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Some properties of otoconia

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[Plates 1 and 2]

Otoconia are dynamic mineral deposits present in the gravity receptors of most vertebrates; fishes often have a single large mass called an otolith instead. Otoconia generally have the appearance of single crystals but contain organic and inorganic components, the mineral being almost exclusively a polymorph of calcium carbonate. The two phases are closely interrelated structurally. Ultra-high resolution transmission electron microscopy of rat otoconia showed them to be mosaic biominerals. The crystallites were 50–100 nm in diameter, had some rounded edges, and were highly ordered into laminae. This suggests that crystallite seeding and growth is organic matrix mediated. Crystallite asymmetry may also indicate piezoelectricity. A further finding of similarities in electron beam diffraction patterns obtained from some frog and rat otoconia could mean that the calcite of mammalian units mimics aragonite. A comparative study showed that turtles, which are close to the stem line for mammals, had calcite-type otoconia in the utricle. Alligators, which share a common ancestry with birds, had this type otoconium in all three gravity receptors, although saccular otoconia had a variety of forms. The nature of the mineral is unknown. The biochemical composition of the organic otoconial material is under study, to learn how mineral deposition is regulated. Proteins of rat otoconial complexes ranged between *ca.* 16500 and over 100000 Da in molecular mass and were similar in saccular and utricular otoconial complexes. Our new analysis of the amino acid composition of the complexes by high performance liquid chromatography showed the complexes to be high in the acidic and low in the basic amino acids. This is comparable to what has already been reported for other biomineralized materials that contain calcite.

Introduction

Otoconia (literally, 'ear dust') are minute, crystalline particles present in the gravity receptors of the vestibular labyrinth in most vertebrates (figures 1–3, plates 1 and 2). In fishes there is greater variation in the form taken by the inner ear mineral deposits, the form is often a single mass ('stone') called an otolith; but mixtures of otoliths and otoconia occur in some species.

Both otoconia and otoliths contain organic material that is continuous with extraneous organic substance, the otoconial (or otolithic) membrane. This membrane links the otoconia together and connects the otolith or otoconial mass to the underlying neuroepithelium, where the sensory (hair) cells are located. It is commonly accepted that the mineral deposits add mass to the organic membranes, increasing their sensitivity to gravitational and other linear acceleratory forces, and that movement of the mass relative to the ciliary tufts of the hair cells signals the direction of applied force.

There are three receptors with mineral deposits in non-placental vertebrates having jaws: the maculas of the saccule, the utricle, and the lagena. Agnatha (jawless fishes) have a single

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vestibular macula; however, in Lampetra (lampreys) the single receptor area can be subdivided into parts homologous with the utricle, saccule and lagena (Lowenstein *et al.* 1968). Placental mammals lack a lagena.

The mineral of otoliths and otoconia is a polymorph of calcium carbonate except in Agnatha, in which the spherical otoconia (figure 1 (*a*)) consist predominately of calcium phosphate in low- or non-crystalline state (Carlstrom 1963). Moreover, the otoconial mineral is generally laid down in the shape of single crystals of aragonite or calcite (figures 2–3), with aragonitic otoconia confined to cold blooded species. Vaterite is rarer, but is found in the otoconia and some otoliths of certain fishes in the class Actinopterygii (Carlstrom 1963). In the gar pike (*Lepisosteus osseus*), the saccular otoconia are shaped like biconvex discs (figure 1 (*b*)) and contribute to the formation of the saccular otolith. Such otoconia were described as vateritic, but gar pike otoliths, which differ greatly in morphology at the three receptor sites, were said to consist of pure aragonite (Carlstrom 1963).

These facts, taken together, begin to pose interesting questions to investigators working in the field of biomineralization. Why should a primitive fish that lacks bone mineral and true teeth use mainly calcium phosphate deposits in its gravity receptors? What were the selective pressures that led other vertebrates with bony skeletons or teeth, or both, which contain deposits of calcium phosphate, to employ a polymorph of calcium carbonate in their inner ear mineral masses instead? Why is it necessary that the otoconia of most vertebrates have an outward morphology that mimics that of single crystals of aragonite or calcite, while the bones and teeth assume shapes totally unrelated to the mineral crystallites of which they are composed? What are the unique mechanisms underlying such 'crystal' formation in a living organism? And, why should the various gravity receptors of a single labyrinth contain mineral deposits of differing appearances? To begin to answer these questions, we must learn more about the functions of otoconia and otoliths, and more about the nature of the organic material that they contain. To this end, we have conducted a series of morphological and analytical investigations to explore the crystallographic nature and calcium binding properties of otoconia. More recently, we began a comparative study of the protein, amino acid and sugar composition of the organic phase of inner ear mineral deposits in species representative of vertebrate phylogeny. Some of our more interesting observations are discussed here.

Calcium binding and structural properties of mammalian otoconia

Several *in vivo* and *in vitro* experiments (Belanger 1953, 1960; Preston *et al.* 1975; Ross 1979; Ross & Williams 1979; Ross *et al.* 1980) have demonstrated that otoconia are dynamic and not merely inert, mass-contributing elements. Under *in vivo* conditions they took up radioactive calcium ions within the same rapid time frame as bone mineral but on a lesser scale, with saccular uptake both more rapid and greater than utricular. They began to give up the sequestered calcium ions more quickly, within 4 days, while levels of radioactive calcium in the bone mineral remained stable over the period of the experiment, 28 days (Ross & Williams 1979). *In vitro* results indicated that uptake was more rapid and greater when living cells did not stand between the calcium ions and the otoconia (Belanger 1953; Ross *et al.* 1980). Moreover, calculation of coefficients of activity of all the ions in the system demonstrated that the otoconia should have dissolved rapidly in the incubating fluid, carbon dioxide-buffered artifical endolymph, but they remained intact (Ross *et al.* 1980). So, both the rate of uptake

and the maintenance of the otoconia in non-ideal solutions strongly suggest that organic material functions in calcium ion binding, exchange and release by the complexes.

The location of the calcium sequestering organic material of the complexes is less easily determined. Is it a part of the organic, otoconial membrane, on the surfaces of the otoconia, or both? This question cannot be answered by studying the surface features of the otoconia themselves. Rat otoconia (figure 2 (a)), like those of other mammals and birds, have the outward form of single crystals of calcite. There are three terminal faces, which have sharp edges where they intersect, and have three-fold symmetry characteristic of single calcite crystals (Ross & Peacor 1976). The end faces are planar in contrast to the side faces, which are rounded and virtual rather than real. A thin layer of organic material may, and probably does, cover the otoconial mineral, even at the terminal faces; but this is difficult to ascertain.

Transmission electron microscopy of sectioned, intact fetal otoconia shows that the organic and mineral phases follow one another precisely (Salamat et al. 1980). There is a central core and a peripheral zone. The size of the core seems to be related to the ultimate size of the otoconium. Furthermore, comparison of the sharp edges observed in sections of mineralized specimens with the more rounded protuberances seen in scanning electron micrographs of entire otoconia strongly suggests that organic material covers the developing crystals. But this does not answer the question of distribution of organic material in or on an adult crystal, since it is well known that diminution of the organic phase generally coincides with maturation of the crystalline phase in other biomineralized substances such as tooth enamel (Deakins 1942; Glimcher et al. 1964a, b; Angmar-Mansson 1971) and bone (Toole & Linsenmayer 1977).

The composite nature of otoconia and the piezoelectric effect

Study of foetal otoconia, nevertheless, does begin to provide evidence that, although otoconia outwardly resemble single crystals, ultrastructurally they are composites of highly ordered crystallites. Conclusive evidence of their composite nature comes from ultra-high resolution transmission electron microscope studies of fragmented otoconia (Parker et al. 1983; Mann et al. 1983), which have shown that the structural units of rat otoconia are crystallites 50–100 nm in diameter (figure 2 (b)). The crystallites were arranged in sheets of highly ordered domains. The domains were slightly out of phase from one layer to the next, thereby forming a 'mosaic biomineral'. Rat otoconial fragments, nevertheless, yielded diffraction patterns typical for specific faces of single crystals of inorganic calcite, predominantly the (001) face. The faces did not correspond, however, to those most commonly produced when pure inorganic calcite (Iceland spar) was similarly fractured. The latter tended to be high index faces. This result, together with the unique morphology of the otoconial subunits (they have some rounded edges) strongly suggest that organic material influences the seeding and growth of the crystallites. This would correspond to a type of 'matrix-mediated' mineralization (Lowenstam 1981). The enigmas remain, however, as to how the organic material can organize the asymmetrical crystallites into the form of single crystals, and why it is important to do so.

One answer to the question of the importance of the single-crystal otoconial form might be that a higher degree of localized sensitivity to linear acceleratory force results when small masses of different sizes are unequally distributed over the appropriate sensory area than would be possible in a system dominated by a single, large mass. But another answer might lie in the very fact that otoconial subunits are asymmetrical. Such asymmetry could confer the property

of piezoelectricity on otoconia (Ross 1983), an attribute already demonstrated for otoliths (Morris & Kittleman 1967). We could not consider this possibility if the otoconia were constructed of unit cells typical of inorganic calcite, which, because of their inherent symmetry, are not piezoelectric. (A piezoelectric crystal is one that becomes electrically charged or polarized when mechanically stressed or, conversely, changes shape in an electric field. Such crystals do not have a centre of symmetry.) Moreover, the proteinaceous organic material of the complexes might be piezoelectric or semiconducting. These concepts have enormous implications for how gravity receptors might work; that is, through hair cell transduction of electric field changes as otoconia are stressed by gravitational and translation linear acceleratory forces (Ross 1983), rather than by actual movement of the otoconia and 'bending' of the ciliary tufts of the hair cells. Still, the piezoelectric property is difficult to prove or disprove experimentally for otoconia, which are of microscopic size (3–50 μm long). Moreover, the use of pooled, dry specimens for such determinations possibly would make them meaningless, because the natural orientations of the crystals would be lost and conductivity could be drastically affected (Rosenberg 1962).

COMPARATIVE STUDIES OF INNER EAR MINERAL DEPOSITS

The ultra-high resolution studies, mentioned above, were done on three different kinds of inner ear mineral deposits: fish (plaice, *Pleuronectes platessa*) otoliths; and frog (*Rana pipiens*) and rat (*Rattus norvegicus*) otoconia. This comparative study was undertaken to determine whether the previous crystallographic results obtained by Carlstrom (1963), who employed X-ray powder diffraction methods, would be verified. His results indicated that otoliths of teleostean fish like plaice were polycrystalline aragonite, while amphibian and mammalian otoconia were described as single crystals of aragonite and calcite, respectively. Our findings demonstrated that inner ear minerals did not correspond either to single crystals or to polycrystalline materials, but were composites of highly ordered crystallites (mosaic biominerals). A further observation was that the diffractograms obtained from fragments of frog otoconia, which are composed of aragonite, sometimes showed single crystal, hexagonal patterns, which were nearly identical to those obtained more commonly from rat otoconia, which contain calcite. This result might indicate that the more stable calcite mimics aragonite. The question then arose whether evolution of the calcitic otoconial form preceded calcite deposition in otoconia of warm-blooded species.

The precociousness of the bird and mammal otoconial configuration is evident in the reptiles we selected for initial study because of their positions in phylogeny. These were turtles, which lie close to the stem line leading to mammals; and alligators, which share a common

DESCRIPTION OF PLATE 1

FIGURE 1. (*a*) Spherical otoconia of the lamprey (*Petromyzon marinus*) gravity receptor. They consist predominantly of calcium phosphate in non-crystalline form. (*b*) The disc-like appearance of the vateritic otoconia of a gar pike (*Lepisosteus osseus*) is shown.

FIGURE 2. (*a*) A scanning electron micrograph of rat (*Rattus norvegicus*) saccular otoconia. (*b*) A single crystallite obtained from fractured rat saccular otoconia. Note the rounded edges of the crystallite and the lattice fringe images (straight lines) that are continuous across it. Amorphous material lies close by.

FIGURE 5. After 10 washings, otoconia show some demineralization. Utricular otoconia, illustrated in (*a*), are less demineralized than the saccular (*b*).
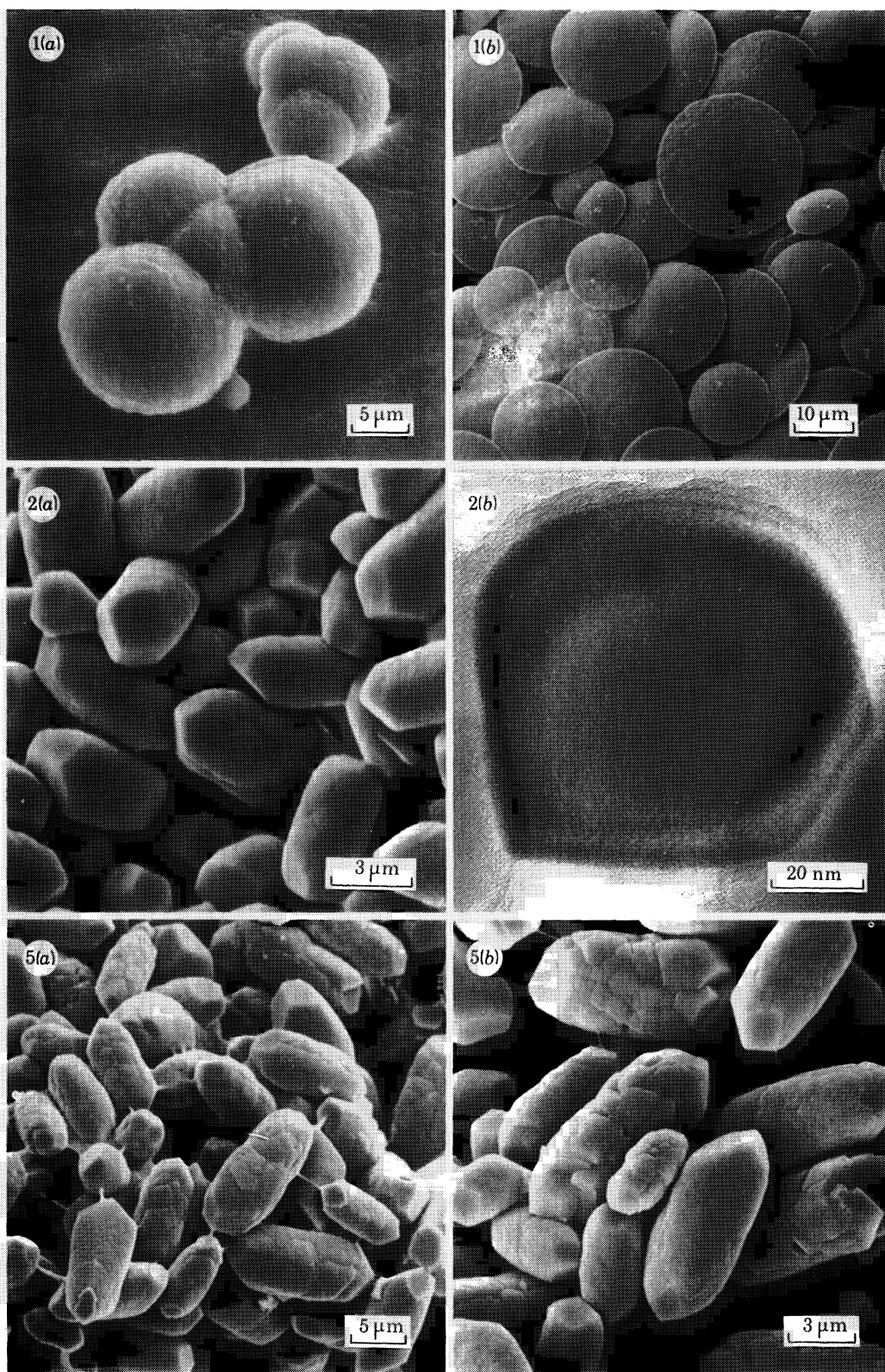
FIGURES 1, 2 and 5. For description see opposite.

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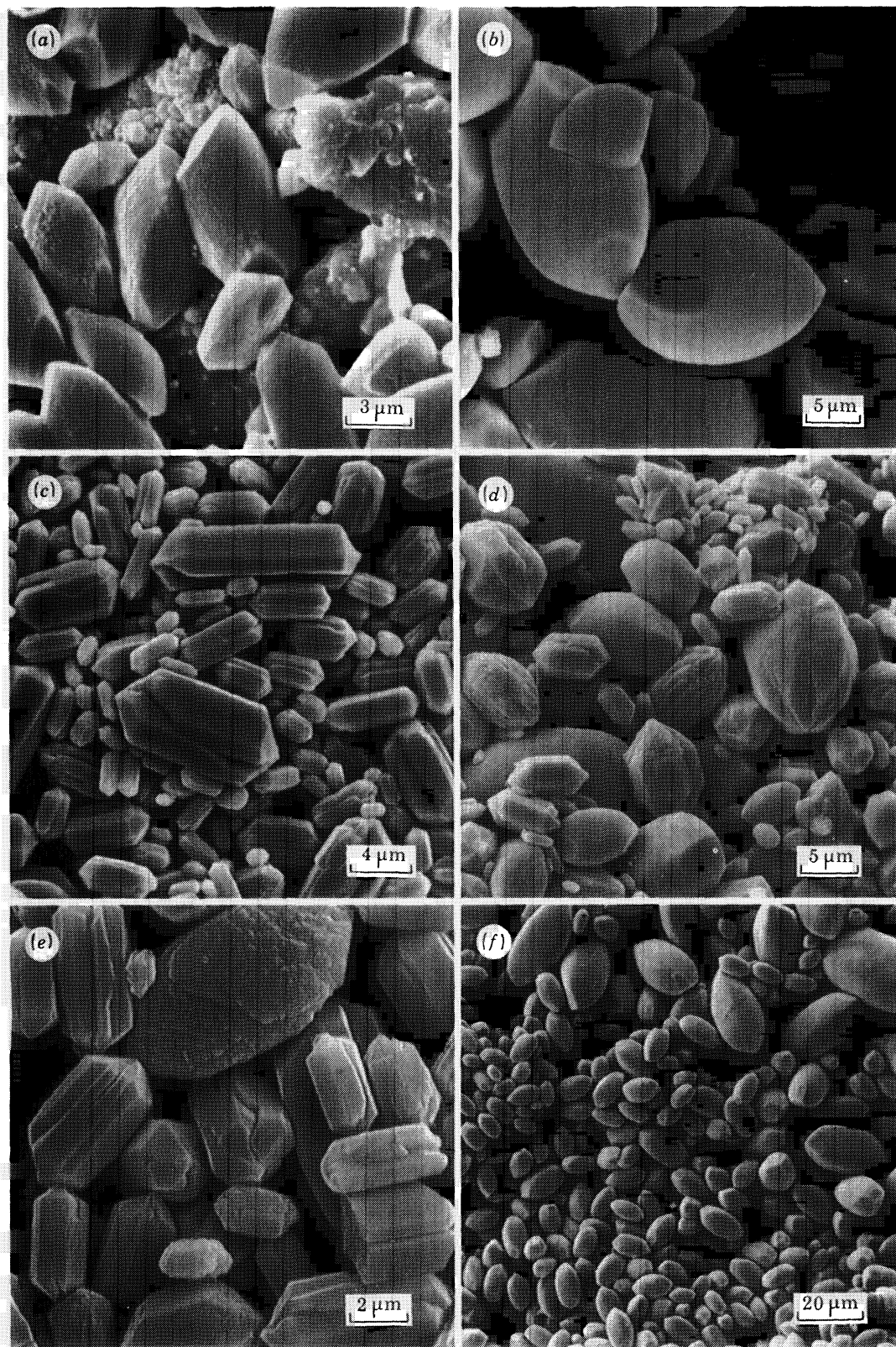
Ross & Pote, plate 2

FIGURE 3. For description see opposite.

ancestry with birds. In the western painted turtle (*Chrysemys picta belli*), hexagonal otoconia with well-defined end faces dominated the saccular and lagenar gravity receptors (figures 3 (*c*), (*e*)). Occasional otoconia with rounded bodies and pointed end faces occurred in these end organs, but the utricular otoconia were almost entirely of this type (figure 3 (*a*)). In the American alligator (*Alligator mississipiensis*) (figures 3 (*b*), (*d*), (*f*)), all the maculas had otoconia that closely resembled those of birds. However, although the utricle and lagena contained only this calcitic type, there was a mixture of otoconial configurations in the saccule.

Presence of the typical, calcitic otoconial morphology cannot be taken as proof of the presence of calcite in the absence of confirmatory, analytical data. In his study, Carlstrom (1963) indicated that he found strong lines for aragonite and just some for calcite in powder diffractograms of turtle otoconia (alligators were not studied). The more precise method of ultra-high resolution transmission electron microscopy may provide additional information on this point, particularly since Carlstrom did not analyse otoconia according to macular origin. If those otoconia of turtles and alligators having the same morphology as calcitic otoconia of birds and mammals should prove to contain calcite, the notion that warm-bloodedness is somehow pertinent to deposition of calcite in gravity receptors will be proven to be incorrect. The finding that such crystals are aragonitic would be no less interesting, because it would lend further support to the concept that the calcitic otoconial form is phylogenetically old.

BIOCHEMICAL ANALYSIS OF THE OTOCONIAL MATRIX

It has become increasingly clear as our research has progressed that detailed knowledge of the biochemical nature of the organic phase of inner ear mineral deposits is essential if we are to understand the complex interactions between organically controlled and purely physical processes that result in a specific biomineralized product. We began our analytical study of the organic material of otoconial complexes (otoconia and otoconial membranes) by employing microdisc gel electrophoresis in sodium dodecyl sulphate (SDS) (Gainer 1971) to separate proteins of rat complexes according to their relative molecular masses. Our results indicated that the proteins of saccular and utricular complexes were identical (Ross *et al.* 1981). A major protein band occurred in the gels at *ca.* 16 500 Da. This is close to the molecular mass reported for calmodulin (16 790 Da by amino acid sequencing; Klee *et al.* (1980)), a well studied, intracellular calcium-modulating protein. Interestingly, the range of masses of the proteins separated under our experimental conditions (*ca.* 16 500–over 100 000 Da) is close to that reported previously for mollusc shells containing calcite (20 000–80 000 Da; Degens (1976)). The range is very different, however, from the one obtained from another inner ear membrane, the tectorial, which is not mineralized. The tectorial membrane yielded a range between 62 000 and 180 000 Da, with a major band at 140 000 Da (Steel 1980).

To prepare ourselves for amino acid and sugar analysis of each of the proteins in otoconial

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DESCRIPTION OF PLATE 2

FIGURE 3. Turtle (*Chrysymys pecti belli*) otoconia are shown in the scanning electron micrographs on the left (*a*), (*c*), (*e*). For comparison, American alligator (*Alligator mississipiensis*) otoconia are depicted alongside (*b*), (*d*), (*f*). (*a*), (*b*) Utricular otoconia; (*c*), (*d*), saccular otoconia; (*e*), (*f*), lagenar otoconia. Otoconia with rounded bodies and three terminal faces occur in the turtle utricle (*a*) and in all three gravity receptors of the alligator (*b*), (*d*), (*f*). The alligator saccule (*d*) contains a variety of otoconia in contrast to the utricle (*b*) and lagena (*f*), in which calcite-type otoconia are present. Compare with figure 2 (*a*).
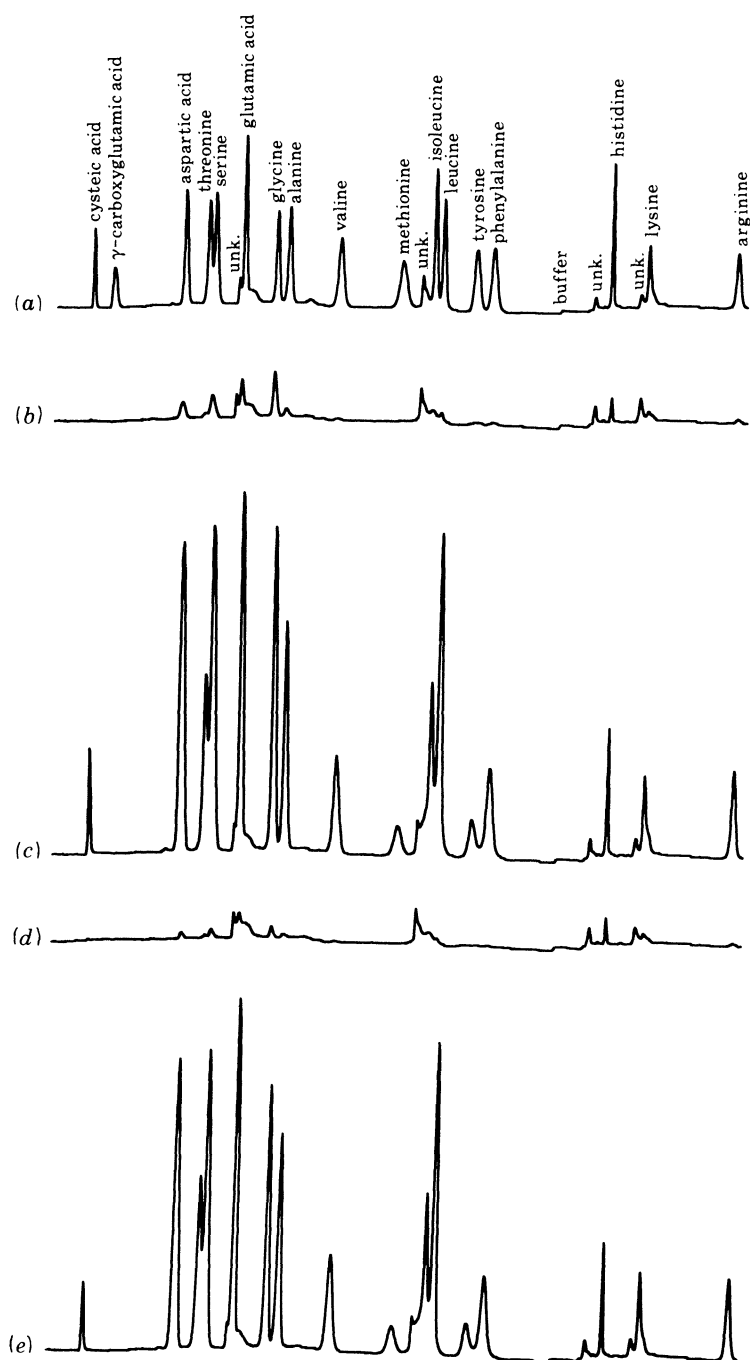
FIGURE 4. This figure illustrates a single series of chromatograms obtained on one day from an amino acid standard, from final wash solutions and from otoconial complexes. The chromatograms shown are actual copies of the originals. The amino acids are named on the standard (*a*) and the remaining chromatograms are aligned for easy comparison. (*a*) Standard, 0.5 nmol of amino acid; (*b*) final wash, utricular complexes; (*c*) utricular complexes, organic material; (*d*) final wash, saccular complexes; (*e*) saccular complexes. The wash fluids approach baseline. The otoconial complexes are high in the acidic (to the left) and low in the basic (to the right) amino acids (unk., unknown). See the text.

complexes, we have begun to develop methodology using high performance liquid chromatography. For the initial phase of this research, we determined the amino acid composition of rat otoconial complexes. Because similar research has not, to our knowledge, been accomplished before; a brief outline of our method follows.

We have employed Kratos high performance liquid chromatography instrumentation, o-phthaldialdehyde (o-PA) for post-column reaction with the amino acids (Roth 1971), and a fluorescence detector theoretically capable of detection in the 10 pmol range. Samples were micro-dissected from the temporal bones under artificial endolymph (not buffered with a continuous stream of CO_2, however) and pooled by origin from ten animals. Because otoconial complexes are subject to contamination during their collection, i.e. from endolymph and blood, the samples were washed while being agitated before hydrolysis. It was determined empirically that ten brief washings, followed by centrifugation to collect the mineral masses, were sufficient to reduce the amino acids in the wash solution to amounts approaching baseline levels (figures 4 (b), (d)). Both the wash solutions and the otoconial complexes were hydrolysed in 6 M normal hydrochloric acid for 24 h at 110 °C, dried under a stream of nitrogen, dissolved in 150 µl sodium citrate buffer (pH 2.2), and a 50 µl portion of the fluid was injected into the column. The resulting chromatograms were compared to standards containing 0.5 nmol of each of 17 common amino acids. Standards were run before and after unknowns were analysed. As a further control, washed otoconial samples were examined by scanning electron microscopy (figure 5, plate 2). The electron micrographs showed that some of the otoconia were undergoing slight demineralization at the end of ten washes (figure 5), but this provided further assurance that our analytical results reflect the amino acid composition of the complexes. It is recognized that soluble proteins may have been removed during the washing process, but this question can be addressed as our work progresses.

Our present findings indicate that the organic fraction of the complexes is high in the acidic and low in the basic amino acids (figures 4 (c), (e)). This is comparable to earlier results obtained in otoliths and shells (Degens 1976). Alanine, glycine and leucine levels are also high. The relative proportions of aspartate, glutamate, threonine and serine appear to be comparable to those found in neogastropod shells (Meenakshi 1971), which are also calcitic.

We have further preliminary findings after basic hydrolysis, which suggest that γ-carboxyglutamic acid is present in the otoconial complexes. These results require further confirmation.

Our findings indicate that rat otoconial complex proteins consist of a mixture of amino acids generally comparable to that reported for other biomineralized materials that contain a polymorph of calcium carbonate. Further study should establish more precisely the extent of similarity, as intra- and extra-otoconial organic materials are separated and analysed. Certainly, we expect the organic material responsible for seeding and protecting the crystallites, and for ordering them into the configuration of single crystals, to be unique and to show some species-dependent differences. Our evolutionary approach may shed light on how otoliths and otoconia came to have their specific mineral content and form. We hope that, additionally, this work will lead to a better understanding of the functions of these mineral deposits, and the role played by the organic matrix in their genesis, maintenance and control.

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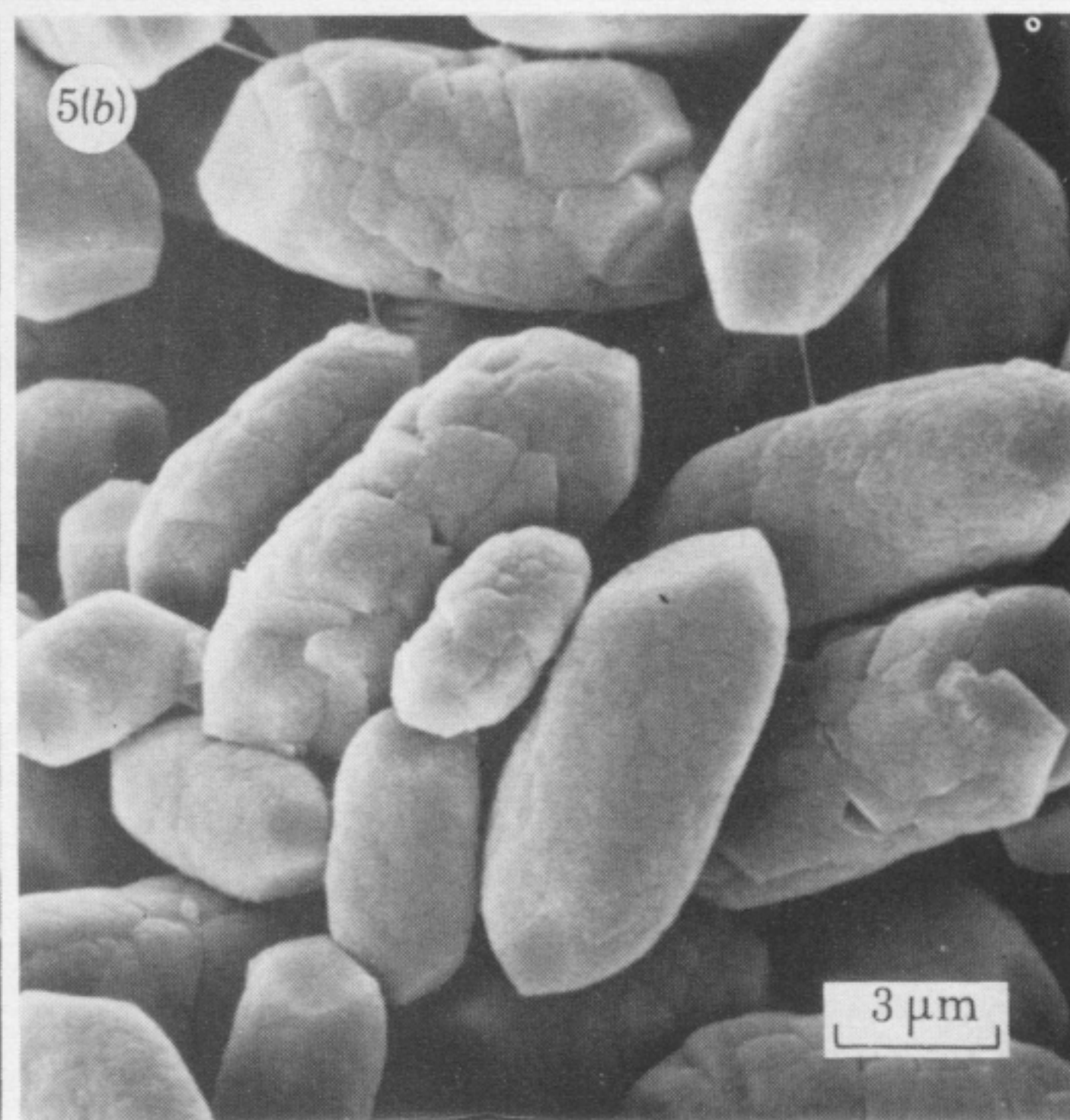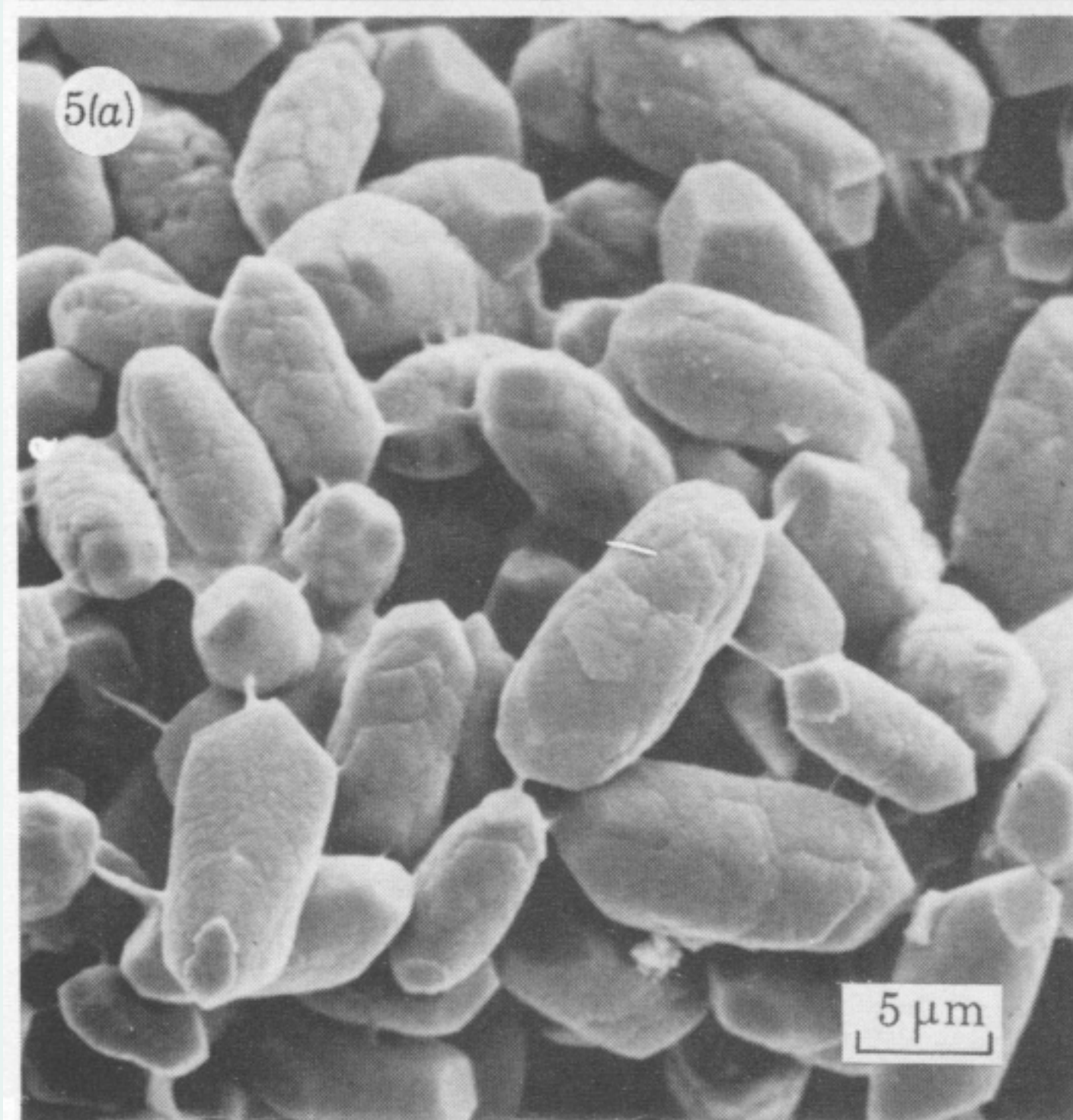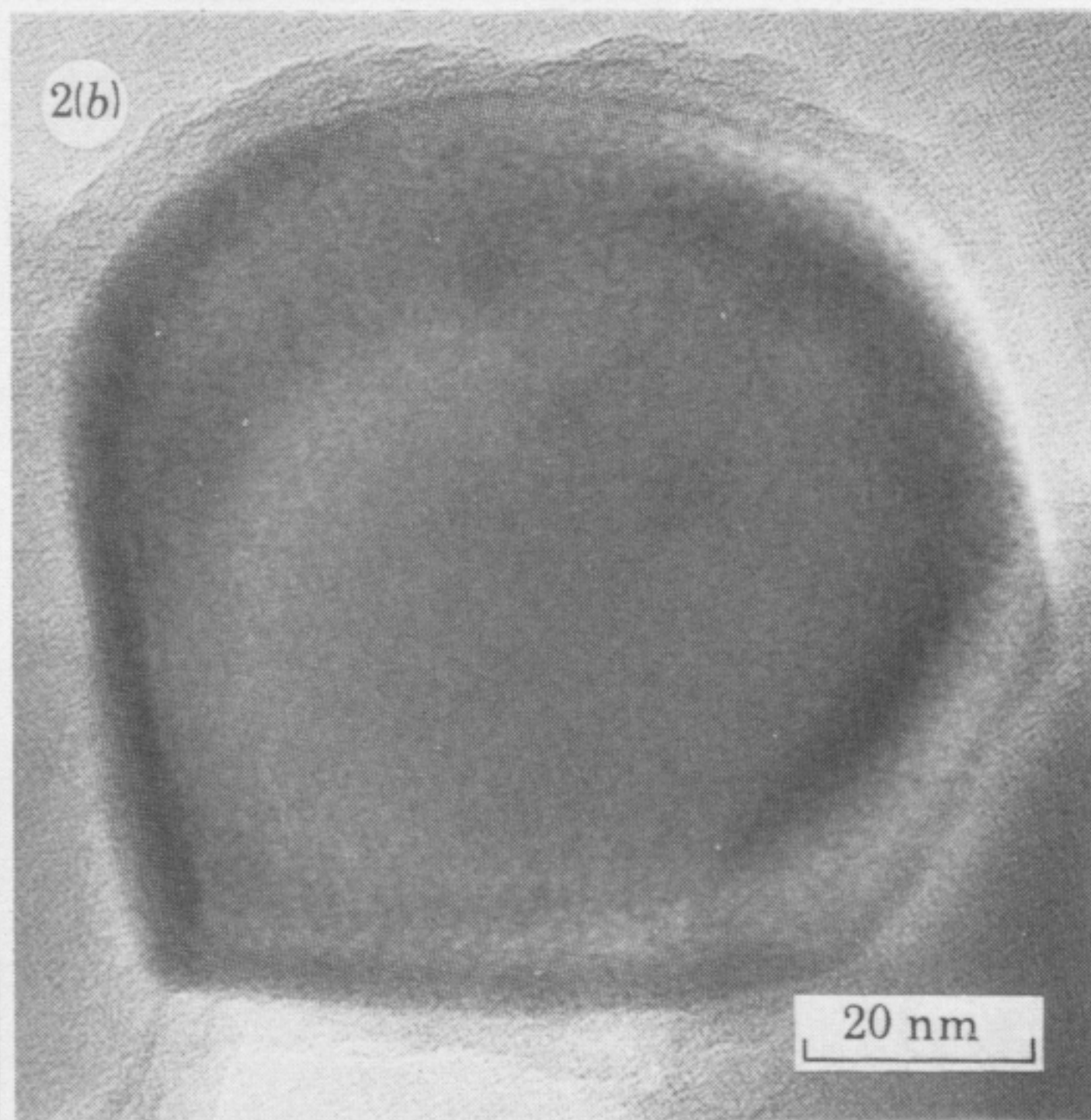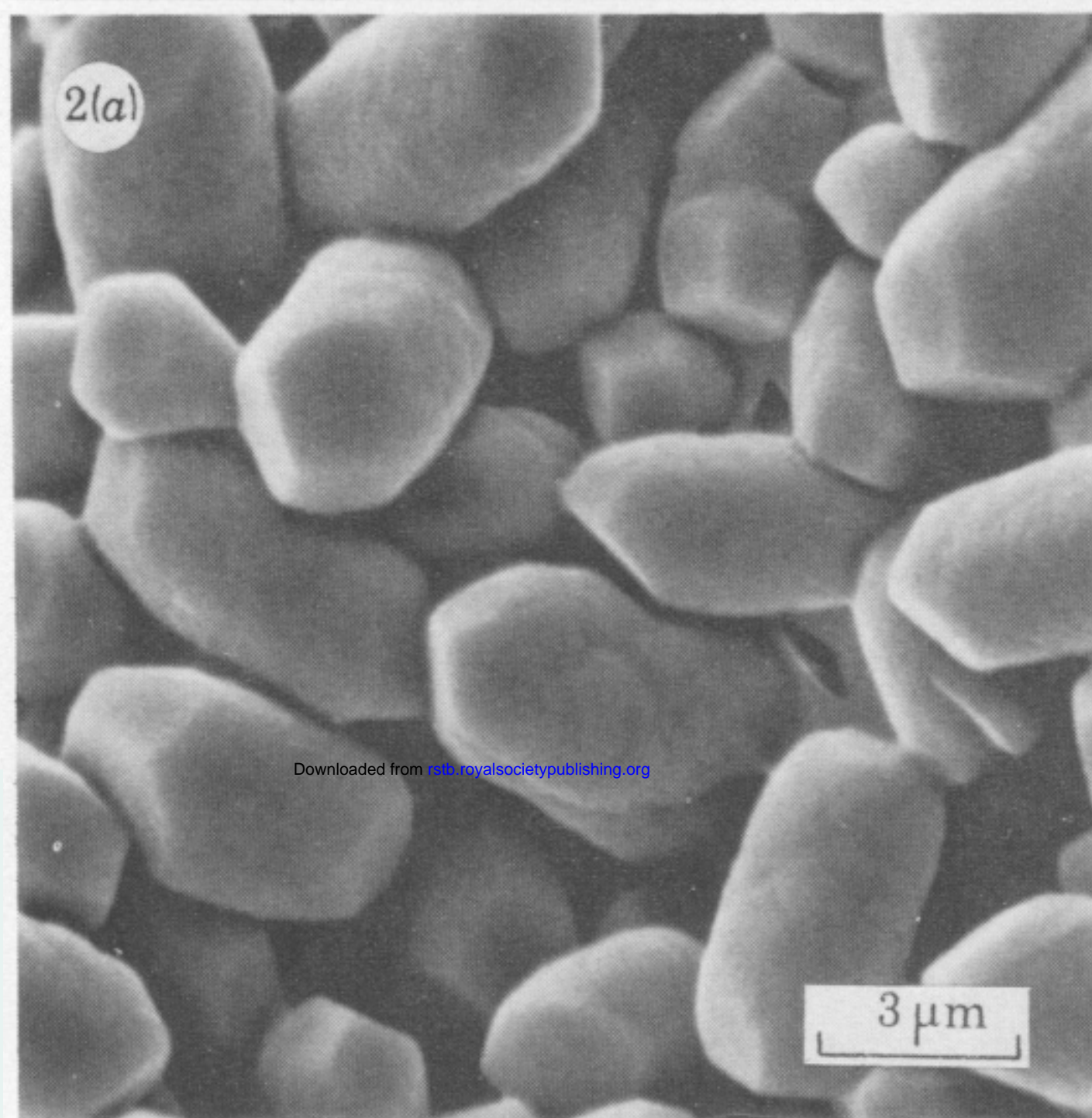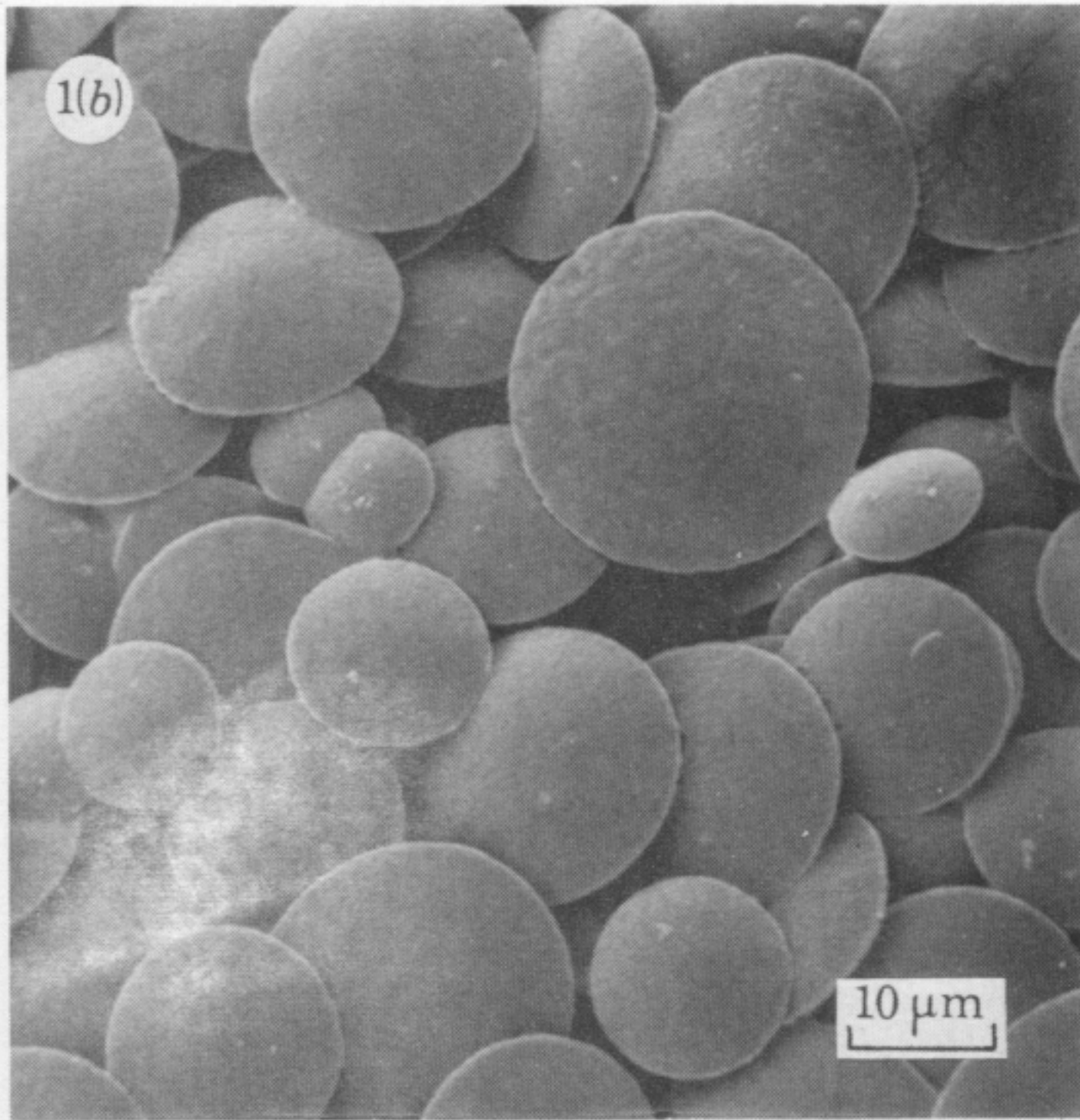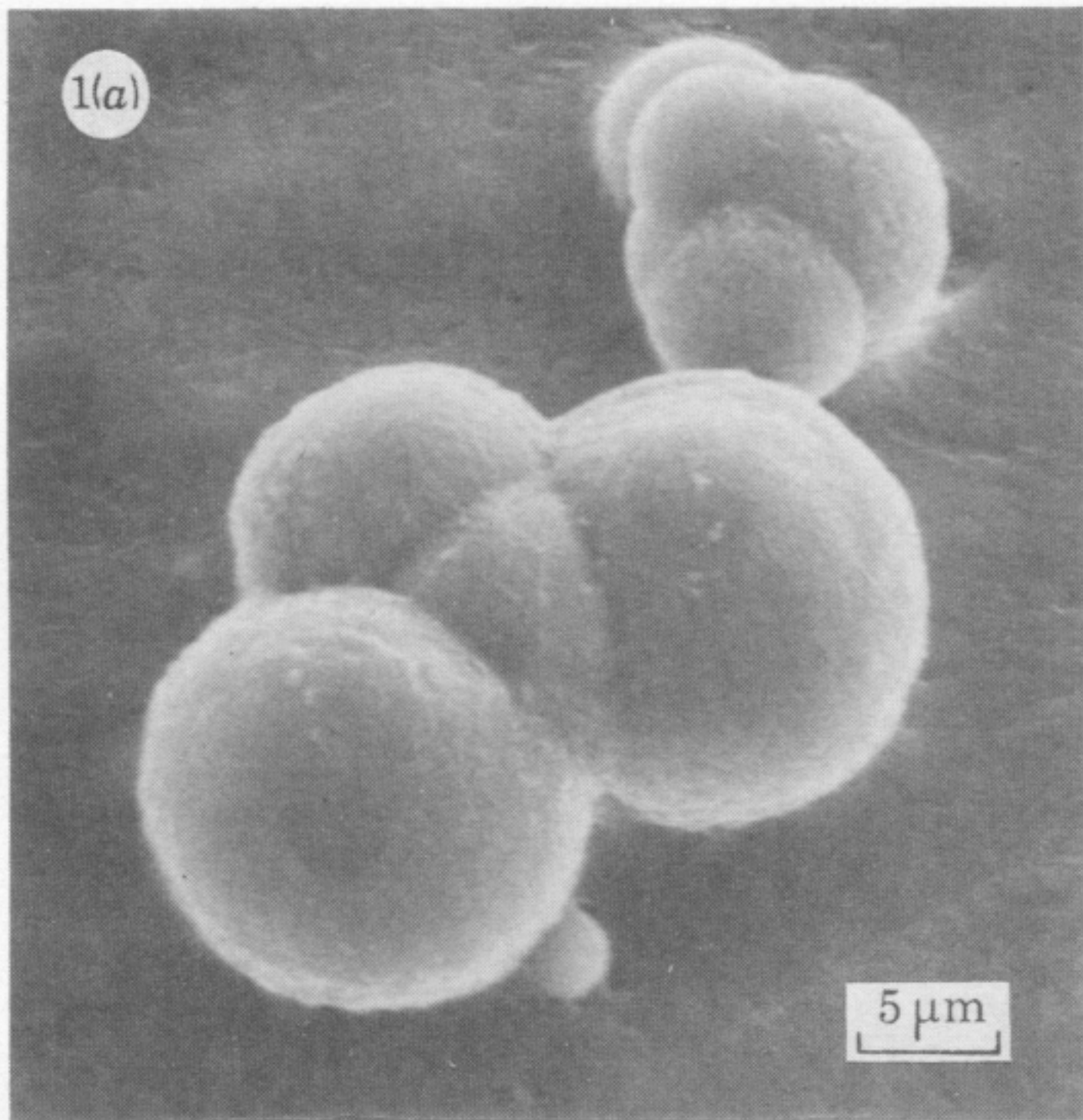
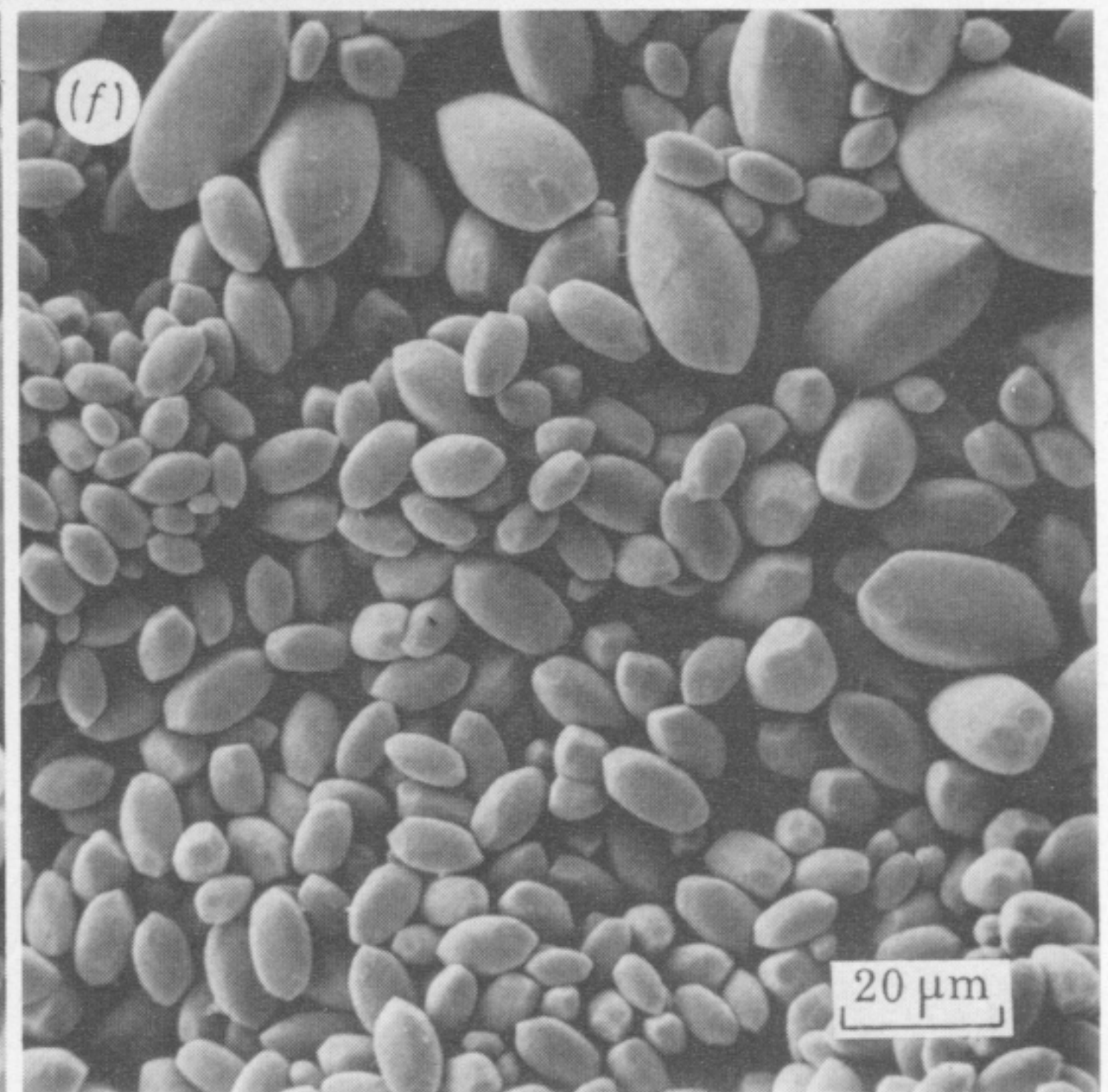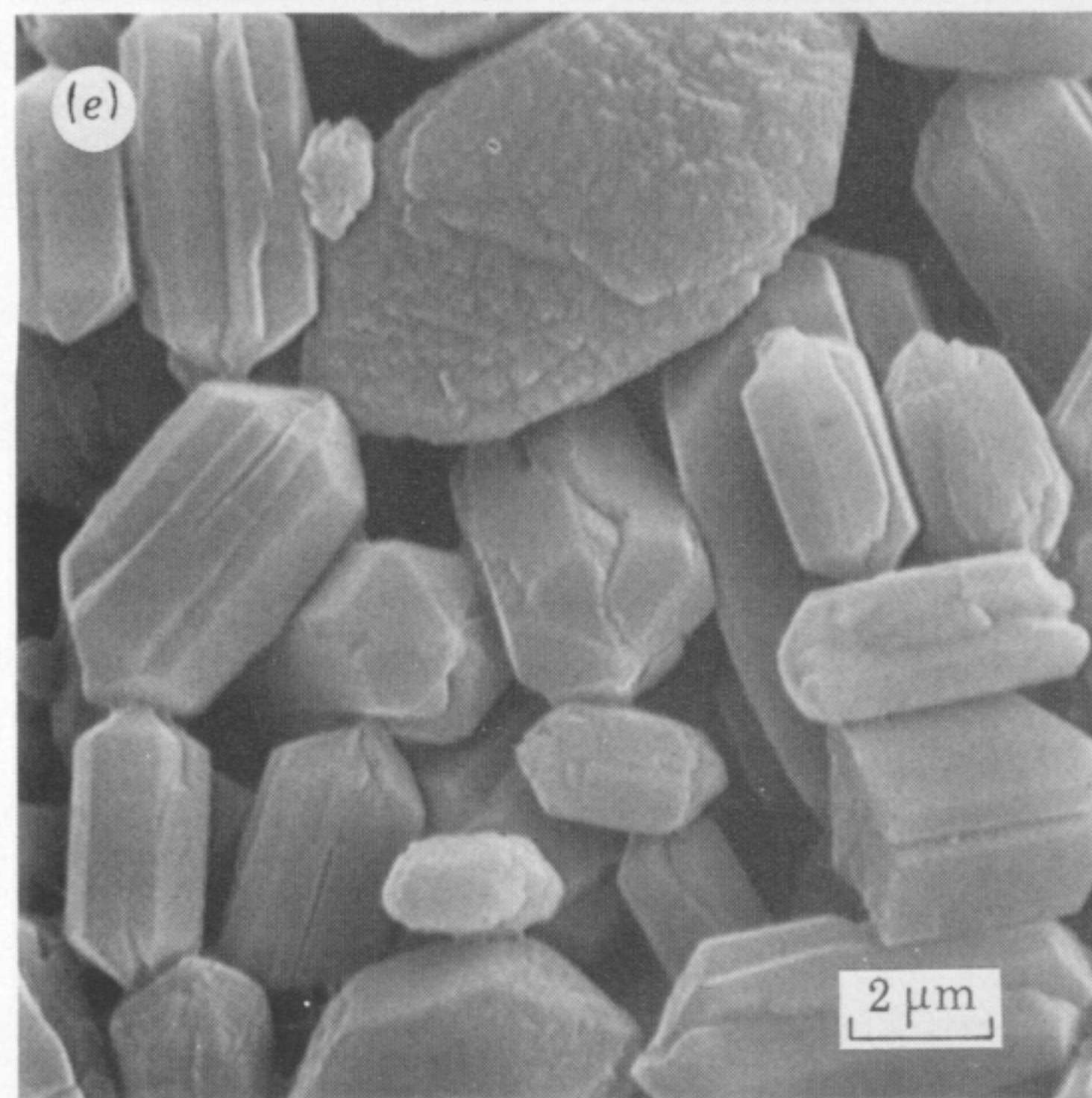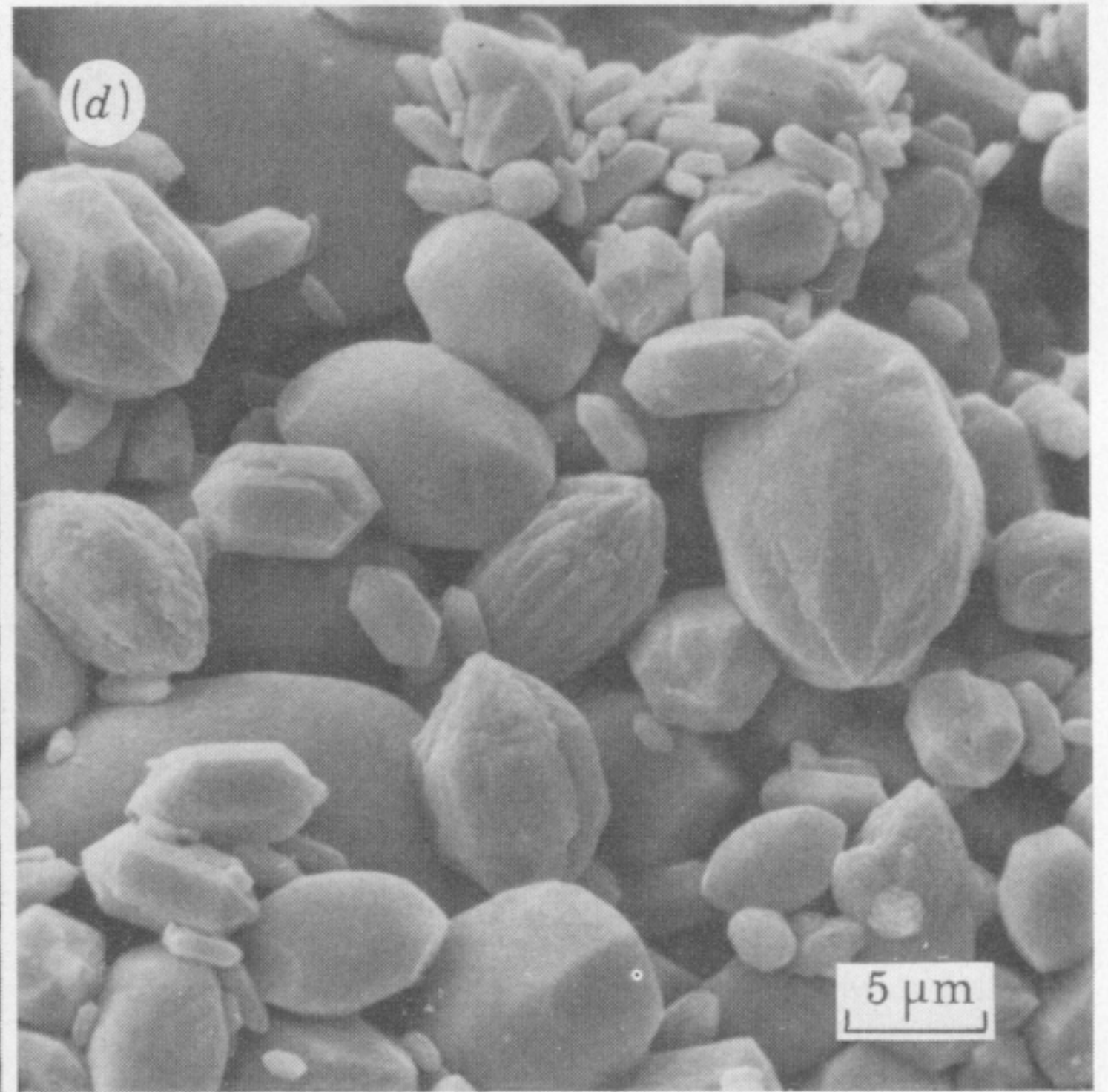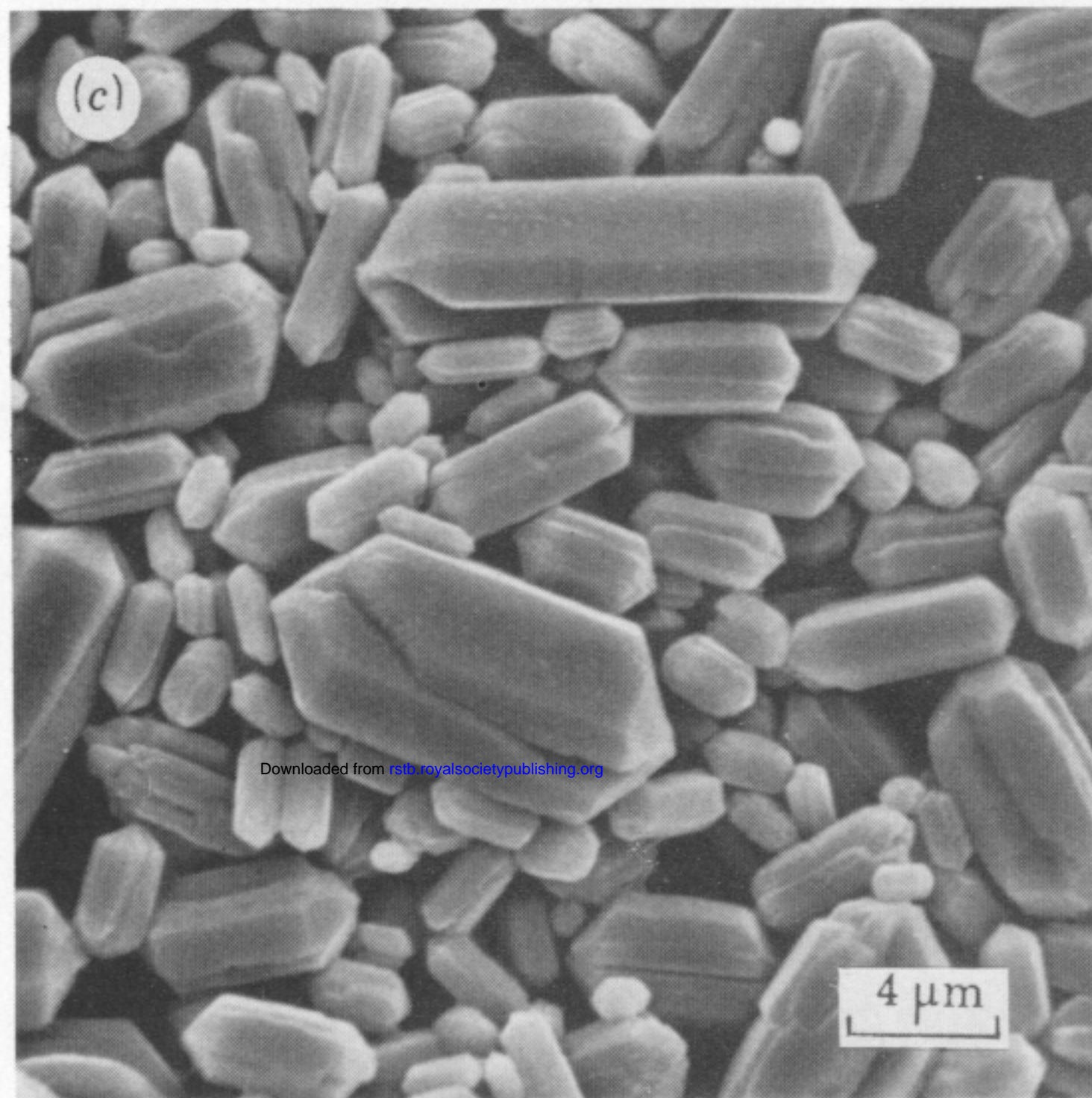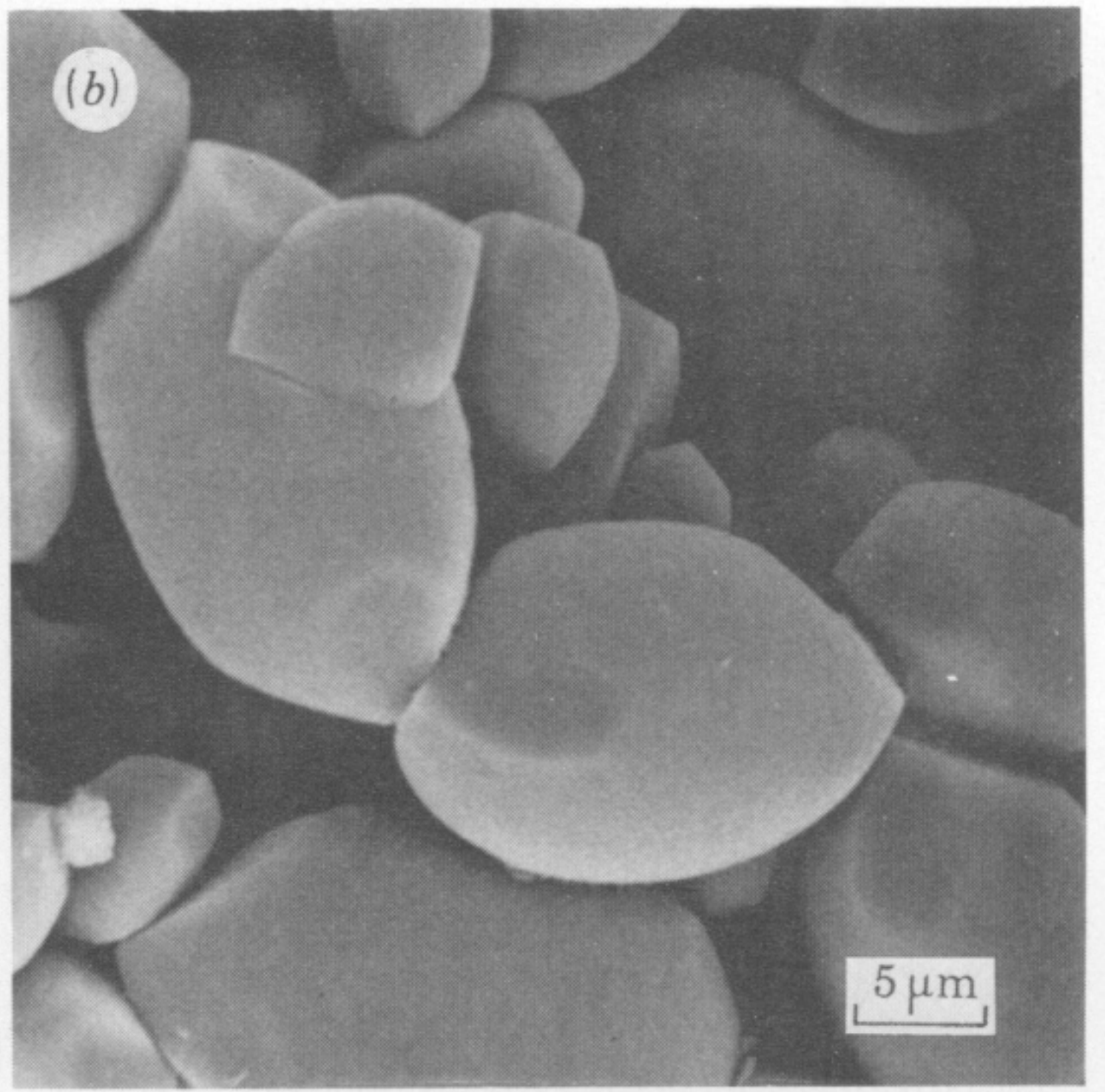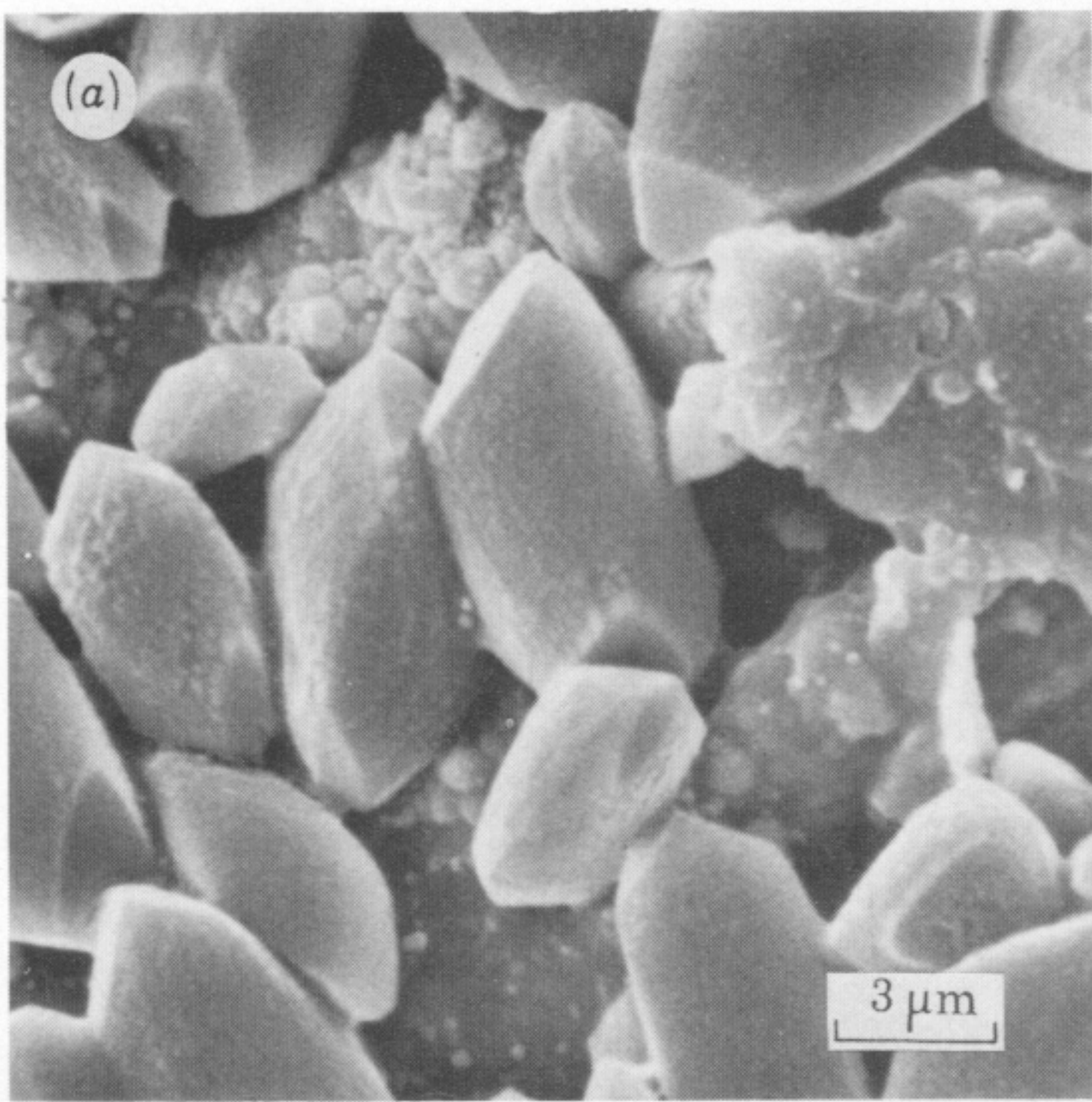FIGURES 1, 2 and 5. For description see opposite.

FIGURE 3. For description see opposite.