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Changes in Microsomal Components Accompanying Cell Differentiation of Pea-Seedling Roots

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This paper describes some changes found in the microsomes of pea roots during growth (see Whaley, Mollenhauer & Leech, 1960, for a review on plant microsomes). The growing-plant root provides a convenient source of material, because growth is largely in one direction and cell division is confined to the apex, so that it is possible to obtain batches of cells in progressive stages of development by cutting serial transverse segments. Small segments of the root tips were used, so that differences between the meristem and the first stages of differentiation could be studied. The experiments depend on the chance observation that the sedimented microsomal pellet may be readily separated into two components, one particulate and the other largely membranous. These components were examined by their protein and nucleic acid contents, by electrophoresis and by electron microscopy.

A preliminary communication of these results has been published (Loening, 1960).

MATERIALS AND METHODS

Preparation of root-tip segments. Pea seeds of the variety Meteor (Sutton and Sons, Reading) were sown in horticultural vermiculite–soft tap water (3:1, v/v) and germinated in the dark at 25° for 48 hr. The roots were then 3–4 cm. long; exceptionally long or short ones were rejected. The roots were cut into three serial segments, the first tip segment being 1·6 mm. long, the second 1·8 mm. and the third basal segment 3·0 mm. To facilitate the cutting of large numbers of tips, a block of four Perspex sheets of thicknesses 1·6, 1·8, 3·0 and 6·0 mm. was drilled with 55 holes of suitable diameter to hold the roots. The block was placed, thinnest sheet downwards, flat on a sheet of glass and the roots were inserted into the holes so that the tips touched the glass. The protruding older parts of the roots were sliced off flush with the Perspex with a microtome blade and the block was inverted. The Perspex sheets were then removed one by one and the exposed segments sliced off. The whole operation took 10–15 min.

The lengths of the segments are chosen so that the first includes the meristem (potentially dividing cells) and the root cap, but few developed or elongating cells. The segment consists therefore of largely undifferentiated tissue. The second segment is a zone of increased metabolic activity, including protein synthesis (see Heyes & Brown, 1956). There is some cell enlargement and cell vacuoles appear. This segment therefore shows the beginnings of differentiation. The oldest part of the second segment and the youngest of the third is the zone with the most rapid rate of cell elongation. Cells of the third segment are very much larger and vacuolated, although the final cell volume is not reached until some 10–11 mm. from the tip. The average weight and number of cells per segment is shown in Table 1.

Table 1. *Weights and numbers of cells of root segments*

Serial segments were cut as described in the text. Cell counts are the averages of six determinations of a batch of 20 segments, by the method of Brown & Broadbent (1950).

Segment	Length (mm.)	Wt. (mg.)	10^{-4} × No. of cells	10^2 × Average wt./cell (μg.)
1	1·6	0·837	10·6	0·79
2	1·8	1·61	8·97	1·8
3	3·0	3·07	6·12	5·0

Preparation of microsomes. The root segments were cooled as soon as cut, to 0–5°, and all subsequent manipulations were done at this temperature. The segments were ground by hand in a glass homogenizer, 165 segments in 3 ml. of a medium at pH 6·9, containing 4 mM-KH_2PO_4, 2·5 mM-tris, 0·01 mM-$MgSO_4$ and 0·5 M-sucrose, being used. The buffer concentration was low since it was found that high ionic concentrations dissolved some microsomal protein and RNA. The Mg^{2+} ion concentration was that used by Webster (1957) for amino acid-incorporation experiments in pea-seedling microsomes. In a few experiments a similar medium at pH 7·5 containing 4 mM-tris (instead of 2·5 mM) was used, and in others 0·4 M-sucrose alone.

The homogenate was centrifuged at 12 500*g* for 30 min. to sediment cell debris, nuclei and mitochondria. The supernatant was centrifuged at 30 000*g* for 30 min. Very little material was sedimented under these conditions, indicating that the sedimentation properties of the mitochondria and microsomes are different and that few intermediate-sized particles are present. The small sediment obtained will be referred to as fraction X and is discussed in more detail below. The remaining supernatant contained a little white floating material and the microsomes had begun to sediment a little, as indicated by schlieren effects on stirring. The whole supernatant was decanted and mixed, and portions (1 ml.) were pipetted into 7 ml. Lustgroid Spinco ultracentrifuge tubes. The tubes were filled with *n*-heptane and centrifuged in the 40·2 rotor at 40 000 rev./min. for 90 min. This gives an average centrifugal force in the 1 ml. sample of about 140 000*g*. The microsomal pellets obtained were almost transparent, colourless or pale yellow from the first segment and yellow from segments 2 and 3. The tubes were inverted to drain and as much as possible of the supernatant liquid was removed with filter paper.

Separation into two microsomal components. If the tubes were left inverted for 5–15 min., the yellow part of each pellet slid down the side of the tube, leaving behind a small colourless firm pellet. Two microsomal components were thus obtained. The yellow sliding pellet will be referred to as 'light' microsomes and the colourless firm one as 'heavy' microsomes. Preparations from the first root segment gave no 'light' microsomes which could be distinguished from traces of the supernatant liquid. 'Light' microsomal pellets from the second root segment were slightly gelatinous and tended to spread out while sliding down the tubes. Those from the third segment retained their circular shape and were so gelatinous that the pellets could be picked up intact with a spatula. The separation of the 'light' and 'heavy' components was hastened, and the 'light' microsomes were collected, by centrifuging the inverted tubes in larger glass tubes at up to 250*g* for about 5 min. The heavy microsomes remained as firm pellets in the Lustroid tubes, although there was a slight streak down the sides of the tubes, most in preparations from the first segment and least from the third. This pellet was similar to the pellets of deoxycholate-treated ribosomes obtained from animal or bacterial cells in physical consistency, transparency and lack of colour.

Determination of ribonucleic acid and protein. Each microsomal pellet was suspended in 1 ml. of 0·5 M-perchloric acid at 0° and the white precipitate was collected by centrifuging at 3000*g* for 10 min. The pellets were extracted with 1 ml. of perchloric acid for 20 min. at 70° and again centrifuged (Littlefield, Keller, Gross & Zamecnik, 1955). The supernatants were removed and diluted with 5 ml. of water. The nucleic acid contents of the extracts were measured spectrophotometrically at 260 mμ in a 1 cm. cell, assuming that 31 μg. of the hydrolysed RNA/ml. gives an extinction of 1·0. This compares with the value used by Ts'o & Sato (1959). The first cold acid extract gave readings about 2% of the total and was neglected. To confirm the value of the extinction coefficient RNA was also determined on a few samples by the orcinol method with correction for sucrose (Slater, 1958), yeast RNA being used as a standard, and by the difference in total nitrogen determinations before and after extraction with hot perchloric acid. The three methods agreed within experimental error except that the extinction at 260 mμ of the 'heavy' microsome samples indicated values 10–15% higher than those obtained by the other methods.

Protein was determined by the Kjeldahl method with 10% selenium in sulphuric acid as the digestion catalyst (Fawcett, 1954). The ammonia was determined by the Conway method in 3 cm. diameter diffusion dishes (Conway, 1957).

Electrophoresis. The cellulose acetate-membrane method devised by Kohn (1958) was used. A buffer at pH 6·7, containing 7·5 mM-citric acid and 35 mM-K_2HPO_4, was found to be the most satisfactory of several tried. A current of 2 mA/5 cm. width of paper (about 100 v/10 cm. length) was applied for up to 3 hr. The paper was then dried at room temperature. RNA was detected by contact printing on to Ilford Document paper 50 with a Hanovia u.v. lamp emitting at 254 mμ. Protein was then stained on the same sheet, up to 0·005% of water-soluble nigrosine (Edward Gurr Ltd., London) in 5% trichloroacetic acid being used. In order to render the microsomal components soluble for electrophoresis, a number of techniques with detergents or ribonuclease were tried. The most satisfactory separations were obtained by suspending the microsomal pellets in about 5 vol. of the electrophoresis buffer containing 0·01% of pancreatic ribonuclease (Sigma Chemical Co.). Digestion of the clear suspension was continued at room temperature for 30–60 min., during which a precipitate appeared. Portions of the suspension were then applied to the acetate membrane.

Electron microscopy. The pellets were fixed in the homogenizing medium containing 1% of osmic acid, for 2–5 hr. at 0°. It was essential not to disrupt the pellets during fixation, otherwise they were easily lost, particularly the 'heavy' microsomes, which did not stain brown or black as much as did the 'light' ones. A pellet of 'heavy' microsomes was recovered by centrifuging the supernatant after

Table 2. *Yields of protein and ribonucleic acid in the cell fractions*

Batches of the three root segments were homogenized and centrifuged as described in the text. 'Debris' refers to all material, including mitochondria, sedimented at 12 500*g* for 30 min.; fraction X, 30 000*g*, and microsomes, 140 000*g*, for 90 min. Figures are the averages of two determinations.

	Protein (percentage of total)				Total protein (mg./g. fresh wt.)
Segment	'Debris'	Fraction X	Microsome	Soluble	
1	32	5·0	15	48	42
2	30	5·4	19	46	37
3	35	6·0	13	45	16
	Ribonucleic acid (percentage of total)				Total nucleic acid (mg./g. fresh wt.)
Segment	'Debris'*	Fraction X	Microsome	Soluble	
1	24	8·5	52	15	13
2	19	8·3	54	18	12
3	22	10·6	37	30	4·6

* Includes DNA.

fixation. The pellets were dehydrated with increasing concentrations of ethanol and left overnight in 1 % of phosphotungstic acid in ethanol (Wakid, 1960). After washing in ethanol they were embedded in methacrylate at 45°, sectioned and examined in the Siemens Elmiscop I at 40 kv and an objective aperture of 30 mμ.

RESULTS

Yields of the cell components. Table 2 shows the percentage distribution of the cell fractions obtained from the three root segments as described above. A large proportion of the RNA of the cell is sedimented with the microsomes, but the percentage falls slightly in the third segment. The yield of fraction X is small, particularly in terms of RNA, and increases slightly in the third segment. This fraction, however, can interfere with the separation of the 'light' and 'heavy' microsome components if it is not removed by centrifuging at 30 000*g*. It causes the 'heavy' pellet to break up when the inverted Lustroid tube is centrifuged at 250*g*, resulting in an apparently increased yield of 'light' microsomes and considerable streaking down the sides of the tubes. Thus although it is possible that fraction X includes some microsomal material, it behaves in a distinct manner during these isolation procedures, and on the basis of total yield it can only include a small proportion of the microsomes. It is believed therefore that the homogenization and centrifuging procedures described are satisfactory for the isolation of the bulk of the microsomes. The exact conditions have been found not to be critical.

Yields of 'light' and 'heavy' microsomes. The fact that the separation of the microsomal pellet into two components occurs in the inverted Lustroid tube under gravity alone, and yet the 'heavy' pellet remains intact at 250*g*, indicates that the two pellets have widely different physical properties. This is confirmed by the electron microscopy described below, in which it is shown that the 'heavy' microsomes consist of ribonucleoprotein particles and the 'light' of a mixture including vesicles. The separation was found to be reproducible and not to be sensitive to small changes in conditions. The amounts of 'heavy' and 'light' microsomes obtained from the three segments were determined in terms of their RNA and protein contents. The results are shown as μg. of RNA or of protein/cell in Table 3. The amount of 'heavy' microsomal protein remains constant at first and falls in the more mature cells of the third root segment, whereas the 'light' increases rapidly with

Table 3. *Yields of 'heavy' and of 'light' microsomes from the three root segments*

Yields are expressed as protein or RNA per cell in the three root segments. Averages of two determinations from one homogenization are given.

	10^5 × Protein (μg./cell)		10^5 × RNA (μg./cell)	
Segment	'Heavy'	'Light'	'Heavy'	'Light'
1	4·72	0·92	5·14	0·78
2	4·85	8·99	7·45	5·28
3	2·48	9·49	4·26	5·12

Table 4. *Ratios of yields of 'light' to 'heavy' microsomes obtained by homogenization in different media*

	Protein ratio of 'light':'heavy' microsomes		RNA ratio of 'light':'heavy' microsomes	
Segment	pH 6·9 medium	Sucrose alone	pH 6·9 medium	Sucrose alone
1	0·19	0·28	0·15	0·16
2	1·8	1·3	0·70	0·79
3	3·8	4·2	1·2	1·6

cell age. Most of this increase normally occurs between the first and second segments and there is little further change in the third. Occasionally an increase between the second and third segments was found, but then the yield of 'light' microsomal protein from the second segment was correspondingly low. These variations are probably caused by slight differences in the conditions of growth, resulting in different lengths of meristem. The results expressed in terms of RNA are similar except for the high RNA content of 'heavy' microsomes from the second segment.

Resuspension and re-centrifuging of the 'light' pellets gave a negligible further amount of 'heavy' and re-centrifuging of the 'heavy' gave no further 'light'. This shows that the separation obtained by this method was complete.

These results are unaffected by changes in the composition of the homogenization medium. A medium containing 1 mM-Mg^{2+} ion and 0·5 mM-Ca^{2+} ion, which would be expected to cause some aggregation of the particles, retarded the separation of the 'light' from the 'heavy' pellet but did not affect the results. Resuspension of the pellets in this medium caused appreciable precipitation of the microsomes. Sucrose alone was used as homogenizing medium by Ts'o, Bonner & Vinograd (1956) and Ts'o & Sato (1959), who isolated microsomes consisting entirely of ribonucleoprotein particles. Table 4 shows the ratios of 'light' to 'heavy' microsomes obtained by homogenization in the pH 6·9 medium and in sucrose alone.

Although small amounts of salts did not seem to affect the isolation of the microsomes, it was possible that material released from the cell vacuoles during homogenization could do so. It is for this reason, as well as convenience in handling small quantities, that a large volume of medium was used for homogenization. As a further check that different materials released by the three segments do not influence the yield of microsomes, the first segment was homogenized mixed with either the second or the third segment. The yields obtained were compared with those calculated from the sum of the tip and basal segments homogenized separately. The results are shown in Table 5. The agreement with the calculated values is good, considering the large differences between the three segments (see Table 4).

It is concluded therefore that the change in the relative amounts of the 'light' and 'heavy' microsomes with the age of the cells is not an artifact due to the homogenizing medium or to the different substances released by the three segments.

Nucleic acid content of the microsomes. Table 6 shows the ratio of RNA to protein in the 'light' and 'heavy' components. The values obtained for the 'heavy' component compare with the highest

Table 5. *Ratio of amounts of 'light' to 'heavy' microsomal protein and RNA obtained from the tip and basal segments mixed, compared with ratios calculated from the sum of segments homogenized separately*

Microsomes were isolated as described in the text: 82 3·4 mm. root tips (segments 1 and 2 together), or 82 each of segments 1 and 3 mixed, per 3 ml. of medium were used. Averages of two determinations are given.

Segments	Yield measured as	Ratio obtained from mixed segments	Ratio calculated from sum of segments (from Table 3)
1 and 2	Protein	0·87	0·98
	RNA	0·37	0·45
1 and 3	Protein	1·4	1·1
	RNA	0·43	0·49

Table 6. *Ratio of ribonucleic acid to protein of the microsome components*

Details are as given for Table 4.

	'Heavy' microsomes		'Light' microsomes	
Segment	pH 6·9 medium	Sucrose alone	pH 6·9 medium	Sucrose alone
1	1·09	0·82	0·85	0·48
2	1·53	1·19	0·59	0·71
3	1·71	1·33	0·54	0·50

reported by Peterman, Hamilton, Balis, Samarth & Pecora (1958) for deoxycholate-treated animal microsomes. Peterman *et al.* reported a probable error in the values and there is likely to be a similar one for the 'heavy' microsomes, as mentioned in the Materials and Methods section. The values are nevertheless considerably higher than those obtained previously for plant tissues. The proportion of RNA increases with the age of the cells in 'heavy' microsomes but decreases in the third segment in 'light'. The net result is a decrease, since the total amount of the 'light' microsomes increases. The differences between the values obtained with the pH 6·9 medium or sucrose alone are probably not significant. The errors become large when the total yield is small, as in 'light' microsomes from the first segment, which were obtained in only just detectable quantities.

Electrophoresis. Fig. 1 shows the electrophoretic patterns obtained from the two microsome components after treatment with ribonuclease. The bands of the nucleic acid components were reproducible whereas the protein patterns varied slightly in different experiments. The 'light' microsomes show two positively charged bands, of which only traces appear in the 'heavy', in addition to some insoluble material. The protein pattern also shows

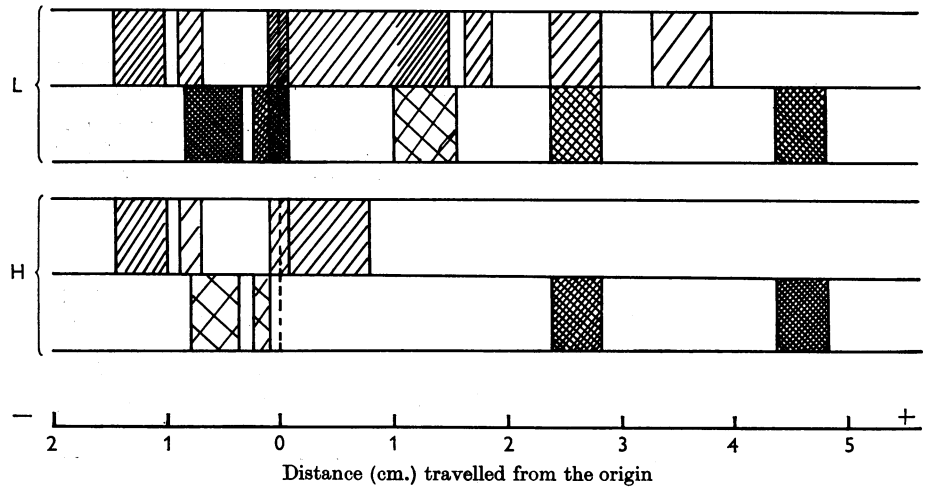


Fig. 1. Electrophoresis of 'light' and 'heavy' microsomes after treatment with ribonuclease. Diagonal shading represents protein fragments, and the cross-hatching, ribonucleic acid fragments. Density of shading represents the approximate strength of the staining or u.v. absorption, the lightest being barely detectable. L, 'Light' microsomes; H, 'heavy' microsomes. Patterns obtained from the three root segments were identical except that the yield of 'light' microsomes from the first segment was too small to use. The most cathodic protein band is the ribonuclease.

a greater complexity in the 'light' than in the 'heavy' microsomes. Except for the insoluble material there is little association between nucleic acid and protein.

These electrophoretic separations are sufficient to show that the two microsome fractions are in part different, the 'light' being more complex. No differences, however, could be detected between preparations from the different root segments.

Electron microscopy. Plate 1 shows (A) an electron micrograph of 'heavy' microsomes, showing that these consist largely of particles about 15 mμ in diameter, very similar to the ribosomes obtained from animal tissues. The particles usually occur in chains or groups, and a dense body, 30–100 mμ in diameter, is frequently associated with each group. Vesicles are very rarely found in preparations of 'heavy' microsomes.

The 'light' microsomes (Plate 1, B and C) vary in appearance in different regions of the pellet. Densely packed zones (B) differ from 'heavy' microsomes in the presence of large numbers of vesicles, 50 mμ or more in diameter. More open zones (C) show very much larger vesicles, some dense bodies and few particles. The particles occur in chains, frequently attached to the dense bodies. The vesicles are 'smooth-surfaced'. In some cases there is a continuous gradation of density between smaller vesicles devoid of contents and the dense bodies, but usually the two are distinct. No systematic differences between the different root segments were found, but the vesicles of the third segment seemed usually to be larger and more numerous than those of the second. 'Light' microsomes of the first segment, obtained in very small amounts, were lost during fixation.

Hodge, Martin & Morton (1957) have also described dense bodies isolated from silver beet, but they found no association of dense bodies with ribosomes.

Fraction X was found to consist of a mixture of vesicular material, including small mitochondria-like particles and some vesicles similar to those found in 'light' microsomes. Ribonucleoprotein-like particles were very rarely found, and this

EXPLANATION OF PLATE 1

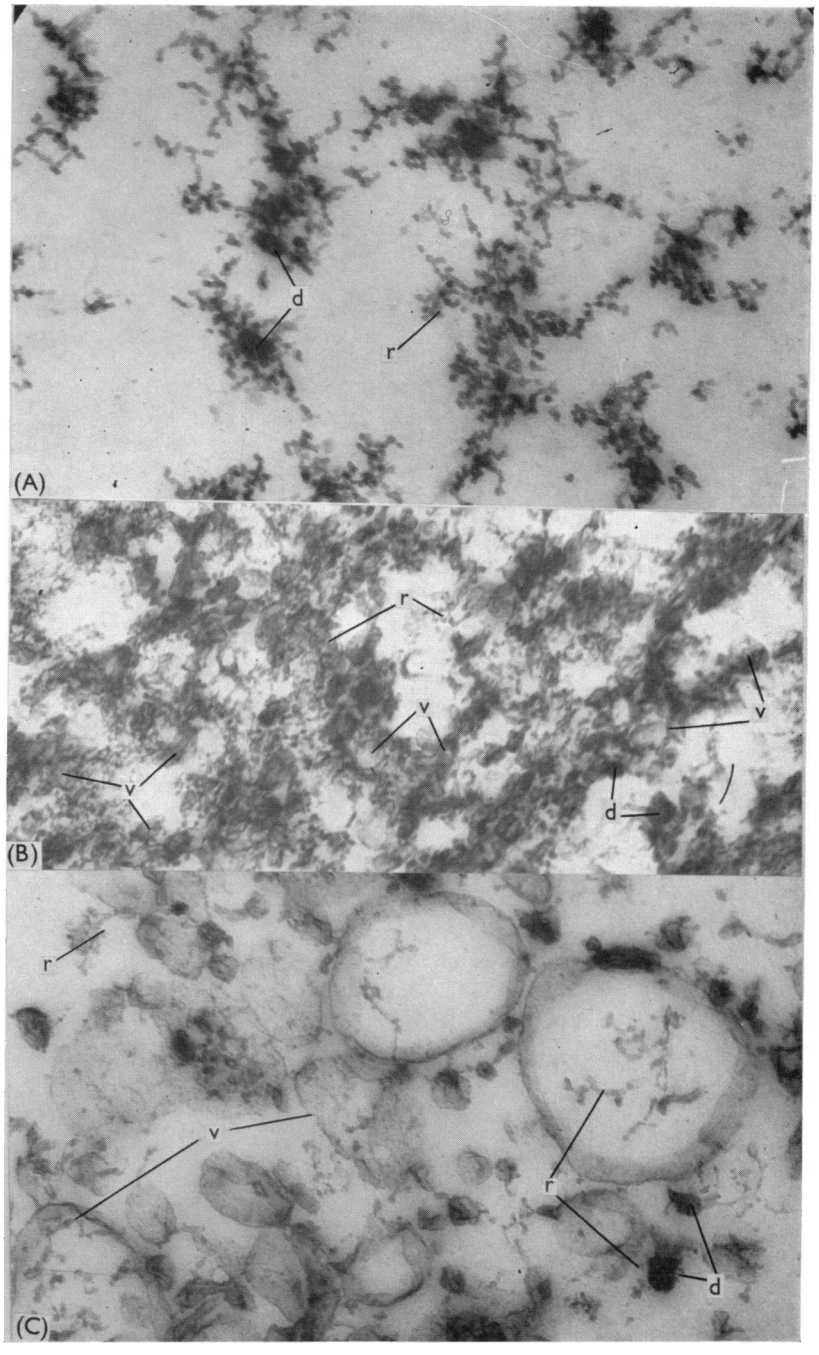
Electron micrographs of fixed and sectioned microsomal pellets (×50 000 approximately).

(A) 'Heavy' microsomes isolated from 6 mm. root tips, showing ribosomes (r) 15 mμ in diameter, frequently occurring in clusters in association with dense bodies (d).

(B) A densely packed region of a 'light' microsome pellet, consisting of vesicles (v) in addition to dense bodies (d) and ribosomes (r).

(C) A more open region of a 'light' microsome pellet showing larger vesicles (v) and a few ribosomes (r) and dense bodies (d).

(B) is from the second root segment and (C) from the third, but both are representative of regions in either pellet.


d
r
(A)
r
v
v
v
d
(B)
r
v
r
d
(C)

fraction is thus quite distinct from the 'heavy' microsomes. This again indicates that the centrifuging procedure was satisfactory.

DISCUSSION

The experiments described show that a particulate and a more membranous fraction of microsomes may be separately isolated from root tips without the use of deoxycholate. Quantitative measurement of these isolated fractions shows that the proportion of membranous material increases with the age of the cell. This confirms the trend indicated by Lund, Vatter & Hanson (1958) and others, but the use of small tip segments has shown that most of the change occurs immediately before the zone of rapid cell elongation.

The separation into the 'light' and 'heavy' microsomes is sharp and reproducible, and the membranes and particles of the 'light' fraction cannot be further separated by the same techniques. It seems therefore that a true fractionation into two microsomal components has been obtained, and this is confirmed by the electron microscopy and electrophoresis. Preliminary experiments have also shown that the 'light' microsomes have a much higher reduced DPN–cytochrome *c* reductase activity than the 'heavy', in agreement with the findings of Palade & Siekevitz (1956) and others that this activity is located in the membranous fraction. The electrophoresis of the ribonuclease-treated microsomes also suggests that the ribonucleoprotein particles present in the 'light' microsomes are in some way different from those in the 'heavy'. It is possible that fraction X includes some 'light' microsomal vesicles, as indicated by the electron microscopy. The yields of fraction X were small, but if added to the yields of 'light' microsomes they would accentuate the results presented in this paper.

It is clear that the morphology and origin of these plant microsomes require further study. Electron micrographs showing the structure of the cytoplasm of young but not of mature cells have been published (e.g. Whaley *et al.* 1960). Evidence indicating which structures give rise to isolated microsomes after homogenization and centrifuging is, however, inconclusive. Nevertheless the results obtained here indicate that there is a synthesis of a membrane and particle complex at the onset of differentiation but not during the subsequent phase of cell expansion. This complex may be regarded as comparable with rough-surfaced vesicles obtained from animal tissues, although the detailed structure is different.

The plasmalemma and tonoplast do not seem to contribute to the isolated microsomes, since there is little increase in the yield when the cells expand. It is possible, however, that the smooth-surfaced vesicles obtained in the 'light' microsomes from the third root segment arise from a breakdown of the cell membranes, whereas similar vesicles from the second segment arise from the precursors of these membranes.

SUMMARY

1. Microsomes were extracted from small serial segments of seedling pea-root tips by homogenization and centrifuging. A 'light' microme pellet consisting of a mixture of vesicles and ribosomes could be slid off the microsomal pellet, leaving a small firm pellet of 'heavy' microsomes consisting largely of ribosomes.

2. The protein and ribonucleic acid contents of the two fractions were determined and expressed as the yield per cell. The meristematic tip segment contained largely 'heavy' microsomes, and the yield of these fell with the age and maturation of the tissue. The yield of 'light' microsomes, however, increased rapidly at the earliest stages of differentiation.

3. The ribonucleic acid to protein ratio in the 'heavy' microsomes was above 1·0 and increased with the age of the tissue, whereas the ratio in the 'light' microsomes was below 0·8 and decreased with age.

4. Electrophoresis of the two fractions after disruption with ribonuclease gave more nucleic acid fragments and proteins in the 'light' microsomes than in the 'heavy'.

5. Electron micrographs of the two fractions are presented.

6. It is suggested that the synthesis of 'light' microsomes is associated with the onset of differentiation.

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New Pigments Derived from Vitamin B_{12} by a Strain of *Aerobacter aerogenes* Present in River Mud

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Micro-organisms have proved very useful in producing not only vitamin B_{12}, but also vitamin B_{12} analogues and some of their derivatives. Thus a number of vitamin B_{12} analogues containing either purines or benziminazoles have been prepared by guided biosynthesis with a vitamin-B_{12}-requiring mutant of *Escherichia coli* (Ford, Holdsworth & Kon, 1955; Bernhauer & Friedrich, 1954). As a result of microbial activity a number of vitamin B_{12} analogues have been isolated from the gut and rumen contents, faeces of ruminants (Kon, 1955; Porter, 1957; Dion, Calkins & Pfiffner, 1952; Lewis, Tappan & Elvehjem, 1952*a*, *b*; Brown, Cain, Gant, Parker & Smith, 1955) and from sewage sludge (Friedrich & Bernhauer, 1953; Neujahr, 1956).

Phosphorylated factor B and guanosine diphosphate factor B have been isolated from cultures of *Nocardia rugosa* (Barchielli *et al.* 1960). A number of intermediates in the biosynthesis of vitamin B_{12} have been isolated from *Propionibacterium shermanii* (Bernhauer, Becher, Gross & Wilharm, 1960); these include nucleotide-free carboxylic acids (amides of cobinic acid) and vitamin B_{12} carboxylic acids. A new group of coenzymes, which are derivatives of vitamin B_{12} or certain analogues, have been prepared from *Clostridium tetanomorphum* (Barker *et al.* 1960*a*). The vitamin B_{12} coenzyme has also been prepared from *P. shermanii* (Barker *et al.* 1960*b*).

Compared with the work quoted above surprisingly little has been reported on the action of micro-organisms on vitamin B_{12} and its analogues. Ford & Porter (1953) have shown that organisms present in calf faeces will induce interconversion between analogues, and micro-organisms from rotten fish were shown to degrade vitamin B_{12} to unidentified products (Mori, Hashimato & Malda, 1953, 1954; Mori & Malda, 1955). In the present work the action of *Aerobacter aerogenes* on vitamin B_{12} is described.

A preliminary account of some of this work has already been given (Helgeland, Jonsen & Laland, 1959).

MATERIALS AND METHODS

Vitamin B_{12}. This was a gift from Glaxo Laboratories Ltd., Greenford, Middlesex.

[^{58}Co]*Vitamin B_{12}*. this was purchased from The Radiochemical Centre, Amersham, Bucks., and had a specific activity of 0·22 μc/μg.

Media. The synthetic medium contained: NH_4Cl, 2 g., $Na_2HPO_4,2H_2O$, 6 g.; KH_2PO_4, 3 g.; NaCl, 3 g.; $MgCl_2,6H_2O$, 0·04 g.; Na_2SO_4, 0·14 g.; water to 900 ml. The pH was adjusted to 7·2 with 10% NaOH before autoclaving. Depending on the experimental conditions, to 9 vol. of media was added either (*a*) 1 vol. of sterile water or (*b*) 1 vol. of a boiled solution containing glucose and vitamin B_{12} or vitamin B_{12} only.

The meat-extract medium contained: beef extract (Difco), 1·5 g.; peptone (Difco) 5 g.; $Na_2HPO_4,2H_2O$, 1 g.; water to a total volume of 500 ml. The pH of the solution was adjusted to 7·8 with 10% NaOH before autoclaving. When required, 0·1 mg. of vitamin B_{12}/ml. was added before autoclaving.

The agar medium contained: meat extract (Difco), 9 g.; peptone (Difco), 30 g.; $Na_2HPO_4,2H_2O$, 6·76 g.; NaCl, 9 g.; water to 1500 ml. The pH of the solution was adjusted to 8·8 with 10% NaOH and agar (37·5 g.) was added before autoclaving.

Subculturing of micro-organism obtained from river mud. A portion (0·1 ml.) of a suspension in 0·9% NaCl solution of mud obtained from a stream in the suburbs of Oslo was used to inoculate 3 ml. of the synthetic medium containing 0·34% (w/v) of glucose and 0·3 mg. of vitamin B_{12}.