potato starch (Badenhuizen & Dutton, 1956). In agreement with these earlier results, our studies on pea starches show that an increase in granular size, gelatinization temperature and percentage of amylose component occurs with increase in maturity.

There are few studies on the actual changes in fine structure of the components during growth. Such studies are important as they may show whether any changes in enzymic activity have occurred. Further, there is no evidence as yet that the physical properties of starch products synthesized *in vitro* are exactly comparable with those of the natural product. Our earlier results (Banks & Greenwood, 1959) indicated that, in potato starch, increase in maturity may result in an increase in molecular size and a decrease in the β -amylolysis limit for the amylose component. This pattern has been confirmed for all the different pea starches studied here.

The starch from smooth-seeded and wrinkled-seeded peas also forms useful material for genetic experiments. Kellenbarger, Silveira, McCready &

Chapman (1951) have already reported cross-breeding experiments that showed that both starch content and the amylose content obeyed Mendelian laws, and that the differences are probably due to a single gene pair. However, our studies suggest that the starch in the wrinkled-seeded pea is probably subjected to abnormal α -amylolytic activity, and the factors controlling this are not yet known.

SUMMARY

1. Starches have been isolated from mature smooth-seeded and wrinkled-seeded varieties of pea, and their physical and chemical properties have been compared.

2. The properties have been examined of: (i) the subfractions of amylose obtained on aqueous leaching of the granules of the mature starches; and (ii) the component amylose and amylopectin obtained on dispersion.

3. The amylose components had properties similar to those of other amyloses. The amylopectins from smooth-seeded peas had chain-lengths of 26–27 glucose units and β -amylolysis limits of 57–58 %, whereas those from wrinkled-seeded varieties had chain-lengths of 35–36 glucose units and β -amylolysis limits of 65–66 %.

4. The amylopectins from wrinkled-seeded peas could be separated by differential ultracentrifuging into normal amylopectins of chain-length 26–27 glucose units, and contaminating short-chain amylose.

5. Molecular-weight values are reported for the amylose and amylopectin components of some mature starches.

6. Starches have been separated from wrinkled-seeded and smooth-seeded varieties of pea at various stages of growth, and their properties have been examined.

7. A comparison has been made of the properties of the components separated from the starches at various stages of growth. With increase in maturity the amylose component increases in molecular size and the β -amylolysis limit decreases; the characteristics of the amylopectins appear to alter very little, but the amount of contaminating short-chain amylose in the wrinkled-seeded peas increases.

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REFERENCES

- Anderson, D. M. W. & Greenwood, C. T. (1955). *J. chem. Soc.* p. 225.
Badenhuizen, N. P. (1959). *Protoplasmatologia*, vol. 2B, section 2b8. Wien: Springer-Verlag.

- Badenhuizen, N. P. & Dutton, R. W. (1956). *Protoplasma*, **47**, 156.
- Banks, W. (1960). Ph.D. Thesis: University of Edinburgh.
- Banks, W. & Greenwood, C. T. (1959). *Biochem. J.* **73**, 237.
- Banks, W. & Greenwood, C. T. (1961). *Chem. & Ind.* p. 714.
- Banks, W., Greenwood, C. T. & Jones, I. G. (1960). *J. chem. Soc.* p. 150.
- Banks, W., Greenwood, C. T. & Thomson, J. (1959). *Makromol. Chem.* **31**, 197.
- Cledenning, A. L. & Wright, D. E. (1945). *Canad. J. Res.* **23 B**, 131.
- Cowie, J. M. G. (1958). Ph.D. Thesis: University of Edinburgh.
- Erlander, S. R. (1960). *Cereal Chem.* **37**, 81.
- Greenwood, C. T. (1960). *Stärke*, **12**, 169.
- Greenwood, C. T. & Thomson, J. (1959). *J. Inst. Brewing*, p. 346.
- Greenwood, C. T. & Thomson, J. (1960). *Chem. & Ind.* p. 1110.
- Harrie, S. & MacWilliam, I. C. (1958). *Cereal Chem.* **35**, 82.
- Hassid, W. Z. & McCready, R. M. (1943). *J. Amer. chem. Soc.* **65**, 1154.
- Jones, I. G. (1959). Ph.D. Thesis: University of Edinburgh.
- Kellenbarger, S., Silveira, V., McCready, R. M. & Chapman, J. L. (1951). *Agron. J.* **43**, 337.
- McConnell, W. B., Mitra, A. K. & Perlin, A. S. (1958). *Canad. J. Biochem. Physiol.* **36**, 985.
- McCready, R. M., Guggolz, J., Silveira, V. & Owens, H. S. (1950). *Analyt. Chem.* **22**, 1156.
- MacWilliam, I. C., Hall, R. D. & Harris, G. (1956). *J. Inst. Brewing*, p. 226.
- Mikus, F. F., Hixon, R. M. & Rundle, R. E. (1946). *J. Amer. chem. Soc.* **68**, 1115.
- Potter, A. L., Silveira, V., McCready, R. M. & Owens, H. S. (1953). *J. Amer. chem. Soc.* **75**, 1335.
- Scheraga, H. A. & Mandelkern, L. (1953). *J. Amer. chem. Soc.* **75**, 179.
- Schoch, T. J. & Williams, C. B. (1944). *J. Amer. chem. Soc.* **66**, 1232.
- Senti, F. R. & Dimler, R. J. (1959). *Food Tech., Champagne*, **63**, 663.
- Whistler, R. L. & Thornberg, W. L. (1957). *J. agric. Food Sci.* **5**, 203.
- Wolf, M. J., MacMasters, M. M., Hubbard, J. E. & Rist, C. E. (1948). *Cereal Chem.* **25**, 312.
- Wolff, I. A., Hofreiter, B. T., Watson, P. R., Deatherage, W. L. & MacMasters, M. M. (1955). *J. Amer. chem. Soc.* **77**, 1654.
- Zimm, B. H. (1948). *J. chem. Phys.* **16**, 1093.

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Biochemistry of Methanol Poisoning

4. THE EFFECT OF METHANOL AND ITS METABOLITES ON RETINAL METABOLISM*

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Methanol poisoning in man, a problem of considerable toxicological interest, is characterized by an initial stage of depression of the central nervous system, which is followed by metabolic acidosis and the specific toxic effect of the oxidation product(s) of methanol on retinal cells which leads to visual degeneration. It has been claimed that swelling of the retinal ganglion cells and the rods and cones, with sparing of the optic nerve and tract, is commonly seen in human cases of methanol poisoning (Fink, 1943; Duke-Elder, 1954). However, very little information is available on the biochemical aspects of this lesion.

It is generally accepted that formaldehyde is the toxic agent in methanol poisoning. The long asymptomatic latent period of 8–36 hr. and the beneficial effects of administered ethanol, which

probably acts by inhibiting the oxidation of methanol (Roe, 1946), indicate that a metabolite of methanol is probably responsible for the various manifestations of poisoning. Further support for this contention comes from the observation that formaldehyde is an extremely potent inhibitor of respiration and glycolysis in ox retina; formate exercises only weak respiratory inhibition, and methanol has no effect even at a concentration of 20 M (Leaf & Zatman, 1952; Potts & Johnson, 1952). These observations on the relative magnitude of effects of methanol and its oxidation products on the metabolism of the retina *in vitro* have been corroborated by studies on the electroretinogram (Praglin, Spurney & Potts, 1955). Potts & Johnson (1952) observed that the enzymic process most susceptible to formaldehyde inhibition was anaerobic glycolysis and stated that the specific site of inhibition is the retinal hexokinase.

Cooper & Marchesi (1959) found that formaldehyde inhibited aerobic glycolysis in ox retinal homogenates with glucose as the substrate, but not

* Part 3: Kini & Cooper (1961).

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