Preparation and characterization of a [^{14}C]cellulose suitable for human metabolic studies

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In six normal subjects administered 5 μCi of an oral dose of a commercially available ^{14}C-labelled cellulose, significant amounts of $^{14}CO_2$ were detected in expired air within 30 min, suggesting that other ^{14}C-labelled non-cellulosic material was present. Chemical and microscopical examination confirmed that starch was the principal contaminant. The commercial preparation was purified using amyloglucosidase (*EC* 3.2.1.3) digestion following gelatinization of the starch by autoclaving. Subsequent administration of the purified cellulose to a further six normal subjects decreased the expired air $^{14}CO_2$ during the subsequent 10 h from 13·0 (SD 4·0) to 4·1 (SD 1·9) %. Administration of the purified product to a further group of four normal subjects, before and after a regimen of increased dietary fibre, showed a cumulative increase in expired $^{14}CO_2$ over 24 h from 7·9 (SD 1·1) to 12·1 (SD 2·6) % on fibre. In six ileostomy subjects the cumulative excretion of $^{14}CO_2$ was greatly decreased compared with normal controls (3·0 (SD 1·14) and 10·5 (SD 3·9) % respectively). In constipated subjects expired $^{14}CO_2$ continued beyond 48 h, in contrast to normal subjects where expired $^{14}CO_2$ at this time was negligible.

Dietary fibre: Digestion of cellulose: Human metabolic studies.

Cellulose occurs naturally as vegetable and fruit fibre and in many foods where processing may have altered its chemical and physical characteristics. The digestive breakdown of cellulose is dependent on its degree of crystallinity, and the action of cellulase enzymes; these factors have been well studied (King, 1966; Rautela & King, 1968; Southgate & Durnin, 1970; Prynne & Southgate, 1979; Ladisch *et al.* 1981). The relative chemical inertness of cellulose, as well as its occurrence in many different forms, results in difficulties in its quantitative measurement. Several studies have been performed to assess the digestion of dietary cellulose within the human gastrointestinal tract, and the results reported have been very variable (Southgate & Durnin, 1970; Milton-Thompson & Lewis, 1971; Prynne & Southgate, 1979), perhaps reflecting inadequacies of older methodology. Suggestions have now been made to standardize methodology for measurement of dietary fibre, including cellulose (Cummings & Englyst, 1987; Englyst *et al.* 1987). Using these methods, cellulose from various food sources was extensively degraded in the human gastrointestinal tract, but large individual variations were found (Nyman *et al.* 1986). Non-starch polysaccharides, including cellulose, are not broken down in the small intestine by human digestive enzymes (Cummings & Englyst, 1987).

Radio-labelled plant cellulose has been available for many years (Conrad *et al.* 1958), but has been little used for human studies. Findlay *et al.* (1981) used a ^{14}C-labelled cellulose and monitored the appearance of $^{14}CO_2$ in expired air. They suggested that cellulose is metabolized in both the small and large bowel, but that ^{14}C-labelled impurities could account for some of the observations. A study using ^{131}I-labelled cellulose showed little breakdown in the human gut (Carryer *et al.* 1982). This is in contrast to earlier results using

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Table 1. *Expired air $^{14}CO_2$ in twelve normal individuals as a percentage of administered dose of either commercially prepared or purified [^{14}C]cellulose**

Subject no.	Time-interval after oral dose 0–4 h	4–10 h	10–24 h	Cumulative 0–24 h
	$^{14}CO_2$ output using the commercial [^{14}C]cellulose			
1	5·0	11·0	7·1	23·1
2	3·8	6·3	8·3	18·4
3	2·0	16·3	6·0	24·3
4	3·6	7·1	10·4	21·1
5	4·9	3·0	10·8	18.7
6	4·5	10·3	15·0	29·8
Mean	3·97	9·00	9·60	22·56
SD	1·12	4·60	3·23	4·24
	$^{14}CO_2$ output using the purified [^{14}C]cellulose			
7	1·9	1·6	3·4	6·9
8	1·3	1·4	3·7	6·4
9	1·7	2·2	7·9	11·8
10	1·6	3·0	10·2	14·8
11	0·8	1·4	6·1	8·3
12	2·6	4·9	7·5	15·0
Mean	1·65	2·42	6·47	10·53
SD	0·60	1·36	2·62	3·87

* For details of procedures, see below.

chemical methods, which suggested that cellulose is extensively digested in the human intestine (Holloway *et al.* 1978; Cummings *et al.* 1979; Prynne & Southgate, 1979). A more recent study, using a ^{14}C-labelled cellulose, confirmed that significant quantities of cellulose are digested in man (Kelleher *et al.* 1984).

The purpose of the present study was to prepare and characterize, from a commercially available ^{14}C-labelled cellulose, a preparation free from non-cellulosic material and retaining the characteristics of natural cellulose, which would be suitable for metabolic studies.

METHODS AND RESULTS

Human studies

Twenty-four volunteer subjects, to whom the purpose and nature of the study were explained in detail, agreed to take part in the study. Approval for the study was obtained from the local Ethical Committee. Sixteen normal subjects, consuming mixed normal diets and without symptoms or history of gastrointestinal disease, and not taking any antibiotics or drugs known to influence gastrointestinal function, were studied. Six patients with an ileostomy following total colectomy and rectal resection, as well as two elderly, constipated patients, were also studied. All subjects, having fasted overnight, were administered a standardized oral dose of the appropriate cellulose preparation as described previously (Kelleher *et al.* 1984). Expired air samples were collected at intervals for a total of 7 d. The results are expressed as disintegrations/min per mmol CO_2 and cumulative excretion of $^{14}CO_2$ expressed as a percentage of the dose administered, assuming an endogenous CO_2 production of 9 mmol/kg body-weight per h (Winchell *et al.* 1970).

Ileostomy effluent and complete faecal collections were obtained for 7 d from the six ileostomy patients and the two constipated, elderly patients respectively.

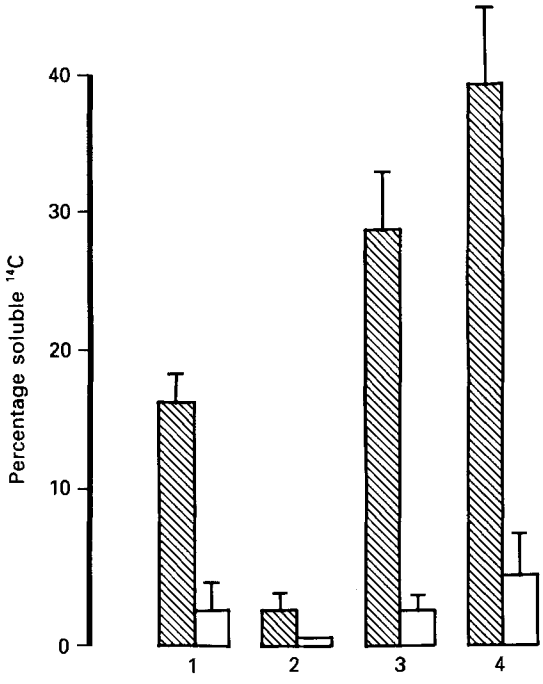

Fig. 1. A comparison of the aqueous solubility of commercial [^{14}C]cellulose (▨) and purified [^{14}C]cellulose (□) under various conditions: (1) solubility in distilled water after 1 h at 37° with constant agitation; (2) the percentage of the soluble fraction from (1) which is dialysable; (3) solubility in 0·001 M-hydrochloric acid treated as in (1); (4) solubility after digestion with amyloglucosidase (*EC* 3.2.1.3) for 5 h at 55°. Values are means, and standard deviations represented by vertical bars.

The commercially available [^{14}C]cellulose was administered to six normal subjects and the rate of excretion of $^{14}CO_2$ in the expired air is shown in Table 1. The pattern of excretion was surprising, in that a large and early peak of $^{14}CO_2$ was consistently detected, corresponding to approximately 20% of the administered dose. This confirmed our previous study that labelled starch was present as a contaminant (Kelleher *et al.* 1984) and that the commercial [^{14}C]cellulose should be further characterized.

Properties of the commercial [^{14}C]cellulose

Duplicate portions of the [^{14}C]cellulose were suspended in 10 ml distilled water and treated as outlined in Fig. 1. All suspensions were gently mixed for 1 h in a water-bath at 37° and then allowed to cool to room temperature. The suspensions were then filtered through 25-mm micropore filters (Type HA, 0·45 μm pore size; Millipore UK Ltd). ^{14}C radioactivity in filtrates was determined using a liquid scintillation counter (model 3375 scintillation counter; Packard) and the micropore filter then dissolved in scintillation fluid (Optiphase 'X'; Pharmacia Ltd, Milton Keynes, Bucks) and radioactivity counted. The percentage soluble ^{14}C was calculated from the ^{14}C activity in the filtrate and that retained by the micropore filter.

The solubility results are shown in Fig. 1. Incubation in distilled water, pH 5–6, produced almost 20% soluble material, which was increased to approximately 30% at pH 2.

The filtrates from the solubility experiments were dialysed overnight against water using Visking tubing (Gallenkamp Ltd). Approximately 2% of the ^{14}C was dialysable (Fig. 1), confirming that the soluble fraction was large-molecular-weight material.

Microscopy, following iodine staining, revealed starch grains in the commercial product (Plate 1*a*). Polarized light microscopy showed similar striation patterns in these starch grains to those seen in starch grains from *Cana indica* sp. leaf material prepared in our own laboratory.

The filtrates were incubated with amylase (*EC* 3.2.1.1; cat. no. A-2771; Sigma Chemical Co., Poole, Dorset) (5 g/l water) and separately with human saliva for 30 min at 37°. The digests were chromatographed on thin-layer chromatography plates (silica gel type G60, Merck; solvent *n*-butanol–acetic acid–water, 50:25:25, by vol.). Following chromatography, the silica gel was scraped from the plates in 5-mm bands, mixed with 10 ml scintillation fluid and the radioactivity determined. The ^{14}C was detected in two areas of the plate with R_f values corresponding to glucose and maltose markers.

It was concluded from these experiments that the commercially available [^{14}C]cellulose was unsuitable for metabolic studies, but that a suitable material would be obtained if the contaminating ^{14}C-labelled starch could be quantitatively removed, without altering the physical characteristics of the cellulose.

Removal of starch from a commercially available [^{14}C]*cellulose*

Various enzymic methods are available for the removal of starch from foods (Rasper, 1981), and the method finally chosen was as follows. The commercial [^{14}C]cellulose was autoclaved at 121° for 20 min in 0·1 M-phosphate-citrate buffer, pH 4·6, to gelatinize starch grains. After washing with hot distilled water, the material was incubated for 5 h at 55° with amyloglucosidase (*EC* 3.2.1.3; cat. no. A-7255; Sigma), 20 g/l 0·1 M-phosphate-citrate buffer (pH 4·6) with continous stirring.

The preparation was then washed copiously with 0·01 M-hydrochloric acid and then water, to remove enzyme and digestion products. The product was then dried under vacuum and finally checked to ensure microbial sterility. This procedure consistently removed 30–40 % of the original ^{14}C activity (Fig. 1). Microscopic examination after iodine staining now revealed only trace amounts of starch fragments (Plate 1(*b*)). It was felt that further treatment of the product would be unrewarding and could lead to degradation of the cellulose. The specific activity of the purified cellulose was 1·5–2·0 μCi/mg.

Properties of the purified [^{14}C]*cellulose*

The morphological features of the purified [^{14}C]cellulose appeared to have been retained when examined by microscopy. The fibrous nature of the material, vascular elements and parenchyma cell material were clearly visible in both the commercial and purified products (Plate 1(*a*,*b*)).

Because of the high specific activity and need to provide portions of the purified product in milligram amounts, the labelled cellulose was ballasted with cellulose powder (cat. no. S-3755; Sigma). The purified [^{14}C]cellulose was ground in a mortar with a little water and ten times its own weight of cellulose powder (Whatman) added. After a water wash, the preparation was dried under vacuum and tested for homogeneity. Weighed portions of the cellulose were suspended in scintillation fluid and ^{14}C activity assessed. There was a linear relation between weights of portions and ^{14}C content, demonstrating that the ballasted [^{14}C]cellulose was homogenous.

The solubility studies, as previously described, were repeated. As seen from Fig. 1, only 0·5–2·3 % of the purified product was now soluble in water, compared with 20–30 % for the commercial material.

Amyloglucosidase digestion of the purified cellulose showed that the product was now almost totally resistant to this enzyme (Fig. 1).

A portion of the purified [^{14}C]cellulose was suspended in distilled water, and the

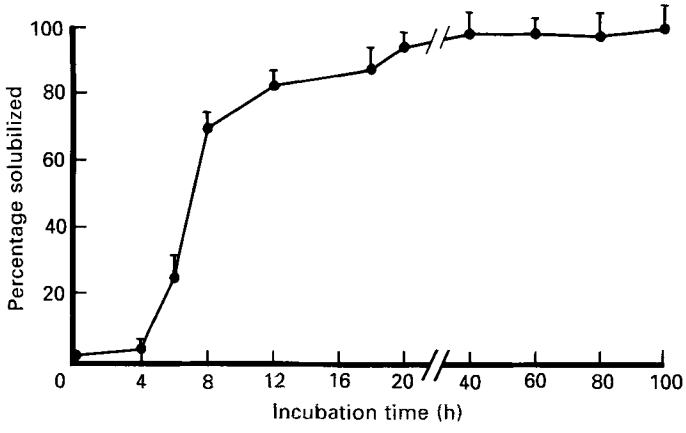

Fig. 2. The in vitro hydrolysis of purified [^{14}C]cellulose with cellulase (*EC* 3.2.1.4) enzyme. The results shown are the means of four experiments, and standard deviations represented by vertical bars. For details of procedures, see below.

suspension was dialysed against water at room temperature overnight, with continuous mixing. Only 0·2–0·3% was found to be dialysable, compared with 2–3% of the commercial cellulose (Fig. 1).

A suspension of the purified [^{14}C]cellulose was filtered through a 5 μm micropore filter and was then examined by electron microscopy. Fragments were seen which ranged from approximately less than 0·1 μm to 5 μm.

A stable suspension of [^{14}C]cellulose was prepared by suspending 0·5 g of the purified [^{14}C]cellulose in 100 ml of distilled water and mechanically shaking for 15 min. The suspension was then centrifuged at low speed (500 *g*) for 15 min. This produced a supernatant fraction containing a stable suspension of particulate cellulose. The suspension (10 ml) was incubated with 30 ml buffered enzyme solution containing cellulase (*EC* 3.2.1.4; cat. no. C2274; Sigma), 20 g/l in 0·1 M-phosphate-citrate buffer, pH 4·9 (containing 0·001 M-sodium azide as an antimicrobial agent) in a closed ultrafiltration cell (Ghose & Kostick, 1970). The cell was equipped with a membrane retaining solutes of molecular weight greater than 10000 (membrane type PM 10, 62 mm diameter; Amicon Corp., USA). The cell was continuously replenished with buffered enzyme solution. Eluate from the cell was removed and ^{14}C activity determined. The purified cellulose was completely digested to products with a molecular weight of less than 10000 using this system (Fig. 2). Further characterization of the eluates, using both column and high pressure liquid chromatography, demonstrated that the ^{14}C was present at retention times identical to cellobiose and glucose markers.

Metabolic studies using the purified [^{14}C]cellulose

Studies in six normal subjects

The breath tests were repeated using the purified [^{14}C]cellulose in six normal volunteers (ages 40–50 years). The individual results are shown in Table 1. Compared with the results obtained with the commercial [^{14}C]cellulose, the large and early peak was now greatly reduced and, from 10 h onwards, ^{14}C excretion was similar for both products. The cumulative excretion of ^{14}C was calculated as a percentage of the oral dose of [^{14}C]cellulose administered and was reduced from 22·6 (SD 4·24) to 10·53 (SD 3·87) % using the commercial and purified [^{14}C]celluloses respectively.

Table 2. *Expired air $^{14}CO_2$ as a percentage of administered dose of purified [^{14}C]cellulose in four normal subjects before and after consumption of Fibogel (ispaghula) fibre (7 g/d for 3 months)**

Subject no.	Time-interval after oral dose 0–4 h	4–10 h	10–24 h	Cumulative 0–24 h
		Pre-ispaghula		
1	1·0	1·6	4·0	7·6
2	2·4	1·2	3·7	7·3
3	1·3	1·9	6·3	9·5
4	1·4	1·8	3·8	7·0
Mean	1·53	1·63	4·45	7·85
SD	0·61	0·31	1·24	1·13
		Post-ispaghula		
1	2·6	2·6	7·0	12·2
2	3·0	3·5	9·2	15·7
3	1·6	1·8	6·1	9·5
4	2·1	2·8	6·1	11·0
Mean	2·33	2·68	7·10	12·10
SD	0·61	0·70	1·46	2·64

* For details of procedures, see below.

The effect of increased dietary fibre

The effect of increased dietary fibre intake on the breakdown of the purified [^{14}C]cellulose was studied in a further group of four volunteers. The [^{14}C]cellulose breath test was first performed while the subjects were on their normal diets. The subjects then consumed additional fibre as Fybogel (Reckitt & Colman Ltd; 7 g), together with their usual diet, for a period of 3 months, after which time the breath tests were repeated. The results are shown in Table 2. It can be seen that increased dietary fibre did influence the breakdown of cellulose, particularly between 10 and 24 h following [^{14}C]cellulose administration. The cumulative 0–24 h excretion of $^{14}CO_2$ increased from 7·85 (SD 1·13) to 12·10 (SD 2·64) % ($P < 0·05$) with increased fibre intake.

Ileostomy subjects

The degradation of the [^{14}C]cellulose was studied in six subjects with an ileostomy following total colectomy and rectal resection. Expired $^{14}CO_2$ was similar to normal subjects over the first 4 h after administration of the cellulose (1·65 (SD 0·60) % in normal controls and 1·12 (SD 0·12) % in ileostomy subjects). Cumulative excretion for 24 h was, however, significantly less in the ileostomy subjects compared with normal controls (3·00 (SD 1·14) and 10·53 (SD 3·87) % respectively). There was negligible CO_2 excretion in the ileostomy subjects from 15 h onwards. Analysis of ileostomy contents showed that a majority of the administered cellulose was excreted by this route (81·0 (SD 12·0) %), compared with 57·2 (SD 13·3) % in faeces from normal subjects, as reported previously (Kelleher *et al.* 1984).

In normal subjects, $^{14}CO_2$ excretion after 48 h was negligible (Table 3). In two elderly, constipated subjects, significant $^{14}CO_2$ excretion continued up to 150 h with a mean of 16·0 %. It was also observed that faecal excretion of ^{14}C was delayed beyond 5 d in these subjects, with a mean of 22 % of the oral dose in these two constipated subjects compared with < 1 % in normal individuals at this time-period (Kelleher *et al.* 1984).

Table 3. *Expired air $^{14}CO_2$ in six normal individuals and in two elderly constipated subjects as a percentage of administered dose of purified [^{14}C]cellulose**

Subject no.	Time-interval after oral dose					Cumulative
	0–4 h	4–10 h	10–24 h	24–48 h	48–150 h	0–150 h
			Constipated subjects			
1	1·5	1·6	5·1	9·0	18·0	35·2
2	0·5	1·3	4·8	6·2	13·0	25·8
Mean	1·0	1·4	4·9	7·6	15·5	30·5
			Normal subjects			
Mean	1·7	2·4	6·5	2·9	0·3	13·8
SD	0·6	1·4	2·6	1·1	0·5	5·4

* For details of procedures, see p. 126.

DISCUSSION

There is little doubt that dietary cellulose is degraded within the human gastrointestinal tract and several studies have now been reported (Southgate & Durnin, 1970; Prynne & Southgate, 1979; Ladisch *et al.* 1981; Nyman *et al.* 1986). Large variations have, however, been documented in these studies which may be partly explained by the differing methodology used to assay cellulose, as well as different sources of cellulose. More recent methods for the determination of non-starch polysaccharides, which include a direct assay of cellulose, will give more specific information (Cummings & Englyst, 1987). However, even using these methods, large individual variations were found when different types of dietary fibre were fed to normal volunteers (Nyman *et al.* 1986). Cellulose and other non-starch polysaccharides are not degraded within the small intestine in man (Englyst & Cummings, 1987).

The use of a purified radio-labelled cellulose offers some advantages, but also disadvantages, in the study of cellulose metabolism in man. The purified cellulose used here, on microscopical examination, showed the fibrous nature of native cellulose with visible vascular elements and parenchymal cell material. However, studies using purified cellulose may not, because of the physical characteristics of the material, give results which can be compared with the degradation of natural plant cellulose (Van Soest, 1973). Even with natural cellulose sources the apparent digestibility varies greatly between different types, thus 90% of cellulose from wheat is excreted in faeces compared with 26% of cellulose from apples and 13% from cabbage (Nyman *et al.* 1986). The [^{14}C]cellulose, as supplied, was stated to be free of lignin and hemicelluloses, but did contain significant quantities of starch. Having removed the starch, it is unlikely that the preparation contained significant quantities of labelled non-cellulosic polysaccharides, particularly since the product was totally hydrolysed by cellulase and the products of this hydrolysis are glucose and cellobiose.

The present results suggest that the commercial source of cellulose labelled with ^{14}C is unsuitable for human metabolic studies, largely because of the high percentage (40%) of ^{14}C-labelled starch contained within the cellulose matrix. The presence of numerous starch granules and their removal with amyloglucosidase treatment leaves little doubt that [^{14}C]starch is the most likely explanation of the large early excretion of $^{14}CO_2$. This validates previous published reservations regarding studies using commercially labelled [^{14}C]cellulose (Findlay *et al.* 1981). It may be possible that this early appearance of $^{14}CO_2$,

using untreated cellulose, is due to rapid transit of the liquid phase of the meal, carrying with it suspended [^{14}C]cellulose, to the colon and being metabolized. However, as $^{14}CO_2$ was observed as early as 15 min after administering the [^{14}C]cellulose, in ileostomy and normal subjects, this seems unlikely. The most probable cause is digestion of residual starch by salivary or pancreatic amylase, followed by absorption and metabolism of the released products.

It has previously been demonstrated that increasing dietary fibre results in increased faecal bulk (Stephen *et al.* 1986), and in normal individuals decreased transit time (Mitchell & Eastwood, 1976). Increasing dietary fibre intake does appear to alter the breakdown of the administered cellulose. The increased excretion of expired $^{14}CO_2$ while on Fybogel suggests that dilution of the administered cellulose by faecal bulking and simultaneous exposure to a larger bacterial population, may be more important than altered transit time in determining cellulose digestion. However, previous studies have shown that prolonging transit time had little effect on cellulose digestion, whereas shortening transit time did significantly reduce apparent cellulose digestion (Stephen *et al.* 1987). The shortened transit time in the study of Stephen *et al.* (1987) was achieved with the laxative Senokot, which is likely to have a more dramatic effect on bowel function than increased dietary fibre and may not have faecal bulking properties. The greatly extended presence of $^{14}CO_2$ in the expired air of constipated patients suggest its potential as a measure of transit time, which is independent of patient observation, though further studies are needed.

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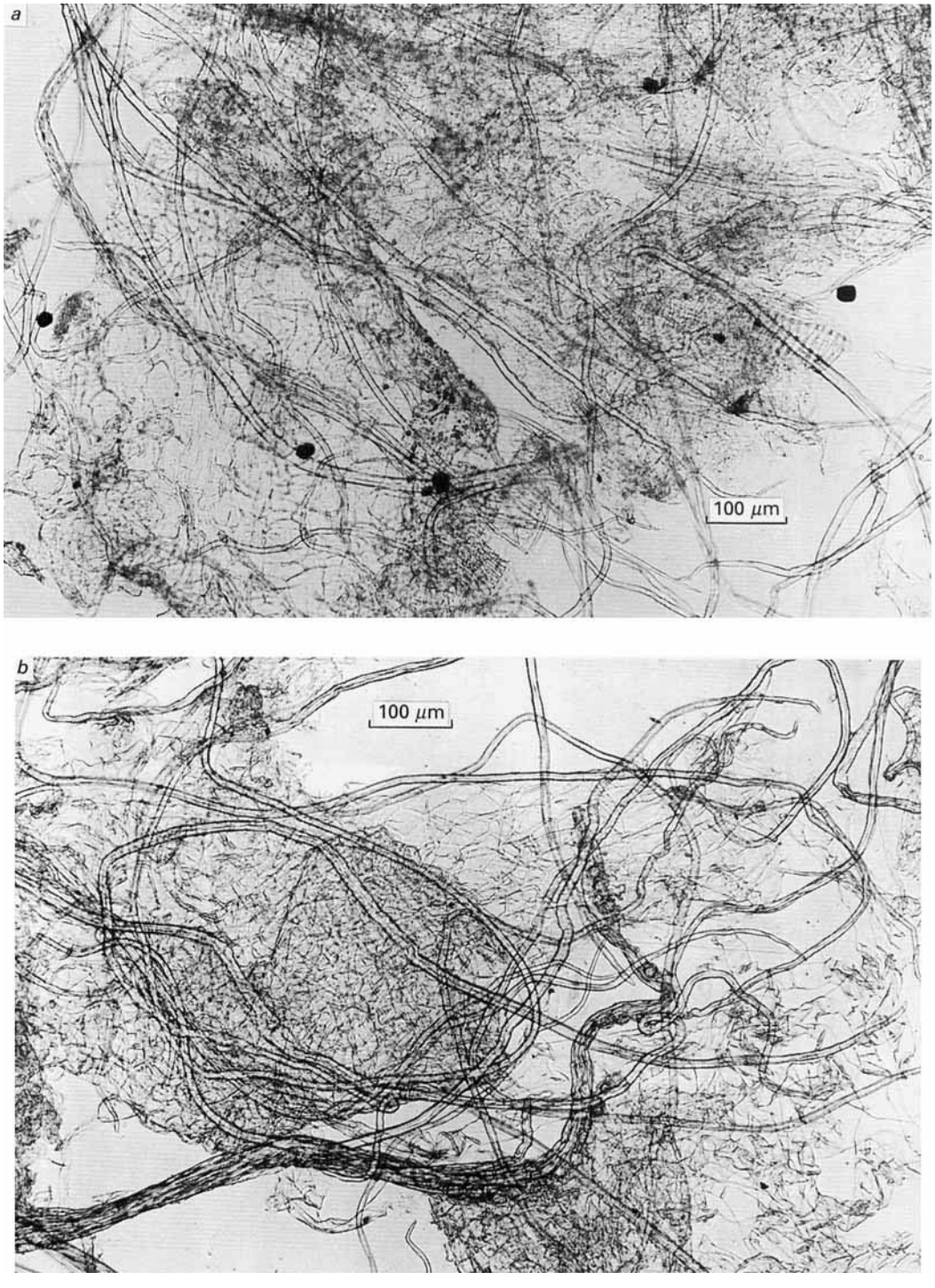

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EXPLANATION OF PLATE

Plate 1. Photomicrographs of iodine-treated suspensions of (*a*) commercial and (*b*) purified [^{14}C]cellulose preparations. For details of procedures, see p. 124. (Magnification × 150.)

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