The Isolation and Composition of Helical Protein Microfibrils from *Hevea brasiliensis* Latex

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1. The microfibrils contained within the lutoid particles of *Hevea brasiliensis* latex obtained from young tissue have been isolated by methods based on low-speed centrifugation, isoelectric precipitation and gel filtration. 2. The isolated microfibrils behave as a single protein having an isoelectric point of about 4 as determined by paper electrophoresis. 3. The only components so far detected in the microfibrils are protein and possibly carbohydrate; nucleic acid appears to be absent. 4. The amino acid composition of the microfibril protein shows no unusual features. 5. In latex from the more mature laticiferous tissues of *H. brasiliensis*, the lutoid particles appear to be devoid of microfibrils or their protein decomposition products.

It has been known for some time that the latex of *Hevea brasiliensis* contains in suspension other particles besides rubber. Homans & van Gils (1948) showed that by low-speed centrifugation latex could be separated into two zones, an upper layer containing most of the rubber particles and a lower yellow layer or 'bottom fraction', containing a high proportion of non-rubber materials. The main component of this fraction consists of bodies named 'lutoids' by Homans & van Gils (1948). Ruinen (1950) demonstrated that the lutoids were discrete, approximately spherical particles of diameter 1–3 μ , and that the yellow colour of the bottom fraction was due to the presence of another type of particle (Frey-Wyssling particle) first described by Frey-Wyssling (1929).

Dickenson (1963), using electron microscopy, has studied in detail the contents of the latex vessel of *H. brasiliensis*. In young latex vessels, the lutoid particles were found to contain bundles of long microfibrils possessing a hollow core. Further details of the structure of the microfibrils were revealed by negative staining, the microfibrils appearing as rigid helical rods of diameter 125 Å, pitch 100 Å and with a hollow central axis of diameter 30 Å.

The present paper reports on the isolation, purification and composition of this new latex component.

MATERIALS AND METHODS

Collection of latex. Fresh *H. brasiliensis* latex was obtained from two sources: small amounts (up to 2 ml./day) were obtained by pricking the branches of 4-year-old

'illegitimate' seedlings and budded trees of clone PB86 growing in the tropical greenhouse attached to these Laboratories. Larger amounts (25 ml./day) were obtained by cutting the stems of 4-year-old buddings (clone RRIC-6) growing in a nursery at the Rubber Research Institute of Ceylon. The latex samples were tapped into 3 vol. of ice-cold 0.11 M-tris-HCl buffer, pH 7.2, containing mannitol (0.4 M) (referred to below as 'tris-mannitol buffer').

Isolation and washing of bottom fraction. Latex samples were centrifuged at 600–800g for 5 min. at 0–5° to give a buff-coloured sediment with a thin layer of yellow Frey-Wyssling particles at the surface, and a white supernatant containing most of the rubber. The supernatant was discarded and much of the rubber adhering to the walls of the centrifuge tube removed with a roll of filter paper. The sediment was washed twice by resuspension in tris-mannitol buffer (5 ml./ml. of latex) and either stored at –15° or used immediately.

Extraction of proteins from bottom fraction. All extraction and purification procedures were performed at 0–5°, except separations on gel-filtration columns, which were carried out at room temperature.

Homans & van Gils (1948) had observed that the addition of water to fresh latex caused rupture of the lutoid particles owing to the decrease in osmotic pressure. Protein extracts were therefore simply prepared by stirring the washed bottom fraction with water or buffer of low osmolality. To aid extraction, the suspensions were sometimes frozen and thawed once.

Freeze-dried bottom fraction. Freeze-dried samples of bottom fraction isolated from latex tapped from the trunks of mature trees were provided by the Rubber Research Institute of Malaya as described by Archer (1960).

Paper electrophoresis. Bottom fraction proteins were separated on Whatman no. 1 paper strips in 50 mm-veronal buffer, pH 8.6, for 16–17 hr. under a potential gradient of 5 v/cm. (Archer & Sekhar, 1955). The strips were dyed with 0.1% bromophenol blue (Kunkel & Tiselius, 1951).

Starch-gel electrophoresis. The vertical technique of Smithies (1959) was employed with a discontinuous buffer system (Poulik, 1957). Starch was purchased from the Connaught Medical Research Laboratories, Toronto, Canada. Gels were prepared in 20mm-tris-0.5mm-boric acid-6.8mm-maleic acid buffer, pH 7.87, and had a water content of 13%. The anode compartment contained 0.1M-tris-50mm-maleic acid buffer, pH 7.44, and the cathode compartment 50mm-boric acid-63mm-borax buffer, pH 8.44. Protein samples (5mg.) were run under an initial potential gradient of 3v/cm. and a constant current of 18.5ma for 16hr. The sliced gel was stained with 0.1% Nigrosine in methanol-water-acetic acid (5:5:1, by vol.).

Spectrophotometry. Absorption spectra were determined with a Hilger Uvispek spectrophotometer.

Dextran-gel column chromatography. Sephadex G-75 (medium grade) and Sephadex CM-50 (medium grade) were purchased from AB Pharmacia, Uppsala, Sweden. The Sephadex G-75 was soaked for 24hr. in 0.11M-tris-HCl buffer, pH 7.2, containing NaCl (1%, w/v), before packing. Sephadex CM-50 was washed several times with 0.5N-NaOH, 0.5N-HCl, water, 0.1M-potassium phosphate buffer, pH 6.6, and finally equilibrated with 5mm-potassium phosphate buffer, pH 6.6.

Amino acid analysis. Hannig's (1959) modification of the automatic procedure of Spackman, Moore & Stein (1958) was used. The sample was dried at 100° for 16hr. and then hydrolysed by heating for 24hr. at 102–103° in a sealed tube with 1000 times its weight of 6N-HCl.

Phosphorus analysis. Phosphorus was determined colorimetrically with ammonium molybdate, after wet oxidation. The samples were dried to constant weight *in vacuo* at room temperature before analysis.

Electron microscopy. The presence of microfibrils was demonstrated by using the negative staining method of Brenner & Horne (1959) as modified by Dickenson (1963). Addition of potassium phosphotungstate (pH 5.3) to bottom fraction extract gave a flocculent precipitate, which was centrifuged off at low speed, and the supernatant was examined for microfibrils. Electron micrographs were prepared by using a Siemens Elmiskop I electron microscope at an instrumental magnification of 40000.

EXPERIMENTAL AND RESULTS

Detection of microfibrils by using paper electrophoresis. Dickenson (1963) had shown that the luteoid particles in mature latex vessels are devoid of microfibrils. Therefore, to see whether the microfibrils could be detected by electrophoresis, a comparison was made between the electrophoretic patterns of the proteins in bottom-fraction extracts from young and old vessels. Washed bottom fraction from 0.5ml. of young branch latex was extracted with 0.5ml. of water, the extract centrifuged at 100g for 3min. and the turbid supernatant used for electrophoresis. Bottom-fraction extract from mature latex vessels was prepared by stirring 50mg. of freeze-dried bottom-fraction solids in 1ml. of 10mm-sodium diethyldithiocarbamate (to inhibit enzymic oxidation of endogenous polyphenols).

The results (Fig. 1) showed that, in the latex

Fig. 1. Paper electrophoresis of proteins of bottom fraction from a young branch and from the trunk of a mature tree: (a) trunk bottom fraction; (b) branch bottom fraction. Electrophoresis was carried out in 50mm-veronal buffer, pH 8.6, at 5v/cm. for 17hr. F, Microfibril protein; H, hevein; B, basic proteins.

from young branches, an extra protein component was present (band F), which was attributed to microfibrils. This conclusion was confirmed by the finding that only this component sedimented when a microfibril-containing extract was centrifuged at 17500g for 1 hr.

Isolation of microfibrils by centrifugation (method I). Preliminary experiments showed that the microfibrils in an extract from young green branches could be sedimented readily at approx. 1700g, owing presumably to their aggregation. Centrifugation at much higher g values was less satisfactory because of extensive microfibril breakdown.

Washed bottom fraction from 2ml. samples of latex was extracted with 1ml. of water, and the extract centrifuged at 100–600g for 2–3min. to give maximum separation between membrane fragments and microfibrils. The residue was re-extracted with 0.4ml. of water and the turbid supernatants were pooled and centrifuged at 1750g for 5min. The sediment was washed twice by resuspension in 0.4ml. of water followed by centrifugation at 5000g. Although clear supernatants could usually be produced by this washing procedure, sedimentation of microfibrils was incomplete. After washing, the microfibrils were freeze-dried. The yield was 1mg.

Isoelectric precipitation of microfibrils (method II). The results shown in Fig. 1 indicated that the microfibrils would probably be isoelectric at a pH a little lower than 4.7, which Archer (1960) had found to be the isoelectric point of the latex protein hevein

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(band H, Fig. 1). Since hevein is readily soluble in the region of pH 4, the possibility of separating the microfibrils from hevein by isoelectric precipitation was investigated. Precipitation of microfibrils from bottom-fraction extracts proved to be maximal at pH 3.5–4.0, and paper electrophoresis showed that no significant precipitation of hevein or basic proteins occurred under these conditions.

For preparative purposes, latex from buddings of clone RRIC-6 was used. Electrophoresis established that microfibrils were present from the graft union upwards and latex was therefore collected from all parts of the stem that could be reached conveniently. Samples (25 ml.) of latex were treated as follows.

A 4 ml. portion of 10 mM-sodium diethyldithiocarbamate was added to the washed bottom fraction and the mixture frozen and thawed once. The suspension was centrifuged at 1000g for 5 min. and the residue re-extracted with 1 ml. portions of 10 mM-sodium diethyldithiocarbamate until a sample of the final extract gave no precipitate at pH 4.0. The extracts were centrifuged at 1800g for 5 min. to remove membrane fragments and the microfibrils precipitated at pH 4.0 with acetic acid. After 1 hr. at 0°, the suspension was centrifuged at 1800g until the supernatant was clear. The sediment was washed twice with 10 mM-sodium diethyldithiocarbamate, then with acetic acid (pH 4.0) and finally with water. Plate 1(a) is an electron micrograph of the preparation. The material was freeze-dried and a sample examined by starch-gel electrophoresis. The remainder was dialysed exhaustively against water and freeze-dried. The yield was 8 mg. The supernatant from the precipitation step was freeze-dried and stored at -15°.

Separation of bottom-fraction proteins by gel filtration. Since Archer (1960, 1963) has shown that the major non-microfibril components of bottom fraction are of low molecular weight, the possibility of purifying the microfibrils by gel filtration was examined.

Washed bottom fraction from 1 ml. of branch latex was extracted with four 0.3 ml. portions of water, and the combined extracts were filtered through glass wool. A 0.2 ml. sample of the filtrate was applied to a column (40 cm. × 1 cm.) of Sephadex

G-75 and the proteins were eluted with 0.11 M-tris-hydrochloric acid buffer, pH 7.2; the extract was resolved into three fractions (Fig. 2). To identify the proteins, the corresponding fractions from three other gel-filtration runs were pooled, dialysed against 50 mM-veronal buffer, pH 8.6, freeze-dried and examined by paper electrophoresis.

Fraction (i) (Fig. 2) contained only microfibril protein, fraction (ii) contained only basic proteins (cf. Fig. 1), and fraction (iii) contained a protein with the same mobility as hevein.

Isolation of microfibrils (method III). The washed bottom fraction from 30 ml. of latex from buddings of clone RRIC-6 was suspended in 10 mM-sodium diethyldithiocarbamate (5 ml.) and filtered through glass wool and a no. 3 glass sinter. An equal volume of 0.2 M-potassium phosphate buffer, pH 8.0, was added to the filtrate and the solution saturated with ammonium sulphate. After standing overnight at -2°, the suspension was centrifuged at 2000g for 10 min. and the residue washed twice with neutral saturated ammonium sulphate solution. The suspension was diluted with 40 ml. of 0.11 M-tris-hydrochloric acid buffer, pH 7.2, containing sodium chloride (1%), filtered through a no. 3 glass sinter and the turbid filtrate chromatographed on a column (43 cm. × 4 cm.) of Sephadex G-75. The proteins were washed through the column with the buffer used to dilute the suspension. The separation is shown in Fig. 3. The turbid microfibril-containing fraction 1 (Fig. 3) was satu-

Fig. 2. Gel filtration of bottom-fraction proteins extracted from 0.2 ml. of young-branch latex on a column (40 cm. × 1 cm.) of Sephadex G-75. The proteins were eluted with 0.11 M-tris-HCl buffer, pH 7.2, containing NaCl (1%). (i) Microfibril protein; (ii) basic proteins; (iii) hevein.

EXPLANATION OF PLATE I

(a) Electron micrograph of microfibrils isolated by isoelectric precipitation: negatively stained with 1% potassium phosphotungstate (magnification × 120,000). (b) Electron micrograph of microfibrils isolated by gel filtration: negatively stained with 1% potassium phosphotungstate (magnification × 120,000). The scale on each photograph indicates 1000 Å.

Fig. 3. Isolation of microfibril protein by gel filtration on a column (43 cm. $\times$ 4 cm.) of Sephadex G-75. The bottom-fraction proteins (from 30 ml. of clone RRIC-6 latex) were eluted with 0.11 M-tris-HCl buffer, pH 7.2, containing NaCl (1%). Fraction 1 was taken for further purification.

Fig. 4. Relative proportions of proteins in bottom-fraction extracts prepared from latices of increasing age. The samples, containing approx. 2 mg. of protein, were analysed by gel filtration as described in the text. ●, Microfibril protein; Δ, basic proteins; ○, hevein.

rated with ammonium sulphate at pH 7 and, after standing for 4 hr. at -2° , centrifuged at 2000g for 15 min. The sediment was washed twice with 0.11 M-tris-hydrochloric acid buffer, pH 7.2, saturated with ammonium sulphate, dialysed against 5 mM-potassium phosphate buffer, pH 6.6, and passed through a Sephadex CM-50 column (3 cm. $\times$ 1 cm.) equilibrated with 5 mM-potassium phosphate buffer, pH 6.6. The microfibril fraction, which passed straight through the column, was stored at -6° . The yield of microfibrils was 20 mg. Plate 1(b) is an electron micrograph of this preparation.

Essentially the same gel-filtration technique was used to recover the microfibrils remaining in the supernatant after precipitation at pH 4.0 (see method II).

Effect of age of laticiferous tissue on microfibril content of bottom fraction. Gel filtration was used to determine the relative proportions of the microfibrils and other major bottom-fraction proteins in latex from tissues of different age. The trunk of a 4-year-old seedling was marked out in ten consecutive intervals of 30 cm., starting at soil level, and 1–2 ml. samples of latex were collected from the areas between the marks. From each sample washed bottom fraction was isolated and extracted four times with water, and the extracts were freeze-dried. The freeze-dried material was dissolved in 0.11 M-tris-hydrochloric acid buffer, pH 7.2, containing sodium chloride (1%), the total proteins were precipitated by saturation with ammonium sulphate and the precipitate was washed with saturated ammonium sulphate solution until the $E_{260\text{ m}\mu}^{1\text{ cm.}}$ value of the washings was less than 0.05. A sample of the precipitate (approx. 2 mg. of protein) was chromatographed on a column (40 cm. $\times$ 1 cm.) of Sephadex G-75. Recovery of proteins from the column was complete.

The relative amounts of microfibrils, hevein and basic proteins in the samples, determined from the areas of the respective peaks on the elution diagrams, are shown in Fig. 4.

General characteristics of the microfibrils. As often observed with asymmetric particles, suspensions of microfibrils had a 'silky' appearance when gently swirled. At a pH of about 13 the suspension became opalescent and at pH < 2 the microfibrils dissolved to give initially a clear solution. Heating the suspension (7 min. at 70°) at neutral pH caused coagulation. Suspensions in 5 mM-potassium phosphate buffer, pH 6.6, could be stored frozen (-6°) for up to 3 months without any obvious change in their electron-microscopic appearance. Microfibrils were also stable structurally for at least 2 weeks when suspended in saturated ammonium sulphate at 0° .

Microfibrils dialysed and freeze-dried showed a less well-defined ultrastructure than those stored as frozen suspensions. This loss of definition was much less marked, however, when the pH 4.0 supernatants obtained in method II were freeze-dried.

Purity of the isolated microfibrils. Microfibrils obtained by methods I and II gave single bands of the expected mobility on paper electrophoresis at pH 8.6, a small amount of material remaining at the origin. Microfibrils prepared by method III behaved anomalously in that essentially all the protein remained at the origin when a suspension of the salt-free freeze-dried material was used. However, when these microfibrils were precipitated at pH 4.0 and then examined electrophoretically at pH 8.6, a single band with the characteristic mobility of the microfibrils was obtained. This unusual behaviour can probably be attributed to the fact that the length of the microfibrils obtained by method III was considerably greater than in the other samples.

Fig. 5. Starch-gel electrophoretogram of microfibril protein purified by isoelectric precipitation (method II). For comparison, the electrophoretogram of a sample of freeze-dried supernatant solids obtained in method II is also shown. (a) Precipitated microfibril protein; (b) freeze-dried solids. Each sample was subjected to electrophoresis in the same starch-gel block for 16 hr. by using the discontinuous buffer system described in the text; 5 mg. of material was applied in each case.

Starch-gel electrophoresis of microfibrils (prepared by method II) showed the presence of small amounts of protein impurities (Fig. 5). The relative mobilities of the microfibrils and of hevein were the reverse of those found by paper electrophoresis, probably because the gel, acting as a molecular sieve (Smithies, 1959), hindered the movement of the microfibrils but not of hevein, which has a molecular weight of only 10000 (Archer, 1960).

Ultraviolet-absorption spectra of the isolated microfibrils. The absorption spectra of the microfibrils were characteristic of protein, but the absorption curves (Fig. 6) were considerably distorted by the strong light-scattering of the suspensions.

Attempts to correct the spectra for scattering by the method of Treiber & Schauenstein (1949) were not successful because plots of $\log \lambda$ against $\log E$ outside the region of true absorption were only approximately linear. It was not possible therefore

Fig. 6. Ultraviolet-absorption spectra of microfibril protein: ●, in 5mM-potassium phosphate buffer, pH 6.6; ○, in 0.1N-HCl; Δ, in 0.1N-KOH. The spectra were measured in 1cm. cells on solutions containing about 0.2 mg. of protein/ml.

to obtain curves sufficiently accurate for the spectroscopic determination of the tryptophan and tyrosine content of the protein.

Amino acid analysis of the isolated microfibrils. A preliminary amino acid analysis of the microfibril protein is shown in Table 1. Duplicate analyses were in good agreement, and the results given are the mean values of two determinations on a single hydrolysate. Cysteine was not determined separately as cysteic acid and the value for this amino acid in Table 1 is therefore too low. Destruction of serine and threonine was corrected for by assuming losses of 10 and 5% respectively on hydrolysis (Rees, 1946). Tryptophan was not determined. The nitrogen content of the protein was 15.3%, of which the amino acid and amide N accounted for 93.5%. Amino acid residues accounted for 87.7% of the dry weight.

Detection of carbohydrate in the isolated microfibrils. The following tests (Dische, 1955) indicated the presence of carbohydrate: (i) the microfibrils gave a cherry-red solution ($\lambda_{\max}$ 560mμ) in the Molisch test, and a brown solution in the indole-sulphuric acid test; (ii) the absorption spectrum of the microfibrils in conc. sulphuric acid showed a maximum at 316mμ; (iii) in the cysteine-sulphuric acid reaction, the microfibril solution had an absorption maximum at 402mμ. The carbohydrate content estimated by the Molisch reaction with glucose as standard (Dische, 1955) was 4%.

Table 1. *Amino acid analysis of microfibril protein*

Amino acid residue	Amino acid N (g./100 g. of protein N)	Amino acid residue (g./100 g. of protein)	Amino acid residue (residues/1000 residues)
Arg	9.8	4.2	34
Lys	9.9	7.0	69
His	3.9	1.9	18
Glu	6.6	9.4	92
Asp	8.6	10.4	115
Thr	4.4	4.9	62
Ser	4.7	4.4	65
Gly	5.5	3.4	76
Pro	3.8	4.0	52
Tyr	4.2	7.5	59
Ala	4.8	3.7	67
Val	3.9	4.2	54
Leu	6.1	7.6	85
Ile	3.9	4.8	54
Phe	4.9	7.9	68
Met	0.8	1.1	11
$\frac{1}{2}$ Cys	1.2	1.3	17
NH ₃	6.5	—	—
Total	93.5	87.7	

Absence of nucleic acid from the isolated microfibrils. The phosphorus content of the microfibril protein was less than 0.01%, thus showing the virtual absence of nucleic acid. This result was confirmed by extraction of the microfibril protein with aqueous phenol by the procedure of Gierer & Schramm (1956).

DISCUSSION

Both isoelectric precipitation and gel filtration (methods II and III) were satisfactory for obtaining microfibril protein in sufficient quantities for chemical characterization. Gel filtration has the advantage that yields are higher, since the losses involved in washing the microfibril suspension are avoided. These losses can be particularly serious in method I (centrifugation method), especially when very small amounts of latex are being handled. However, the centrifugation method is useful when small samples only are required, e.g. for electron microscopy. The paper and starch-gel electrophoresis results indicate that all three methods of isolation yield microfibril protein of about the same degree of purity.

Although the amino acid analysis was only of a preliminary nature, the high weight and nitrogen recoveries obtained indicate that the microfibrils are probably composed entirely of protein. With the exception of tryptophan, for which no data are

available, the microfibrils contain all the amino acids commonly found in proteins, and the amino acid composition itself does not possess any unusual features. At one stage of this investigation it was thought that the microfibrils might be built up from sub-units consisting of hevein. Comparison of the amino acid analysis of the microfibril protein with that of hevein (Archer, 1960) rules out this possibility. Besides protein, the only other material detected in the microfibril preparations was carbohydrate, which may, however, be present as an impurity.

Gel-filtration analyses and paper-electrophoretic examination of extracts of bottom fraction from latices drawn from tissues of different age has confirmed Dickenson's (1963) observation that lutoid particles in sufficiently old latex vessels are devoid of microfibrils. Only basic proteins and hevein were found in extracts of bottom fraction prepared from the latex of old tissue, hevein comprising about 80% of the total protein in such samples. The marked differences between the amino acid composition of hevein and the microfibrils and the fact that hevein has been found in substantial amounts in bottom fraction from very young laticiferous tissue makes it most unlikely that this protein is an end product of microfibril decomposition. Thus it appears that, as the lutoid particles age, the microfibrils disappear without being converted into other proteins that remain in the particle, indicating that the lutoid particles may well be a site of proteolytic activity. The disappearance of the microfibrils suggests that they may provide a store of protein that is utilized by the developing tissue. However, this concept of their role does not take into account their highly orientated helical structure. It seems more likely that the microfibrils have a more specific function that may become apparent when further information is available about the role of the lutoid particles themselves in the metabolism of the latex vessel.

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REFERENCES

- Archer, B. L. (1960). *Biochem. J.* **75**, 236.
 Archer, B. L. (1963). In *The Chemistry and Physics of Rubber-like Substances*, p. 54. Ed. by Bateman, L. London: Maclaren and Sons Ltd.

- Archer, B. L. & Sekhar, B. C. (1955). *Biochem. J.* **61**, 503.
- Brenner, S. and Horne, R. W. (1959). *Biochim. biophys. Acta*, **34**, 103.
- Dickenson, P. B. (1963). In *The Chemistry and Physics of Rubber-like Substances*, p. 43. Ed. by Bateman, L. London: Maclaren and Sons Ltd.
- Dische, Z. (1955). *Meth. biochem. Anal.* **2**, 303.
- Frey-Wyssling, A. (1929). *Arch. Rubbercult. Ned.-Ind.* **13**, 392.
- Gierer, A. & Schramm, G. (1956). *Z. Naturf.* **11B**, 138.
- Hannig, K. (1959). *Clin. chim. Acta*, **4**, 51.
- Homans, L. N. S. & van Gils, G. E. (1948). *Proc. 2nd Rubb. Tech. Conf.* p. 292.
- Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
- Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.
- Rees, M. W. (1946). *Biochem. J.* **40**, 632.
- Ruinen, J. (1950). *Ann. bogor.* **1**, 27.
- Smithies, O. (1959). *Advance. Protein Chem.* **14**, 65.
- Spackman, D. H., Moore, S. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
- Treiber, E. & Schauenstein, E. (1949). *Z. Naturf.* **4B**, 252.