isolate the two fractions of leucocidin is necessarily the most efficient. Many other fractionation procedures than those described in this paper were tried but gave poor recoveries of the activity. It is now clear that this may have been due to separation of the fractions and not to irreversible inactivation. Nevertheless the method of purification described here permits the production of highly purified materials in reasonable yield and these are suitable for assessing the significance of leucocidin in staphylococcal pathogenicity. The separation of leucocidin into two fractions will permit controlling of such biological experiments; any effect possessed by one component, if it is due to an action on the white blood cell, must be multiplied manyfold by addition of the other.

SUMMARY

1. A medium suitable for large-scale production of leucocidin in high titre by *Staphylococcus aureus* has been developed.

2. An assay method for leucocidin suitable for routine use, based on the ability of leucocidin to prevent reduction of phenol-indophenol-2:6-dichlorophenol by macrophages has been developed.

3. Crude leucocidin can be partially purified by fractionation with ammonium sulphate and on hydroxylapatite.

4. On columns of cation-exchange resins leucocidin separates into two main fractions. There is loss of activity during the separation which is regained by mixing the fractions.

5. The nitrogen contents, ultraviolet-absorption and amphoteric behaviour of the fractions of leucocidin are characteristic of proteins.

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The Starch of the Tuber and Shoots of the Sprouting Potato

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Relatively few chemical investigations have been carried out on the amylose content of the potato-starch granule during growth (see Greenwood, 1956). Meyer & Heinrich (1942) isolated starch from the leaves, shoots and tubers and reported the amylose contents to be 22, 73 and 37 % respectively. The percentage of amylose determined by potentiometric iodine titration of the starch in the growing tuber was shown by Halsall, Hirst, Jones & San-

some (1948) to remain constant at 17 % for two varieties. These authors also found the average length of unit chain of the amylopectin components (calculated from periodate oxidation results on the whole starch) to be constant at 24–26 anhydroglucose units. There is, however, evidence that the percentage of amylose may vary with the botanical variety of the tuber. Anderson & Greenwood (1955) have found small variations in iodine affinity of

3.94–4.03 %, representing changes of 20–21 % of amylose, and Doremus, Crenshaw & Thurber (1951) have reported variations of 17.5–21.7 % in 22 varieties of sweet potato.

In the work reported here we have studied the physical and chemical characteristics of the starch isolated from the shoots and tubers of the sprouting potato in comparison with those of the original tuber starch.

EXPERIMENTAL

Isolation of starches. Starch was isolated immediately after harvesting the tubers (var. Home Guard) by extraction with 0.01 M-HgCl₂ (to inhibit enzymic activity), sedimentation of the granules after filtration and deproteinization by shaking with toluene and 4 % NaCl soln. as previously described (Cowie & Greenwood, 1957a). Some tubers were then kept at room temperature in semi-darkness until sprouts of up to 3 in. in length were formed. These were removed and the starch in them was isolated as described above. The residual flaccid tubers were similarly extracted. Although no quantitative estimate was made, the yield of starch from all three sources was high.

Estimation of granular size distribution. Size distributions were obtained by enlarging photomicrographs (four or five fields per sample) to give a final magnification of about 350 diameters and by measuring with a rule (to the nearest millimetre) the size of at least 500 granules for each starch. In view of the ellipsoidal nature of potato-starch granules, each was characterized by the length of its major axis (d). Number-average and weight-average particle diameters were calculated from $\sum n_i d_i / \sum n_i$ and $\sum n_i d_i^3 / \sum n_i d_i^2$ respectively, where n_i is the number of granules of diameter d_i . (In the latter case, this assumes that the ratio major:minor axis was constant for all granules.)

Gelatinization temperatures. The number of granules gelatinized at a particular temperature in water was determined with a microscope and Kofler electrically-heated microscope stage as described by Schoch & Maywald (1956).

Determination of phosphorus. Percentages of phosphorus in the starches and their components were determined by wet oxidation of the polysaccharide with perchloric acid (Smith, 1953), followed by colorimetric estimation of the phosphomolybdate complex (Fogg & Wilkinson, 1958). Weights of polysaccharide taken were: amylose, 500 mg.; starch, 150 mg.; amylopectin, 100 mg. Results were reproducible to ± 5 % for the starch and amylopectin and to ± 15 % for the amylose.

Aqueous leaching and fractionation. Portions of the

starches were leached with water at 60° for 10 min. under N₂ and the amylose was recovered as the butan-1-ol complex. Fractionation was achieved by complete dispersion of the starch in water by boiling for 1 hr. under N₂, followed by the addition of thymol to precipitate the amylose. The amylose was purified by reprecipitation as the butan-1-ol complex. These methods have been given in detail elsewhere (Cowie & Greenwood, 1957b).

Characterization of the fractionation products. The purity of the amylose and amylopectin components was obtained by potentiometric iodine titration with a semi-micro differential apparatus (Anderson & Greenwood, 1955). Titration conditions were: [I⁻], 0.01 M; [total free I₂]_{max.}, 10 μ M; temperature, 20°. Limiting viscosity numbers $[\eta]$ were obtained graphically from $[\eta] = \lim_{c \rightarrow 0} \eta_{sp}/c$, where η_{sp}

is the specific viscosity; concentrations (c) are expressed in g./ml. Measurements were made on the components dissolved in M-KOH at 22.5° with a modified Ubbelohde viscometer; detailed procedures are given by Cowie & Greenwood (1957a). A Spinco model E analytical ultracentrifuge was used to obtain the sedimentation constants (S_{90}) of the amylopectin components dissolved in water. The procedure and methods of evaluating the data have been described by Greenwood & Das Gupta (1958). Number-average degrees of polymerization for the amylose fractions were calculated from the relation: number-average degree of polymerization = $7.4 \times [\eta]$ (Cowie & Greenwood, 1957b). The percentage conversion of samples of both amylose and amylopectin into maltose under the action of purified soya-bean β -amylase (Peat, Pirt & Whelan, 1952) was determined as described previously; the enzyme was free from maltase and α -amylase activity and contained no Z-enzyme (Bryce, Cowie, Greenwood & Jones, 1958). [Z-Enzyme (see Peat, Thomas & Whelan, 1952) is capable of effectively removing barriers to β -amylolysis found in amylose.] Average lengths of unit chain for the amylopectins were calculated from the results of a potentiometric estimation of the formic acid released on oxidation with NaIO₄ at 2° (Potter & Hassid, 1948).

RESULTS AND DISCUSSION

Physical characteristics of the starches

Potentiometric-titration experiments indicated small but reproducible differences between the iodine affinity of the different starches. Corresponding percentages of amylose are shown in Table 1. The starch from the shoots with 16.7 % of amylose differs significantly from the other two.

Table 1. *Physical properties of the starches*

Average gelatinization temperature is the temperature at which 50 % of the granules are gelatinized.

Starch source	Iodine affinity (mg. of I ₂ bound/100 mg. of starch)	Amylose* (%)	Av. granular diam. (μ)		Av. gelatiniza- tion temp.	Phosphorus (%)
			No.	Wt.		
Original tuber	3.94	20.2	42	55	61°	0.093
Shoots	3.25	16.7	14	19	61.5°	0.071
Sprouted tuber	3.96	20.3	40	51	62.5°	0.071

* Calculated from [(iodine affinity $\div$ 19.5) $\times$ 100]; Cowie & Greenwood (1957b).

Fig. 1 shows the number- and weight-percentage distribution curves for the granules. Calculated averages are given in Table 1. The two tuber starches possess granules of similar size and differ only in that the proportion of large granules (i.e. $> 80 \mu$) is greater in the unsprouted tuber starch. This difference is emphasized in the weight-distribution curves. The size-distribution curves for the shoot starch are completely different, the average granular size being only about a third of

that for both tuber starches. This suggests that the shoot starch may be immature.

Gelatinization curves are shown in Fig. 2, and the average gelatinization temperatures are given in Table 1. The curves are typical and illustrate the range of about 5° over which gelatinization of a starch occurs. The differences observed between the starches are again reproducible but are small and not of obvious significance. Granule size, the percentage of amylose present, the molecular weight of the components and the corresponding micellar organization of the granule may all influence gelatinization. An increase in amylose content raises the average gelatinization temperature (Savage, Deatherage, MacMasters & Senti, 1958). On the other hand, granular size is also important, as in any experiment large granules are seen to gelatinize first, whereas a few small ungelatinized granules often remain when the process is otherwise complete. For the two tuber starches, which have the same amount of amylose, the effect of the very large granules in the original starch may account for the differences in average gelatinization temperature. Although the shoot starch contains less amylose, the granular size is small. The observed gelatinization temperature must be accounted for by the interplay of these two effects, although the whole phenomenon is very complex.

The phosphate content of the original tuber starch appears to be higher than that for the other two starches. [Phosphorus occurs in potato starch as phosphate on C_6 of the glucose unit (Posternak, 1950).]

Aqueous leaching and fractionation

Our previous work (Cowie & Greenwood, 1957b) has shown that when potato starch of different botanical varieties is dispersed and fractionated under careful oxygen-free conditions, an amylose with a degree of polymerization of 3000–4000 is obtained. Further, aqueous leaching at 60 – 70° results in the preferential extraction of an amylose fraction of relatively low number-average degree of polymerization (~ 2000). Table 2 shows that the original starch gave results in agreement with this, which supports the suggestion of Cowie & Greenwood (1957b) that the molecular size of amylose is to some extent independent of the botanical variety of potato. Essentially similar results were obtained for the starch from the sprouted tuber. Although these tubers had obviously undergone great changes in physical state, surprisingly little enzymic modification or degradation of the granular starch has occurred. However, the number-average degree of polymerization of the aqueous-leached and total amyloses from the shoot starch are both significantly smaller, whereas the percentage conversion of the total amylose into

Fig. 1. Size-distribution curves for (a) number percentage and (b) weight percentage of granules. ---, Starch from original tubers; —, starch from shoots; ..., starch from sprouted tubers.

Fig. 2. Percentage of granules gelatinized in water as a function of temperature. O, Starch from original tubers; ●, starch from shoots; Δ, starch from sprouted tubers.

Table 2. *Properties of the fractions obtained from aqueous leaching and dispersion of the starches*

Granules were leached with water at 60° for 10 min., or dispersed in water at 100° for 1 hr.; both experiments were carried out in N₂.

	Original tuber		Shoot		Sprouted tuber	
	Leach	Dispersion	Leach	Dispersion	Leach	Dispersion
Amylose						
Percentage of total amylose*	45	> 95	45	> 95	40	> 95
Purity (%)*	> 98	> 98	—	> 98	—	> 98
Phosphorus (%)	—	0.005	—	0.001	—	0.004
[η] (g./ml.)	270	450	210	315	270	435
Degree of polymerization	2000	3300	1600	2300	2000	3200
β -Limit†	—	83	—	94	—	83
Amylopectin						
Purity (%)*	89	99.5	89	99.5	88	99.0
Phosphorus (%)	—	0.111	—	0.089	—	0.097
[η] (g./ml.)	—	160	—	160	—	160
β -Limit†	—	56	—	56	—	57
Chain length	—	24	—	24	—	24
External chain length‡	—	16	—	16	—	16

* Calculated from iodine titrations.

† Percentage conversion into maltose on β -amylolysis.

‡ Calculated from [(chain length $\times$ β -limit) + 2.5] (Peat, Whelan & Thomas, 1952).

Fig. 3. Relation between the inverse of the sedimentation coefficient (S_{20}^{-1}) and the concentration (c) for the amylopectins dissolved in water. $\circ$, Amylopectin from original tuber starch; $\bullet$, independent runs on amylopectin from shoot starch (see text); $\triangle$, amylopectin from sprouted-tuber starch.

maltose on β -amylolysis is very much higher. The amount of phosphorus in this amylose was significantly lower.

On dispersion and fractionation the amylopectins obtained from all the starches were identical within experimental error in phosphorus content, average length of unit chain, percentage conversion into maltose with β -amylase and calculated average exterior chain lengths (see Table 2). As far as could be determined there was also little difference in the molecular size. Measurements of the molecular size of amylopectin are difficult (see Greenwood, 1956). Although the limiting viscosity numbers were identical, this is not conclusive as viscosity measurements are not particularly sensi-

tive for detecting small changes in size for such highly branched molecules. Sedimentation measurements are more satisfactory in theory, but are complicated by the high concentration-dependence observed for the sedimentation coefficient (Greenwood & Das Gupta, 1958). Previously, we have made the measurements in alkaline solution. Here we have studied aqueous solutions, and the results of typical measurements are shown in Fig. 3. In agreement with Erlander & French (1958) it was found that the graph S_{20}^{-1} versus c (where S_{20} is the sedimentation coefficient corrected to water at 20°) was linear and allowed extrapolation to infinite dilution. However, the accuracy of such an extrapolation is limited in view of the high values of S_{20} involved. It was found that independent runs on the same sample gave curves with the same intercept but different slopes (see Fig. 3). This is thought to be due to small variations in the salt content of the solvent altering the ionization of the phosphate groups and so influencing intermolecular entanglements. Sedimentation in 0.2M-NaCl was unsuccessful as aggregation occurred as shown by the presence of two or more sedimenting peaks on the schlieren pattern. This agrees with the light-scattering experiments of Witnauer, Senti & Stern (1955), who observed aggregation in the presence of salt of concentrations greater than mM. These authors suggested that the salt destroyed the repulsion between the ionized phosphate groups.

The problem of the ultracentrifuging of amylopectin is complicated, and only a limited number of investigations have yet appeared in the literature (Greenwood & Das Gupta, 1958; Erlander & French, 1958; Stacy & Foster, 1957). Further studies are being made. At most, it can be said

that S_{20} for the potato amylopectins studied here in aqueous solution is large and probably at least 500×10^{-13} c.g.s. units.

Utilization of the starch granule

The similarity in physical and chemical properties of both tuber starches suggests that when granules are utilized enzymically they are individually completely broken down to soluble material rather than being partially attacked. Furthermore, the extremely large granules may well be utilized first, a fact which may be related to their looser organization as shown by gelatinization studies. Both starches contain about 20 % of amylose. This value differs radically from that of 37 % found by Meyer & Heinrich (1942). However, these authors did not isolate whole granules and their procedure would preferentially extract amylose from the plant tissues.

Nature of the barrier to β -amylolysis

The starch from the shoots contained 16 % of the linear component, in contrast to the 73 % reported by Meyer & Heinrich (1942). As the starch is unlikely to have been transported to the shoots in granular form, the possibility exists that it may be immature. Also, the molecular size of the corresponding amylose is considerably smaller than for the tuber starches and the barrier to β -amylolysis (see, Peat, Pirt & Whelan, 1952) is more limited. The nature of the barrier to β -amylolysis is uncertain. It may be an artifact introduced during the isolation of fractionation of the starch (see, Baum, Gilbert & Scott, 1956). Equally well it may be native to the amylose. In the latter case the possibility of phosphorus giving rise to the barrier must be considered (compare, however, Peat, Thomas & Whelan, 1952). The amount of phosphorus in all the amyloses is very small (see Table 2) and consequently its estimation is difficult. Also, the presence of an amylopectin impurity of about 1 % would cause a considerable error in the given phosphorus contents. Nevertheless, it is thought significant that the shoot amylose, which has the highest conversion into maltose on β -amylolysis, should have the lowest phosphate content. The problem is, however, highly complex and is unlikely to be solved until the specificity of Z-enzyme is finally elucidated [cf. Peat, Thomas & Whelan (1952), Hopkins & Bird (1953) and Neufeld & Hassid (1955)].

SUMMARY

1. Starch has been isolated from the shoots and tubers of sprouting potatoes, and its physical and chemical properties have been compared with those of the starch from the original tubers.
2. Although the two tuber starches were similar,

that from the shoot was profoundly different and possessed a lower iodine affinity and smaller granular size.

3. The amylose fractions separated from the different starches varied both with regard to molecular size and β -amylolysis limit, whereas the amylopectin fractions appeared to be similar.

4. The sedimentation of amylopectin has been studied.

5. The nature of the barrier to β -amylolysis is discussed.

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