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Small-angle Neutron Scattering Studies of Starch Granule Structure

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SUMMARY

Starch granules from the potato, wheat, barley, millet, waxy maize, mung bean, smooth pea and wrinkled pea have been examined as slurries in D_2O by the technique of small-angle neutron scattering (SANS). All starches except that from wrinkled peas exhibit a Bragg peak at 100 Å approximately but this disappears on gelatinization. The Bragg peak is believed to arise from alternating amorphous and crystalline regions. By the use of different D_2O/H_2O mixtures, the isopicnic point was shown experimentally to occur at 52% D_2O w/w which was close to that calculated theoretically (50·4% D_2O w/w). Examination of native and defatted wheat starches in an isopicnic D_2O/H_2O mixture showed evidence of a lipid peak at 152–167 Å. On the basis of this evidence and that from Debye-Scherrer broadening of the 100 Å peak it is proposed that the lipid occurs radially. Further evidence in support of these dimensions and conclusions comes from Guinier analyses of the gelatinized starch granules. Using the racemose model for amylopectin, then each raceme was found to fit optimally the SANS scattering data when it was assumed to be either a squat cylinder or ellipsoid with dimensions of 150 × 60 Å.

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INTRODUCTION

The structure of the starch granule, which is the major source of metabolic energy for the human body, presents many enigmatic features despite being the subject of investigation for the past century. The supermolecular structure of this polymer spherulite is still a matter of debate and, therefore, not surprisingly, it is not apparent why starches from separate botanical sources display differing gelatinization behaviour.

The granule itself is a chemical and physical mixture. Two principal polysaccharide components are present: the linear amylose which is an α-(1→4)-glucan of DP = 1000–5000 and the branched amylopectin which contains short, linear α-(1→4)-glucan chains of DP = 15–20 with α-(1→6) branch points. Cereal starches also contain lipid as a minor component. Despite the percentage by weight of lipid being small ($\leqslant$1%), as a molar percentage it is large and is thought to exert a marked effect on gelatinization properties (Hart & Blanshard, 1982). The physical state is one of admixed crystalline and amorphous regions, the former being, it is believed, due to the branched amylopectin. There is no firm evidence about the state or position of lipid in the native granules.

Although considerable effort has been expended in exploring the crystalline structure of starch granules by wide-angle X-ray scattering techniques (French, 1983) the corresponding use of small-angle X-ray or neutron scattering to study long range order has been very limited (SAXS – Sterling, 1962) or non-existent (SANS).

The purpose of this study was to examine in a preliminary fashion the architecture of the granule using small-angle neutron scattering, to determine what evidence there is for long range order and to investigate whether the lipid component in cereal studies could be detected at this low concentration.

Theory of neutron scattering in semicrystalline polymers

By analogy with Bragg's law for crystal lattices, a periodicity within a scattering sample leads to a maximum in the intensity of the scattered neutrons. The intensity, I, is plotted against a scattering vector, Q, which is defined as $Q = 4\pi \sin\theta/\lambda$ where 2θ is the scattering angle and

λ the neutron wavelength. Using the Bragg relationship $n\lambda = 2d \sin\theta$ (and, since, for first order scattering $n = 1$), then if d is the repeat distance, it is evident that

$$d = 2\pi/Q \tag{1}$$

Therefore the repeat spacing may be directly found from the SANS plot of I versus Q.

At small angles ($R_g Q < 1$), Guinier's law applies and the scattering intensity for a particle is given by

$$I(Q) = I_0 \exp(-R_g^2 Q^2/3) \tag{2}$$

where I_0 is the scattering intensity at zero angle and R_g is known as the radius of gyration. A plot of $\ln I$ versus Q^2 (the so-called Guinier plot) permits one to calculate R_g from the slope.

These basic scattering phenomena are observed with both neutrons and X-rays but the information derived by their study can be extended by the exploitation of some of the distinctive features of neutrons. Although in X-ray diffraction the intensity of the diffracted X-ray is dependent on the electron density within the regular lattice planes which give rise to the reflections (atoms in these planes having the larger atomic numbers scatter more), in neutron scattering an atom has a coherent scattering length b_j and a molecule with n atoms of coordinates r_j has a scattering length density given by

$$\rho(r) = \frac{1}{v} \sum_{j=1}^{n} b_j \delta(r - r_j) \tag{3}$$

The contrast, $\bar{\rho}$, between a particle and its solution (or matrix) determines the intensity of scattering. If a material appears homogeneous in terms of neutron scattering it will not of course scatter. This contrast is given by:

$$\bar{\rho} = \bar{\rho}_p - \rho_s \tag{4}$$

where $\bar{\rho}_p$ is the mean scattering length density of the particle and ρ_s that of the solvent (Stuhrmann, 1982).

The great advantage of neutron scattering for biological systems is that the coherent scattering lengths of different isotopes may be different and, in particular, those of the isotopes of hydrogen (protium

and deuterium) are markedly different. Therefore with mixtures of H_2O and D_2O a very wide range of ρ_s values and thus of contrast can be obtained. The practical value of the above theory will be illustrated in the observation of scattering by lipid in the wheat starch granules.

EXPERIMENTAL

Isolation and modification of starch granules

Samples of both potato, wheat and millet starches were kindly supplied by Dr M. O. Ahmed. In all cases the method of isolation that had been employed was that of Adkins and Greenwood (1966) with minor modifications. A solution of 0·01 M mercuric chloride was used to inhibit enzyme activity, while residual surface protein was removed by prolonged shaking of the granules in an aqueous saline suspension composed of 1 volume of toluene and 8 volumes of saline (specific gravity = 1·07407). The starch granules were freeze dried. The large and small barley starch granules had been kindly supplied by Dr A. W. MacGregor (Canadian Grain Commission, Winnipeg).

Wheat starch was lintnerised, to varying degrees, by exposure to 2·2 N HCl (1 g HCl 100 ml^{-1}) at ambient temperature (*c.* 20°C) for the specified times.

The defatted wheat starch sample was prepared by Soxhlet extraction with methanol for 30 h. A sample of this was refatted using stearic acid according to the method of Ohashi *et al.* (1980).

Preparation of starch samples for SANS

The experiments were performed on either the D11 spectrometer at the Institut Laue-Langevin, Grenoble, France, or on the 7HIR spectrometer at the Atomic Energy Research Establishment, Harwell, England. Details of the apparatus have been published elsewhere (Ibel, 1976; Baston & Harris, 1978).

Samples were prepared as slurries in H_2O, D_2O or H_2O/D_2O mixtures. Normally samples were vacuum-dried in a desiccator and then slurried again with the same solvent, to ensure that exchange had occurred. To

determine whether this drying cycle had affected the results, one sample of potato starch was slurried three times with 100% D_2O, left to exchange for 1 h after each slurrying and the excess D_2O decanted off; no drying was performed in this instance.

The final slurries were placed in either 1 or 2 mm quartz cells which were sealed to prevent loss of water. Corrections for non-linearity of the detector were made using D_2O as an incoherent scattering standard.

SANS experiments

The following series of experiments were conducted:

(i) A range of starches (potato, wheat, millet, barley, waxy maize, smooth and wrinkled pea and mung bean) were examined, principally as slurries in D_2O, a few samples in H_2O and some in the dry state using the 7H1R spectrometer at Harwell. The run time on average was 7 h.

(ii) Samples of millet and potato starches in D_2O were heated *in situ* in the cell until gelatinization had occurred as evident through the sample becoming translucent. The sample was thereafter examined using the 7H1R spectrometer. Again the run time was 7 h.

(iii) The contrast variation behaviour of a series of samples containing slurries of potato starch in D_2O/H_2O mixtures over the range 100–0% D_2O was studied. These samples were examined using the D11 spectrometer, each exposure time being approximately 10 min. After subtraction of background the intensities of the scattering maxima were corrected for monitor efficiency. The square root of intensity was plotted against solvent composition and a straight line obtained which passed through zero on the $\sqrt{I(Q)}$ axis at 52% D_2O on the composition axis. Thus 52% D_2O (w/w) was found to be the isopicnic point (Fig. 1).

(iv) Wheat starch slurries of both native and defatted granules were examined in an isopicnic (52% D_2O : 48% w/w H_2O) mixture using the D11 spectrometer. The run time of each was approximately 4 h. The same experiment was repeated on the 7H1R spectrometer at Harwell with run times of 24 h.

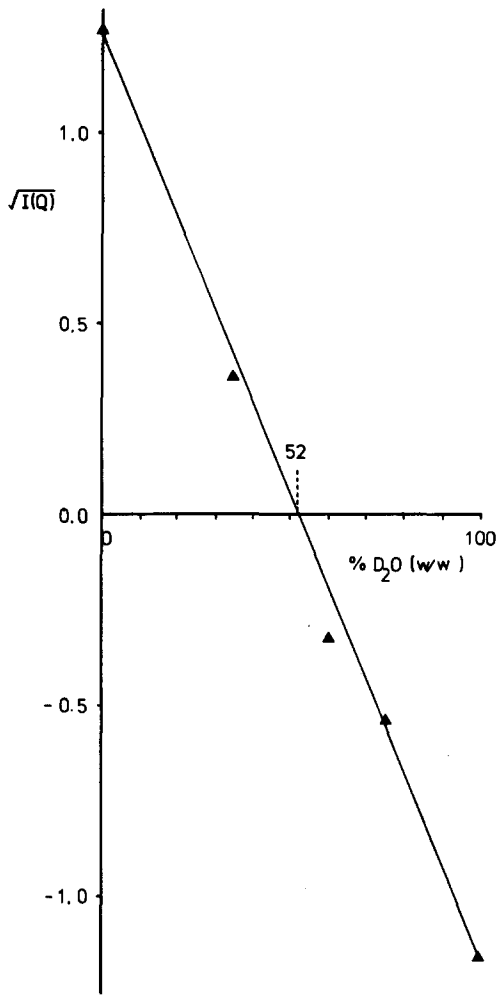

Fig. 1. Plot of the square root of the corrected scattering intensity of SANS from slurries of potato starch in H_2O/D_2O mixtures versus composition of the mixtures.

RESULTS AND DISCUSSION

(i) The nature and significance of the Bragg peak at *c.* 100 Å

Native starch granules of potato, wheat, millet, barley (both large and small granules), waxy maize, smooth pea and mung bean all showed

Bragg peaks in their SANS pattern when examined as slurries in D_2O. No peak, however, was observed with wrinkled pea starch. Similarly, starches in H_2O also showed the same peak though with a reduced intensity. However, when investigated in the dry state no peak was evident. Examples of the results for potato and wheat slurries in D_2O are shown in Fig. 2. The repeat spacings obtained from the Q values at the peak positions are listed in Table 1 and in some cases a Guinier plot was possible using the scattering intensities at very low angles; the ensuing radii of gyration are also listed in Table 1.

It can be seen that in general the value is about 100 Å, but there is a significant difference between that of the potato starch, which has a

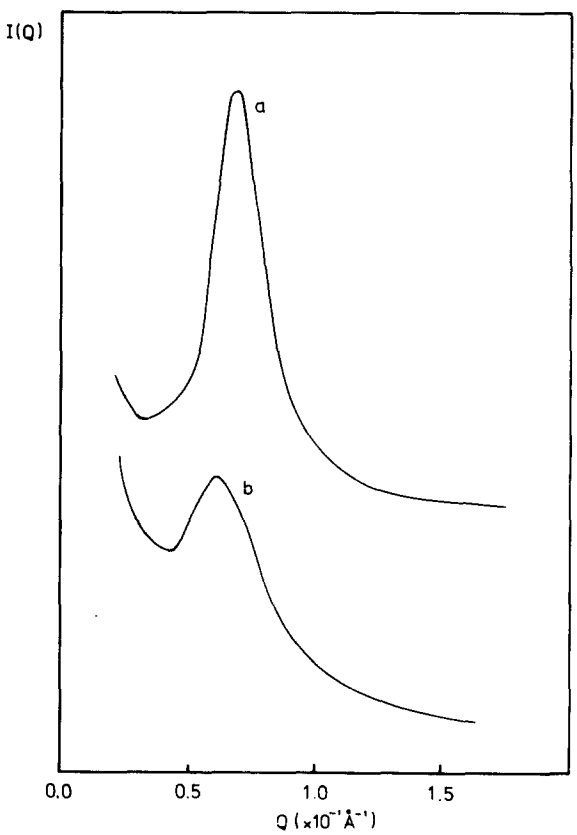

Fig. 2. SANS from (a) potato and (b) wheat starch granules in D_2O.

TABLE 1

Bragg Distances, d, and Radii of Gyration, R_g, Calculated from SANS Data Collected at AERE, Harwell and ILL, Grenoble from Various Starches

Material	*d (Å)*		R_g *(Å)*
Potato starch granules (in H_2O and D_2O)	92	50	(one Guinier plot obtained)
Gelatinized potato starch in D_2O	–	48	
Dry potato starch granules	–	57	
Wheat starch granules in D_2O	101, 103	55	
Wheat starch granules grown under diurnal light conditions	102	–	
Wheat starch granules grown under continuous light conditions	102	–	
Large and small barley starch granules	105	–	
Millet starch granules	100	–	
Gelatinized millet starch granules	–	53·1	
Dry millet starch granules	–	92	
Amylopectin solution	–	61·6	
Waxy maize starch granules[a]	101	–	
Smooth pea starch granules[a]	101	–	
Mung bean starch granules[a]	101	–	
Wrinkled pea starch granules[a]	–	–	

[a] Results kindly obtained by Dr D. L. Wild, Nottingham University.

repeat distance of 92 Å, and those of wheat, barley, waxy maize, smooth pea, millet, and mung bean where the d value is some 10 Å higher. Within these cereal starches there is some variation, but this is confined to within a 5 Å range (millet, 100 Å; wheat, 101–103 Å; barley (both large and small granules), 105 Å). The Bragg peak of the potato starch sample which had been exchanged with D_2O but without involving freeze-drying was identical both in its intensity and position. The absence of the Bragg peak in wrinkled pea starch is most interesting but reflects its rather different behaviour and structure (Colonna *et al.*, 1982).

It was only after conducting these experiments that our attention was drawn to the work of Sterling (1962) who had employed small-angle X-ray scattering to study starch granules and observed this same

periodicity and modest differences between potato and wheat starches.

The nature of this periodicity and the reasons for its variation in different species are not immediately clear. It is, however, interesting that although a periodicity is observed in the granules when the granules are presented as slurries in D_2O, or H_2O, no such peak is observed in the dry state (when dehydrated over P_2O_5, wide-angle X-ray crystallinity also vanishes, though both WAXS, SAXS and this periodicity reappear on sufficient hydration). Our conclusion is therefore that the 100 Å periodicity depends on a differential scattering density, presumably arising from a higher concentration of water (either D_2O or H_2O) in regions which alternate with a periodicity of 100 Å. It is interesting to note that recent models of amylopectin (Robin *et al.*, 1975) have on the basis of biochemical evidence suggested that the amylopectin chains (which are responsible for the crystalline regions) are of the order of 60 Å in size. If we assume that the amylopectin chains are largely radially oriented and that amylose is present to a level of 25% and is largely present in the intervening amorphous regions, then a repeat distance of 90–100 Å can be readily foreseen. Such a view is, however, in conflict with the results of Kassenbeck (1978) who used a scanning electron microscope to study enzymically treated granules and reported a repeat distance of 70 Å. Similarly Yamaguchi *et al.* (1979) have observed a periodicity of 70 Å along the molecular axis.

French (1983) has attempted to reconcile the SAXS results of Sterling (1962) with those of Kassenbeck (1978) and his own group by postulating that the 100 Å figure is the mean of the 147 Å dimensions reported by Hizukuri & Nikuni (1957 – see below) and this 70 Å figure. We believe, however, that the sharpness of the Bragg peak at 90–100 Å precludes the suggestion that it is the smeared average of two peaks at 70 and 147 Å. Furthermore, since the preparation of samples for electron microscopy is known to introduce artefacts, there is justification for treating the 70 Å figure with some reserve, and it is therefore proposed that the 100 Å peak represents a distinct periodicity which we would suggest is in the radial direction.

(ii) The use of contrast variation for locating the position of lipid

The fact that an isopicnic point could be determined using the contrast variation technique gave support to the hypothesis that the appearance

of the Bragg peak with slurries of granules in D_2O and H_2O, but not when dry, was due to the differential penetration of solvent into the crystalline and amorphous regions. It is also of some interest that the observed isopicnic point coincided very closely with what was calculated as the probable value from other data (see Appendix).

Because of the very low mass concentration of lipid, as long an exposure as was feasible on the D11 spectrometer was arranged. Since intuitively we expected the lipid to be closely associated with the amylose in the amorphous regions, it was anticipated that a residual periodicity at 100 Å would appear. In fact it proved impossible to observe any Bragg peak on either the native or defatted granules. However, on subtracting the scattering of defatted granules from those of the native granules, a Bragg peak (Fig. 3) was observed with a maximum at 167 Å. A second experiment at AERE, Harwell yielded a *d* value of 152 Å though the peak was less clearly defined. It is interesting, however, that refatted starch showed no trace whatsoever of a Bragg peak even after using the above subtraction method.

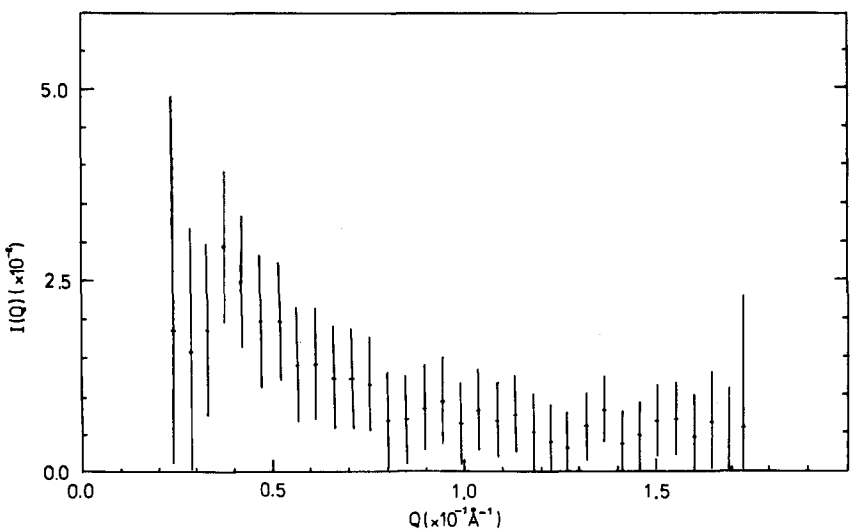

Fig. 3. SANS pattern after subtraction of SANS of defatted wheat starch granules from that of native wheat starch granules, both measured as slurries in an isopicnic mixture of D_2O/H_2O (52%/48%).

It is not surprising in view of the very low lipid contrast that the statistics are less than ideal; only longer exposure times on an instrument such as the D11 could improve the situation. One possible alternative explanation for the appearance of the 160 Å periodicity is that the Bragg peak of the defatted starch has shifted relative to the native starch. This point was kindly checked by Dr Ann Maconnachie using the D17 instrument at Grenoble. No shift in the Bragg peak on defatting was observed.

If, however, we accept the veracity of these results, then some explanation must be given. It does not seem likely that the lipid is occurring in alternate radial periodicities as 2×100 Å is too far removed from 152–167 Å. A more likely explanation arises from those observations already made by Hizukuri & Nikuni (1957) using the Debye–Scherrer formula to calculate crystallite size. One of the problems in such an approach is to establish in a powder diffraction pattern whether an observed peak is discrete or complex in character. Hizukuri & Nikuni (1957) used the broadening of the 16 Å Bragg spacing of potato starch which is quite discrete from other peaks, and which from Wu & Sarko's work (1978) we now know is the d_{100} spacing. If the amylopectin chains are arranged radially then this spacing is in the tangential direction. Unfortunately no such discrete peak is available in the *A* powder pattern of wheat starch, but, if we assume that the crystalline size is similar then we may envisage (Fig. 4) that the lipid is positioned radially between the racemes of the amylopectin chains, with a net distance between them of approximately 150–170 Å.

This, of course, raises a number of interesting points, which are yet unresolved, as to the nature of the lipid and its function, if any.

(a) Is it, for example, residual membrane, since certainly it is believed to be a lysolecithin? If so, it is perhaps more conceivable that a residual membrane or template in that position could assist in laying down the antiparallel double helices postulated by Wu & Sarko. However, on other grounds it is still difficult to envisage a single raceme yielding an antiparallel packing and even more so two opposing racemes with interdigitating double helices producing an absolute crystallinity which has been estimated at 30% (Cleven *et al.*, 1978).

(b) Is the lipid present as a separate entity or is it complexed with amylose or more probably the outer chains of the amylopectin?

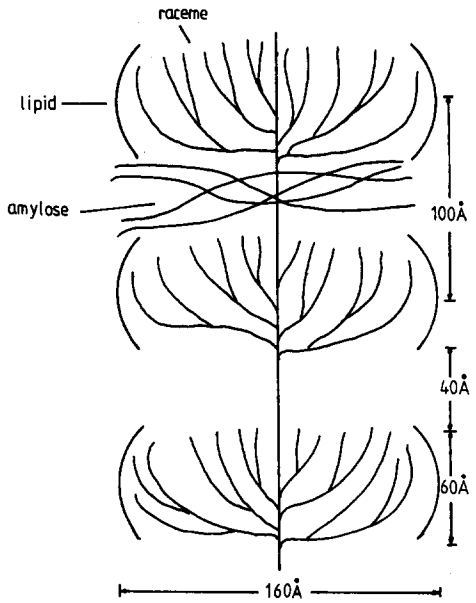

Fig. 4. Schematic diagram of portion of amylopectin molecule illustrating the 100 Å radial periodicity with amylose interdigitating in the 40 Å amorphous region. Lipid is evident, radially disposed with a tangential periodicity of approximately 160 Å.

In that there is an excess molar concentration of lipid for the amylose present, the involvement with the myriad chains of an amylopectin molecule would appear possible and indeed likely. Furthermore, evidence we have already published (Hart & Blanshard, 1982), and that forthcoming, abundantly demonstrates that the removal of some (though perhaps not all) the lipid leads to an enhanced rate in gelatinization as measured by the dynamic small-angle light scattering technique. In addition, Morrison (personal communication) believes that it is impossible to remove all the lipid without destroying crystallinity. Only future work can establish or disprove that claim. We do, however, consider that the lipid appears to delay diffusion (or 'waterproof') in amylopectin crystallites and modify the gelatinization process.

(iii) The evidence from Guinier analysis

It has already been mentioned that in certain instances the radius of gyration can be calculated from the scattering data of the native starch granules in D_2O or H_2O. This has also been performed for an amylopectin solution.

More interestingly, however, is the scattering behaviour of both millet and potato starch, since in both instances the Bragg peak completely disappears and a somewhat featureless curve replaces it from which radii of gyration of 48 Å for potato and 53 Å for millet were calculated.

It is, however, possible and indeed desirable to fit such data to an appropriate model and in fact a number have been tried, including a sphere, a cylinder and an ellipsoid. The appropriate expressions for these are given below (Guinier & Fournet, 1955).

1. Sphere of radius R:

$$I(Q) = \left[3\left(\frac{\sin QR - QR\cos QR}{Q^3R^3}\right)\right]^2 = \phi^2(QR)$$

2. Cylinder of revolution of diameter $2R$ and height $2H$:

$$I(Q) = \int_0^{\pi/2} \frac{\sin^2(QH\cos\theta)}{Q^2H^2\cos^2\theta} \times \frac{4J_1^2(QR\sin\theta)}{Q^2R^2\sin^2\theta} \sin\theta \, d\theta$$

where J_1 is a Bessel function and Q is half the scattering angle.

3. Ellipsoid of revolution, axes 2a, 2a, 2va:

$$I(Q) = \int_0^{\pi/2} \phi^2(Qa\sqrt{\cos^2 Q + v^2\sin^2\theta})\cos Q \, d\theta$$

It is obvious that to fit the experimental data requires both the choice of the correct model and also the appropriate values of the parameters. It is immediately evident from Fig. 5 that a sphere would not be satisfactory but an ellipsoid (with a squat cylinder as a near second best) gives a remarkably good fit between theoretical and experimental data. What is, however, most encouraging is that the dimensions of the ellipsoid coincide very closely with what has already

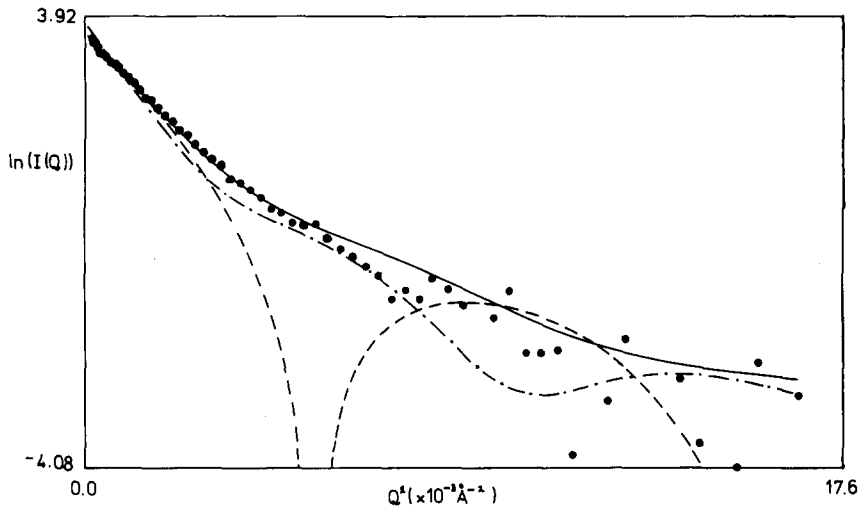

Fig. 5. A Guinier plot from SANS data for gelatinized potato granules in D_2O (●). Also shown are theoretical Guinier plots expected for ellipsoids (——), cylinders (— · — ·) and spheres (— — —). The dimensions of these model scattering particles are given in the text. Radius of sphere = 61·6 Å.

been calculated, namely that a raceme (i.e. the crystalline portion of the amylopectin chain) when considered as an ellipsoid has a major axis diameter of 150 Å and a minor axis diameter of 60 Å. It will be seen that a squat cylinder with a diameter of 150 Å and a height of 60 Å behaves very similarly. It appears, therefore, that gelatinization of the starch granule, particularly at higher concentrations, though resulting in a loss of birefringence and X-ray crystallinity, does not lead to a major disturbance in the dimensions of the amylopectin molecule.

It is gratifying too to note that the radius of gyration, where measurable, of the native starches is similar, but the intrusion of the Bragg peak prevents a similar model fitting exercise as was performed with the gelatinized systems.

A number of questions have been raised in this paper which urgently require answering. It would be desirable, firstly, to confirm the existence of the 150–170 Å lipid peak and, secondly, to establish the nature of the lipid in such a position. Is it, for example, present by itself or as a complex, presumably as V-amylose, with the poly-α-(1→4)-glucan chains of either amylopectin or amylose?

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APPENDIX

The coherent scattering lengths of the nuclei of interest in this work (hydrogen, deuterium, carbon and oxygen) are tabulated below (Jacrot, 1976); the b values are given in units of 10^{-12} cm

$$b_H = -0.3742 \qquad b_C = 0.6651$$

$$b_D = 0.6671 \qquad b_O = 0.5804$$

Summation according to the molecular formula then gives the total scattering lengths of the relevant molecules or, in the case of the carbohydrates, of the monomer units

$$\bar{b}_{H_2O} = -0.168 \qquad \bar{b}_{C_6H_{10}O_5} = 3.1506$$

$$\bar{b}_{D_2O} = 1.9146 \qquad \bar{b}_{C_6H_7D_3O_5} = 6.2745$$

(Note: Only the three protons on the C2, C3 and C6 hydroxyl groups can be exchanged on the carbohydrate.)

If we take the volume of a water molecule as 30 Å^3 and use the partial specific volume of amylopectin (0·62 cm^3 g^{-1} – Banks *et al.*, 1972) we may then use the volumes of carbohydrate monomer units ($V_{C_6H_{10}O_5} = 166.76$ and $V_{C_6H_7D_3O_5} = 169.85$) to calculate the contrast of any aqueous solution of carbohydrate.

For a solution in water having a mole fraction X of D_2O

$$\bar{b}_{water} = \frac{-0.168}{30} + \frac{(1.9146 - (-0.168))\,X}{30}$$

$$= 0.0056 + 0.0694X$$

$$\bar{b}_{carbohydrates} = \frac{3.1506}{166.76} + \left(\frac{6.2745}{169.85} - \frac{3.1506}{166.76}\right)X$$

$$= 0.0189 + 0.0180X$$

These values are equal at the isopicnic point

$$\therefore X = 0.477$$

Thus the isopicnic mixture is calculated to contain 47·7% D_2O on a molar basis or 50·4% w/w.