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# ERGOTHIONEINE AND HERCYNINE IN *HEVEA BRASILIENSIS* LATEX

C. H. TAN and B. G. AUDLEY

The Natural Rubber Producers' Research Association, Tewin Road, Welwyn Garden City, Herts.

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**Abstract**—The readily sedimentable fraction (bottom fraction) of *Hevea brasiliensis* latex has been analysed for phenols and betaines. The only phenol detectable was tyrosine, but three betaines, trigonelline, ergothioneine, and hercynine, were identified. Ergothioneine has also been detected in bottom fraction from mature specimens of *H. Benihamana* and *H. spruceana*. In the bottom fraction of latex from mature trees, the amount of ergothioneine present on a dry weight basis is comparable with the ergothioneine content of the ergot fungus; latex serum contains much smaller amounts. Ergothioneine could not be detected in latex from young specimens of *H. brasiliensis* growing in England, but was present in latex obtained from young *H. brasiliensis* growing in Ceylon.

## INTRODUCTION

THE LATEX of *Hevea brasiliensis* is known to contain in suspension other particles besides rubber. On centrifugation at low speeds, the latex separates into an upper layer containing most of the rubber particles, and a lower layer or "bottom fraction". The main components of this fraction are the so-called lutoid particles,[1] which are approximately spherical bodies about 3 $\mu$ in diameter, with a fluid interior, and bounded by an osmotically sensitive membrane.[2, 3] It is known that the bottom fraction contains a phenolase[4, 5] and the initial objective of the present work was to identify the endogenous phenolic substrates. The only phenolic compound detected was tyrosine, but in the course of the investigation it was found that the bottom fraction of latex from mature trees contains the betaines ergothioneine (2-thiol-histidine betaine), hercynine (histidine betaine), and trigonelline. Ergothioneine is widely distributed in fungi,[6] but hitherto has been found in only one higher plant,[7] whilst hercynine has not previously been detected in higher plants. In this paper the evidence for the above findings is described, and the results of analyses of latex fractions for the three betaines, reported.

## RESULTS

Freeze-dried bottom fraction (1 g) was extracted with light petroleum (b.p. 40–60°) to remove tocotrienols and other lipid materials which reduce Folin–Ciocalteau reagent.[8] The residual solid was then extracted in a Soxhlet extractor successively with ether, methanol

[1] L. N. S. HOMANS and G. E. VAN GILS, *Proc. 2nd Rubber Technol. Conf.* p. 292 (1948).
[2] J. RUINEN, *Ann. Bogor* **1**, 27 (1950).
[3] P. B. DICKENSON, In *Proceedings of the Natural Rubber Producers' Research Association Jubilee Conference, Cambridge* 1964 (edited by L. MULLINS), p. 52. Maclaren and Sons, London (1965).
[4] L. N. S. DE HAAN-HOMANS, *Trans. Inst. Rubber Ind.* **25**, 346 (1950).
[5] R. C. H. HSIA, *Trans. Inst. Rubber Ind.* **34**, 267 (1958).
[6] D. B. MELVILLE, *Vitamins Hormone* **17**, 155 (1959).
[7] D. B. MELVILLE and S. EICH, *J. Biol. Chem.* **218**, 647 (1956).
[8] P. J. DUNPHY, K. J. WHITTLE, J. F. PENNOCK and R. A. MORTON, *Nature* **207**, 521 (1965).

and water. Materials which reduced Folin–Ciocalteau reagent were present only in the methanol and water extracts and these compounds were not extractable into ether even at low pH. In order to ascertain whether phenolic glycosides were present, a 2 g sample of freeze-dried bottom fraction was extracted with boiling water and the extract refluxed with 1 vol. of 5N $H_2SO_4$ for 1 hr. The resulting brown solution was then extracted continuously with ether for 24 hr. The concentrated ether extract gave only a very weak Folin–Ciocalteau reaction indicating the absence of phenolic glycosides in bottom fraction. To test for the presence of *o*-dihydroxyphenols, 100 mg of bottom fraction solids were extracted with boiling water, and a saturated solution of lead acetate added to the extract until no further precipitate formed. The precipitate was washed with water, decomposed with $H_2S$, and the lead sulphide removed by centrifugation. The supernatant was concentrated *in vacuo* and chromatographed in solvent 1. No spots were detected in u.v. light or by spraying with diazotized *p*-nitroaniline, indicating the absence of *o*-dihydroxyphenols.

The nature of the reducing compounds extractable from bottom fraction by methanol was investigated by paper chromatography. 20 mg of freeze-dried bottom fraction were extracted with 0·2 ml of boiling methanol and 10–20 $\mu$l samples of the extract run on Whatman No. 4 paper in solvent 1. Several spots could be discerned in u.v. light and by spraying with diazotized *p*-nitroaniline. The $R_f$ values of the more strongly staining and u.v. absorbing spots are given in Table 1.

TABLE 1. $R_f$ VALUES IN SOLVENT 1 OF THE MAJOR DIAZOTIZABLE AND U.V.-ABSORBING COMPOUNDS PRESENT IN METHANOL EXTRACTS OF *H. brasiliensis* BOTTOM FRACTION

| Designation | Ultraviolet absorption | Colour with diazotized *p*-nitroaniline | $R_f$ | Identity |
|---|---|---|---|---|
| S1 | Nil | Faint purple | 0·17 | Not identified |
| S2 | Nil | Purple | 0·29 | Histidine and hercynine |
| S3 | Strong | Strong orange–red | 0·41 | Ergothioneine |
| S4 | Strong | Pale red | 0·46 | Trigonelline |
| S5 | Nil | Strong purple | 0·66 | Tyrosine |

Several other faint red spots were also visible. To determine whether any of the diazotizable compounds were aromatic amino acids (tryptophan, histidine, or tyrosine), a similar chromatogram was sprayed with ninhydrin. Numerous spots were discernible, many of which coincided with the spots in Table 1 and with the fainter diazotizing spots. Comparison with marker amino acids allowed S2 (Table 1) to be tentatively identified as histidine, and S5 as tyrosine; tryptophan appeared to be absent. The similarity between the patterns obtained by spraying with ninhydrin and diazotized *p*-nitroaniline, suggested that the compounds responsible for the fainter spots seen on spraying with the latter reagent could be aliphatic amino acids. The reaction between diazotized *p*-nitroaniline and a range of amino acids was therefore examined. It was found that all the commonly occurring amino acids tested formed coloured spots. $\alpha$-Alanine, $\beta$-alanine, glycine, arginine, aspartic acid, glutamic acid, serine, threonine, methionine, valine, leucine, isoleucine and phenylalanine gave pale-red spots and tryptophan a browny-yellow spot. The limit of detection was about 5 $\mu$g of amino acid per $cm^2$, less in the case of tyrosine and histidine. Cysteine and glutathione also

gave pale-red spots but the limit of detection was about 10 $\mu g/cm^2$. The colours had only faded slightly six months after spraying. These results supported the idea that aliphatic amino acids, not phenols, were responsible for many of the faint red spots seen when chromatograms of bottom-fraction extracts were sprayed with diazotized *p*-nitroaniline. The identification of the major spots is described below.

The presence of tyrosine in bottom-fraction solids, was confirmed by isolation of the amino acid in crystalline form. The isolated material ran as a single spot with the same $R_f$ as tyrosine in solvents 1, 2 and 3. The light-absorption maxima in 0·1 N HCl were at 223 nm and 274·5 nm ($\epsilon$ 8240 and 1460 respectively) and the minimum at 245 nm. In 0·1 N NaOH the maxima were at 240 nm and 293·5 nm ($\epsilon$ 1090 and 2370 respectively) and the minimum at 269 nm. These values are in excellent agreement with the data given for tyrosine by Beaven and Holiday.[9]

S2 was shown to contain histidine by chromatographing the eluted material with a histidine marker in solvents 2 and 3 and in 72% (w/w) phenol. S2 ran in the same position as histidine in all three solvent systems.

The material S4 (Table 1) eluted with water from preparative scale chromatograms (in solvent 1) of bottom-fraction extracts, was freed of traces of S3 by further paper chromatography in solvent 3. After being thus purified, S4 reacted only faintly with ninhydrin and diazotized *p*-nitroaniline. Its absorption spectra in 0·1 N HCl and 0·1 N NaOH were identical ($\lambda_{max}$ 264 nm, shoulder at 270 nm), and the presence of a basic functional group was indicated by its retention on Dowex-50 $H^+$. These data were consistent with S4 being trigonelline (N-methyl nicotinic acid), a compound previously found in *Hevea* latex by Altman.[10] The identification was confirmed by paper chromatography, S4 and trigonelline having identical $R_f$ values in seven solvent systems (Table 4). S4 also gave a red precipitate with Kraut's reagent and reacted positively in the colour tests described for trigonelline by Giulano.[11] The amounts of trigonelline present in four different samples of bottom fraction were 2·7, 5·2, 3·0 and 4·9 mg/g of solids, respectively.

Substance S3 was found to reduce potassium ferricyanide, ammoniacal silver nitrate, 2:6-dichloroquinonechloroimide and Folin–Ciocalteau reagent. After purification by two-dimensional chromatography in solvents 1 and 2, S3 did not react with ninhydrin, indicating the absence of a free amino group. However, some other basic group was apparently present since the compound was retained by Dowex-50 $H^+$. The u.v. absorption spectrum in water of a sample of S3 purified by two-dimensional chromatography showed a maximum at 258 nm, and in alkali (pH 12) there was a hypsochromic and hypochromic shift to a maximum at 245 nm, with marked broadening of the peak; $\lambda_{max}$ in ethanol was at 264 nm. These data suggested that S3 might be ergothioneine and further evidence that this was the case was obtained by applying the Hunter reaction,[12] which is virtually specific for this compound.[13] When a spot of S3, on paper, was sprayed with buffered diazotized sulphanilic acid, a lemon-yellow colour was produced which slowly turned magenta on further spraying with 10 N NaOH. A sample of authentic ergothioneine hydrochloride behaved in the same way. The identification was confirmed by isolating a crystalline sample of S3 and determining its i.r. and NMR spectra. These were identical with the corresponding spectra for ergothioneine. The NMR data for ergothioneine are given in Table 2.

[9] G. H. Beaven and E. R. Holiday, *Advan. Protein Chem.* **7**, 319 (1952).
[10] R. F. A. Altman, *Rubber Chem. Technol.* **14**, 664 (1941).
[11] R. Giulano, *Ann. Chim. Farm.* April, 46 (1939).
[12] G. Hunter, *Biochem. J.* **22**, 4 (1928).
[13] A. Lawson, H. V. Morley and L. I. Woolf, *Nature* **167**, 82 (1951).

TABLE 2. NUCLEAR MAGNETIC RESONANCE DATA FOR ERGOTHIONEINE; THE SPECTRA WERE DETERMINED IN $D_2O$ WITH TRIMETHYLSILANE AS EXTERNAL STANDARD

| Ergothioneine isolated from bottom-fraction solids | Synthetic ergothioneine hydrochloride | Assignment |
|---|---|---|
| 3·15s | 3·08s | $=CH$ |
| 5·99dd (5·5, 9·5) | 5·75dd (5·5, 10·0) | $CH—N$ |
| 6·62s | 6·57s | $-N(CH_3)_3+$ $CH_2$ |
| 5·21 | 5·18 | Water and exchangeable protons |

s, singlet; dd, double doublet: coupling constants are shown in brackets.

The above finding suggested that hercynine, a precursor of ergothioneine in *Neurospora crassa*,[14] might be present in latex. Betaine-containing extracts of equal amounts of freeze-dried bottom fraction and serum were therefore prepared using Kraut's reagent, and these were chromatographed in solvent 1 with marker hercynine. On examination in u.v. light at least six compounds were detected in the serum solids extract and two in the bottom-fraction extract. On spraying with diazotized *p*-nitroaniline, an orange–yellow spot of the same $R_f$ as hercynine was revealed on the chromatogram of both extracts, in an area devoid of u.v. absorption. The spot appeared more intense on the chromatogram of the serum solids extract, and this material was used for isolation of the compound suspected of being hercynine, by means of preparative scale paper chromatography. The isolated material was found to run with the same $R_f$ as hercynine in seven solvent systems (Table 3).

TABLE 3. $R_f$ VALUES OF HERCYNINE AND TRIGONELLINE: DESCENDING CHROMATOGRAPHY ON WHATMAN NO. 4 PAPER; TEMP. 20°. THE SOLVENT COMPOSITIONS GIVEN ARE BY VOLUME

| Solvent | $R_f$ | |
|---|---|---|
| | Hercynine | Trigonelline |
| Butan-1-ol–acetic acid–water (4:1:5) | 0·22 | 0·42 |
| Butan-1-ol–propionic acid–water* (20:10:14·1) | 0·46 | 0·55 |
| Propan-1-ol–0·2 N ammonia soln. (3:1) | 0·42 | 0·45 |
| Propan-1-ol–0·1 N acetic acid (3:1) | 0·23 | 0·39 |
| Butan-1-ol–ammonia soln. (sp. gr. 0·88)–water (100:3:18) | 0·05 | 0·05 |
| *t*-Butanol–methyl ethyl ketone–water–ammonia soln. (sp. gr. 0·88) (4:3:2:1) | 0·72 | 0·73 |
| *t*-Butanol–methyl ethyl ketone–acetic acid–water (8:6:3:3) | 0·64 | 0·78 |

* Used at 25°.

[14] A. ASKARI and D. B. MELVILLE, *J. Biol. Chem.* **237**, 1615 (1962).

The unknown also had the same mobility as hercynine on paper electrophoresis. Further evidence that the isolated material was hercynine, was obtained by treatment with hot 50% KOH, which breaks down hercynine to urocanic acid. When this degradation was carried out with the unknown, and the neutralized reaction mixture run on paper in solvent 1, a diazotizable and u.v. absorbing spot with the same $R_f$ as marker urocanic acid was found. Since hercynine has the same $R_f$ as histidine in solvent 1, it appeared that S2 (Table 1) was in fact a mixture of histidine and hercynine. A sample of S2 was therefore electrophoresed on paper with histidine and hercynine markers. On spraying with diazotized *p*-nitroaniline, it could be seen that two compounds, corresponding in mobility to histidine and hercynine, were present.

*Detection of Ergothioneine in Latex Fractions by Gel-filtration*

The observation that ergothioneine could be cleanly separated by gel-filtration from most other u.v. absorbing materials in latex, afforded a simple and relatively rapid means of carrying out analyses of latex fractions from various sources. Figure 1(*a*) shows a typical

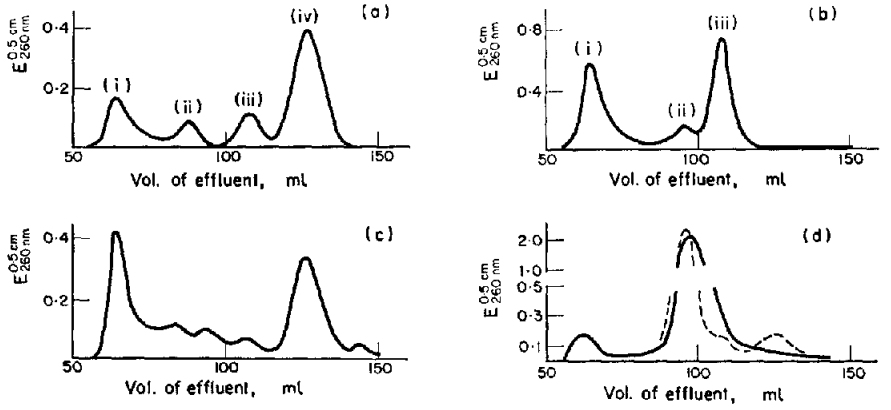


FIG. 1. GEL-FILTRATION OF BOTTOM-FRACTION EXTRACTS FROM VARIOUS LATICES ON A COLUMN (55 CM × 2 CM) OF SEPHADEX G-15. THE ELUTING AGENT WAS WATER AND 2-ML FRACTIONS WERE COLLECTED.

(*a*) Extract from 25 mg of bottom-fraction solids: (i) protein; (ii) unidentified material; (iii) trigonelline; (iv) ergothioneine. (*b*) Extract of unwashed bottom fraction from latex (2 ml) obtained from the base of the stem of an 8-yr-old tree growing in the tropical greenhouse: (i) protein; (iii) trigonelline. (*c*) Extract of unwashed bottom fraction from 1 ml of clone RRIC 7 latex. (*d*) Extract from 50 mg of freeze-dried serum; full line, no ergothioneine added; broken line, 60 μg of ergothioneine hydrochloride added; the large peak is due to the nucleotides and other u.v.-absorbing compounds present in serum.

separation of the boiling-water soluble components of freeze-dried bottom fraction on a Sephadex G-15 column (55 cm × 2 cm), using water as the eluting agent. Ultraviolet absorption spectra at neutral pH and at pH 12, of fractions from peak (iv) indicated that ergothioneine was the only u.v.-absorbing compound present, and paper chromatograms, in solvents 1 and 3, of pooled freeze-dried material from the peak showed a single u.v.-absorbing spot with the same $R_f$ as ergothioneine. Similar examination of peak (iii) showed that the major u.v.-absorbing compound present was trigonelline. Peak (ii) was not identified. Peak (i) is probably attributable to the heat-stable protein hevein.[15] Examination of extracts of

[15] B. L. ARCHER, *Biochem. J.* 75, 236 (1960).

bottom fraction from mature *Hevea Benthamiana* and *H. spruceana* (growing in Malaya) and young (4-yr- and 8-yr-old) specimens of *H. brasiliensis* (growing in Ceylon) gave very similar results, ergothioneine being readily detected in all cases. In contrast, the elution diagram obtained with extracts of bottom fraction from an 8-yr-old tree growing in the tropical greenhouse, showed no peak in the ergothioneine region. The major low-molecular-weight u.v.-absorbing compound present (peak iii) was trigonelline (Fig. 1(*b*)).

A sample of serum solids (50 mg) was next examined, and although no definite peak could be seen in the ergothioneine position on the elution diagram, u.v. absorption was present in this region (Fig. 1(*d*)). However, further examination of this material failed to reveal any ergothioneine. In order to see at what level the compound could have been detected, 60 $\mu$g of ergothioneine hydrochloride was added to an aqueous extract of 50 mg of serum solids and the mixture chromatographed. The ergothioneine peak was readily detected (dotted line, Fig. 1(*d*)). Thus the serum solids sample must have contained considerably less than 1 mg of ergothioneine/g.

The elution diagram for serum (2 ml) obtained from latex tapped from the base of the stem of a young (8-yr-old) tree growing in the tropical greenhouse showed a small peak in the ergothioneine position, but no ergothioneine was detected on examination of the material from this peak by absorption spectroscopy and paper chromatography.

Karunaratne and Nadarajah,[16] as a result of an investigation of the phenols of bottom fraction from various clones of *H. brasiliensis*, suggested that clone RRIC 7 and clone RRIM 513 contained, besides tyrosine, aminophenols. This suggestion was based on the finding that paper chromatograms of bottom-fraction extracts from these clones showed spots, which reduced potassium ferricyanide and also reacted with ninhydrin. From our results it appeared likely that at least one of the spots found by these workers was a mixture of an amino acid and ergothioneine. Samples of bottom fraction from clones RRIC 7 and RRIM 513 were therefore analysed by gel-filtration. The elution pattern found with the clone RRIC 7 material (Fig. 1(*c*)) indicated that ergothioneine was present and this was confirmed by the methods described above. The small amount of material eluting after ergothioneine was pink in colour and may have been the oxidation product of some phenolic material. Analysis of clone RRIM 513 gave the same result, except that the pink band was absent.

At this stage of the investigation it was considered that the latex ergothioneine might have arisen from the micro-organisms which proliferate at the cut ends of latex vessels of trees which are regularly tapped. Bottom fraction from a mature tree which had never previously been tapped was therefore analysed. The results were almost identical to those found with bottom fraction from tapped mature trees, indicating that the ergothioneine in latex does not originate from micro-organisms on the tapping panel.

The results of all the above analyses were confirmed by using Kraut's reagent in conjunction with paper chromatography of the recovered betaines. By using this method with large (5 g) samples of freeze-dried serum, it was found that very small quantities of ergothioneine, and considerable quantities of trigonelline, were present in this material.

Since gel-filtration of bottom-fraction extracts could be used to obtain ergothioneine free of other u.v.-absorbing materials, it was possible to estimate the ergothioneine content of aqueous extracts of bottom fraction by u.v. spectrophotometry. In order that the results might be compared with those obtained by the colorimetric method, the molecular extinction coefficient was determined of the sample of ergothioneine hydrochloride used as the colorimetric standard. In water at 258 nm the value of $\epsilon$ found was 14,900 (previously reported

[16] S. W. KARUNARATNE and M. NADARAJAH, *J. Rubber Res. Inst. Ceylon* **40**, 1 (1964).

values are 16,000 and 14,500).[17] The symmetrical shape of the elution peak (Fig. 1(*a*)), suggested that recovery of ergothioneine from the column would be complete and this was confirmed by experiment. For the analysis, a 25 mg sample of freeze-dried bottom fraction was extracted in a centrifuge tube with three 1-ml portions of boiling water, the insoluble material removed by centrifugation, and the extract clarified by filtration. The filtrate and washings were then chromatographed on Sephadex G-15 as previously described. Approximately 2-ml fractions were collected and the extinction values of each fraction determined at 258 nm. The results obtained by this method, and by colorimetric analysis of several different samples of bottom-fraction solids, are recorded in Table 4.

TABLE 4. AMOUNTS OF WATER-EXTRACTABLE ERGOTHIONEINE IN FOUR DIFFERENT SAMPLES OF FREEZE-DRIED BOTTOM FRACTION

| Sample No. | Ergothioneine content (mg/g of solids) | |
|---|---|---|
| | Determined by u.v. spectrophotometry | Determined colorimetrically |
| 1. | 6·6±0·4 (6) | 9·0±0·9 (3) |
| 2. | 9·7±0·2 (3) | 8·2±0·6 (3) |
| 3. | — | 8·4±1·0 (3) |
| 4. | — | 9·5 (1) |

## DISCUSSION

The identification of tyrosine as the only water-soluble phenolic compound present in *Hevea brasiliensis* latex bottom fraction, has thrown little further light on the role of the phenolase present. There is little evidence from studies with other tissues, that phenolases are involved in terminal respiration, and it has been suggested that the essential role of this enzyme in plants is in the *ortho*-hydroxylation reactions necessary in the synthesis of such commonly occurring plant products as lignins, tannins, flavonoids, and certain alkaloids.[18, 19] Though tyrosine appears to be a precursor of the above types of compound, there is little indication that the *ortho*-hydroxylation of tyrosine itself is a step in their biosynthesis.[20] Also, no examples of the above types of compound have been detected in *Hevea* latex.

Some difficulty was experienced in interpreting chromatograms of latex extracts containing phenolic compounds and amino acids, since aliphatic, as well as aromatic amino acids, were found to react with diazotized *p*-nitroaniline to give stable coloured products (although the colour reaction was weaker in the case of the aliphatic compounds). The reaction between aliphatic amino acids and diazotized *p*-nitroaniline is not unexpected since it is known that these amino acids react with diazotized aniline and diazotized *p*-arsanilic acid,[21] and that ammonia or ammonium acetate converts diazotized *p*-nitroaniline into *pp'*-dinitrodiazoaminobenzene.[22]

[17] E. C. STOWELL, In *Organic Sulphur Compounds*, Vol. 1 (edited by N. KHARASCH), p. 462. Pergamon Press, Oxford (1961).
[18] H. S. MASON, *Advan. Enzymol.* **16**, 105 (1955).
[19] E. E. CONN, In *The Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 399. Academic Press, New York (1964).
[20] A. C. NEISH, In *The Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 295. Academic Press, New York (1964).
[21] A. N. HOWARD and F. WILD, *Biochem. J.* **65**, 651 (1957).
[22] H. T. BUCHERER and S. WOLF, *Ber. Deut. Chem. Ges.* **42**, 881 (1904).

Prior to the work reported here, the only higher plant material known to contain ergothioneine was dried oats, in which Melville and Eich[7] found 17 $\mu$g of ergothioneine per g. Table 3 shows that the amount of ergothioneine in latex bottom-fraction solids is even greater than the highest value reported for the ergothioneine content of the causative fungus of ergot, *Claviceps purpurea* (5·3 mg/g of sclerotia).[17] On the basis of Cook and Sekhar's[23] data for bottom-fraction solids content of freshly tapped latex (1·8 g/100 ml), the ergothioneine content of latex itself would range from 12 to 17 mg/100 ml. These figures, which do not include the small amount of ergothioneine in the latex serum, may be compared with the ergothioneine content of rat blood (2·0–5·2 mg/100 ml) and pig seminal fluid (29–256 mg/100 ml).[17]

It is established beyond reasonable doubt that higher animals cannot synthesize ergothioneine, and therefore must obtain it from the diet.[6] The situation in plants is less clear, only the oat plant having been examined in this respect. When oat seedlings were reared in nutrient solutions containing high activities of [$^{35}$S] sulphate, very small amounts of labelled ergothioneine could be isolated from the plants, but the possibility that contaminating micro-organisms were responsible for the synthetic activity could not be completely ruled out (Eich and Melville, quoted by Melville[6]). The absence of unequivocal evidence that higher plants can synthesize ergothioneine has led Melville[6] to suggest that higher plant as well as animal ergothioneine is of exogenous origin. This view is consistent with the finding that oat seedlings readily take up ergothioneine through their roots (Eich and Melville, quoted by Melville[6]), and that many of the fungi commonly occurring in soil can synthesize ergothioneine.[24]

Our finding, that ergothioneine is present in the latex of *Hevea* spp. growing in Malaya and Ceylon, but is not detectable in *H. brasiliensis* growing in a tropical greenhouse in England, is in accord with the above views of Melville, since differences in the numbers and activity of ergothioneine-synthesizing fungi, in the soils in which the trees were growing, would not be unexpected. Melville[6] has suggested that the variability of the ergothioneine content of dried oats found by Eich and Melville, might also be accounted for in this way. On the other hand, latex is unusual both cytologically[3] and biochemically, and the possibility that it can synthesize ergothioneine cannot be ruled out.

Evidence has also been given that *H. brasiliensis* latex contains hercynine, a compound which has been established as an ergothioneine precursor in *Neurospora crassa*. However, the presence of this compound in latex does not necessarily indicate that the latex can synthesize ergothioneine, for hercynine has also been detected in the seminal fluid of the boar.[25] In both cases it is possible that hercynine is a degradation product of ergothioneine, or an end-product of histidine methylation.

The metabolic role of ergothioneine is poorly understood at present,[6] but further studies of its origin and metabolism in *H. brasiliensis* and other laticiferous plants may throw some light on this problem. At the same time, a better understanding may be obtained of the functional significance of the lutoid particles, of which the latex bottom fraction is mainly composed.

## EXPERIMENTAL

*Latex and latex fractions.* Latex obtained from 8-yr-old *Hevea brasiliensis* growing in a tropical greenhouse attached to these laboratories was first separated by low-speed centrifuging into an upper layer and bottom

[23] A. S. COOK and B. C. SEKHAR, *J. Rubber Res. Inst. Malaya* **14**, 163 (1953).
[24] D. B. MELVILLE, D. S. GENGHOF, E. INAMINE and V. KOVALENKO, *J. Biol. Chem.* **223**, 9 (1956).
[25] D. ACKERMANN, P. H. LIST and H. G. MENSSEN, *Z. Physiol. Chem.* **314**, 33 (1959).

fraction.[26] The upper layer was then centrifuged at 175,000 *g* for 2 hr to produce a clear aqueous phase (latex serum). Samples of freeze-dried *H. brasiliensis* bottom fraction and serum, isolated from latex tapped from the trunks of mature trees[15,27] and unwashed bottom fraction from mature *H. Benthamiana* and *H. spruceana* were obtained from the Rubber Research Institute of Malaya. Unwashed bottom fraction from the *H. brasiliensis* clones RRIC 7 and RRIM 513, from a mature specimen of *H. brasiliensis* never previously tapped, and from young *H. brasiliensis*, was provided by the Rubber Research Institute of Ceylon. The unwashed bottom-fraction samples were all obtained as slurries containing 1% sodium dithionite to prevent oxidation of phenolic components, and were stored at −20°.

*Paper chromatography.* Chromatograms were run on Whatman No. 3 or No. 4 paper. The main solvents used were: (1) butan-1-ol–acetic acid–water (4:1:5, by vol.); (2) ethanol–water–diethylamine (77:22:1, by vol.); (3) butan-1-ol–propionic acid–water, made by mixing just before use, equal volumes of butan-1-ol–water (623:42, by vol.) and propionic acid–water (62:79, by vol.). Chromatograms with solvent (3) were run at 25°, others at room temperature. For the detection of phenolic materials and ergothioneine, the following reagents were used; diazotized *p*-nitroaniline, 2,6-dichloroquinonechloroimide, ammoniacal silver nitrate, potassium ferricyanide, Folin–Ciocalteau reagent[28,29] and buffered diazotized sulphanilic acid.[30] Amino acids were detected by spraying with ninhydrin, and compounds absorbing in the u.v. by means of a Hanovia chromatolite.

*Alumina chromatography.* Aluminium oxide (Savory and Moore) was washed with water and after removal of fine particles, was activated by heating at 220° for 4 hr. Ethanol required for alumina chromatography was prepared as recommended by Melville, Horner and Lubschez.[31]

*Dextran-gel chromatography.* Sephadex G-15 (medium grade) was purchased from AB Pharmacia, Uppsala, Sweden, and was soaked in water for about 1 hr with intermittent stirring, before packing into columns.

*Ion-exchange resins.* The ion-exchange resins used were: Amberlite IRA-410 (standard grade), Zeo-Karb 225 (100–200 mesh) and Dowex-50 (200–400 mesh). They were washed several times with acid, alkali, and water, before conversion to the appropriate ionic form.

*Paper electrophoresis.* Paper electrophoresis of hercynine and histidine was carried out using the apparatus of Archer and Sekhar.[27] The conditions were those recommended by Ackermann, List and Menssen,[25] except that 0·1 M sodium phosphate buffer, pH 6·4, was substituted for the pyridine–acetic acid system used by these authors.

*Spectroscopy.* Ultraviolet absorption spectra were determined with a Hitachi-Perkin Elmer 139 spectrophotometer, i.r. spectra with a Hilger H-800 double-beam i.r. spectrophotometer and NMR spectra with a Varian HA 100 NMR spectrometer.

*Kraut's Reagent.* To prepare Kraut's reagent ($KBiI_4$), 6 g of $(BiO)_2CO_3$ were dissolved in 30 ml of conc. HCl, 150 ml of water added, and the solution mixed with 80 ml of water containing 30 g of KI.[32]

*Precipitation of betaines.* Extracts containing betaines were deproteinized by adding ethanol to a final concentration of 80% (v/v), followed by centrifugation. The supernatant was dried *in vacuo* and the residue taken up in a small volume of water. Kraut's reagent was added and the resulting precipitate washed twice with water. By shaking a suspension of the washed complex with Amberlite IRA-410 acetate, the betaines could be recovered in solution.

*Colorimetric estimation of ergothioneine.* The method adopted was based on that described by Melville, Horner and Lubschez.[31] 200 mg of freeze-dried bottom fraction were extracted three times with boiling water and the pooled extracts taken to dryness *in vacuo*. The residue was treated with a small volume of ethanol–water–98% (w/w) formic acid (75:25:1, by vol.) and the soluble material chromatographed on a column (15 cm × 1·8 cm) of alumina, using the same solvent for elution. Ergothioneine first appeared after 25 ml of the solvent had passed and was completely eluted after 65 ml had passed. This fraction was dried *in vacuo*, and the residue taken up in water and shaken with a small amount of Amberlite IRA-410 acetate. After about 10 min, the resin was filtered off and washed, and the filtrate and washings made up to 10 ml.

Ergothioneine was estimated on aliquots of this solution by the method of Hunter[33] using a Spekker colorimeter fitted with a Spekker 604 filter. The overall recovery was about 93 per cent. The efficiency of the preliminary purification procedure in eliminating substances which might have interfered in the colorimetry was checked by carrying out several determinations with ergothioneine hydrochloride as an internal standard. No significant interference was observed.

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*Estimation of trigonelline.* A 1 g sample of freeze-dried bottom fraction was extracted several times with boiling water and the combined extracts concentrated *in vacuo* to about 5 ml. 20 ml of ethanol was added and the mixture centrifuged. The residue was washed several times with 80% (v/v) ethanol and the washings and supernatant combined and concentrated. The resulting solution was made up to 10 ml and trigonelline determined using the method of Sarrett.[34] Using pure trigonelline as an internal standard, it was shown that no substances interfering with colour development were present.

*Isolation of tyrosine.* After removal of lipid with light petroleum, 10 g of freeze-dried bottom fraction were extracted for 72 hr with methanol. The solvent was evaporated and the resulting residue washed several times with cold water. The insoluble material remaining was taken up in boiling water, the mixture centrifuged, and the clear supernatant cooled in ice. The white crystals which formed were recrystallized three times from water; yield 6 mg. (Found: C, 59·6; H, 6·3; N, 7·5. Calc. for $C_9H_{11}NO_3$: C, 59·6; H, 6·1; N, 7·7%.)

*Isolation of ergothioneine.* 5 g of freeze-dried bottom fraction were extracted twice with boiling water and the combined extracts (40 ml) chromatographed on a column of Zeo-Karb 225H$^+$ (15 cm × 1·8 cm). After washing with 150 ml of water, ergothioneine was eluted with 0·1 N ammonia solution.[35] The crude ergothioneine was taken up in a small volume of ethanol–water–formic acid (75:25:1, by vol.) and chromatographed on a column of alumina (16 cm × 1·8 cm).[31] The fraction containing ergothioneine gave on removal of solvent a white solid which, after extraction with several portions of cold ethanol, was crystallized three times from aqueous ethanol; yield 20 mg; m.p. 255–258°.

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