Phytochemistry, 1974, Vol. 13, pp. 2057 to 2063. Pergamon Press. Printed in England.

THE MECHANISM OF OPERATION OF CLIMATIC FACTORS IN THE BIOGENESIS OF TEA FLAVOUR

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(*Revised received* 21 *March* 1974)

Key Word Index—*Camellia sinensis*; Theaceae chlorophyll; terpenoid biogenesis; acetate or leucine precursor; solar radiation.

Abstract—The relative amounts of chlorophyll, leucine, α-alanine and α-ketoisocaproic acid in tea leaves, and those of carotenoids, β-ionone, dihydroactinidiolide, theaspirone, β-amyrin, acetate, trans-2-hexenal and phenlacetaldehyde in processed black tea have been found to vary with climatic conditions, and indicate the mechanism of flavour development in tea. These variations are related to the degree of CO_2 fixation, and the extent of intra- as compared with extra- chloroplastidic biosynthetic reactions. Evidence is discussed that extra-chloroplastidic biogenesis of terpenoid compounds from leucine leads to the development of tea flavour, whereas intrachloroplastidic fixation of CO_2, and the consequent derivation of terpenoids from acetate results in the production of teas without flavour. The leucine pathway for terpenoid biogenesis operates in conditions of climatic stress which limit CO_2 fixtation in the leaf, whereas the acetate pathway predominates in conditions of vigorous growth.

INTRODUCTION

THE DELICATE and economically valuable flavour of tea (*Camellia sinensis*, var. *assamica*) is a seasonal characteristic, and the reasons for its ephemeral nature have long been sought to be understood. In Sri Lanka (Ceylon), there are two well-defined seasons during which Ceylon tea flavour is outstanding. These are Jan/Feb in the Dimbula District, and July/Aug/Sept in the Uva District. The characteristics common to both of these seasons are cool (t 20°) dry, windy, bright, cloudless days, followed by cold (t 6–10°), clear nights. In these climatic conditions growth is slow, and 2 consecutive weeks of such weather are generally necessary before flavour becomes manifest. Rain, even slight, arrests the development of flavour, and then a further continuous period of suitable, dry weather is necessary for flavour to reappear. The famed 'first' and 'second flush' Darjeeling flavour is also confined to that short period of time when the bushes are recovering from their winter dormancy, and here again growth is slow, and the weather bright, cool and dry.

The foregoing observations and other characteristics of tea aroma are all explicable by an extension of the findings of Goodwin[1] that the terpenoids of photosynthesizing organisms and leaves are normally derived from acetate, and that the relative amounts of the different terpenoids depend on whether intra- or extra-chloroplast reactions predominate. This was established as being due to the compartmentalization of enzymes within and outside the chloroplast, and the impermeability of the chloroplast membrane to mevalonic acid. Goodwin's results together with additional reactions which are now

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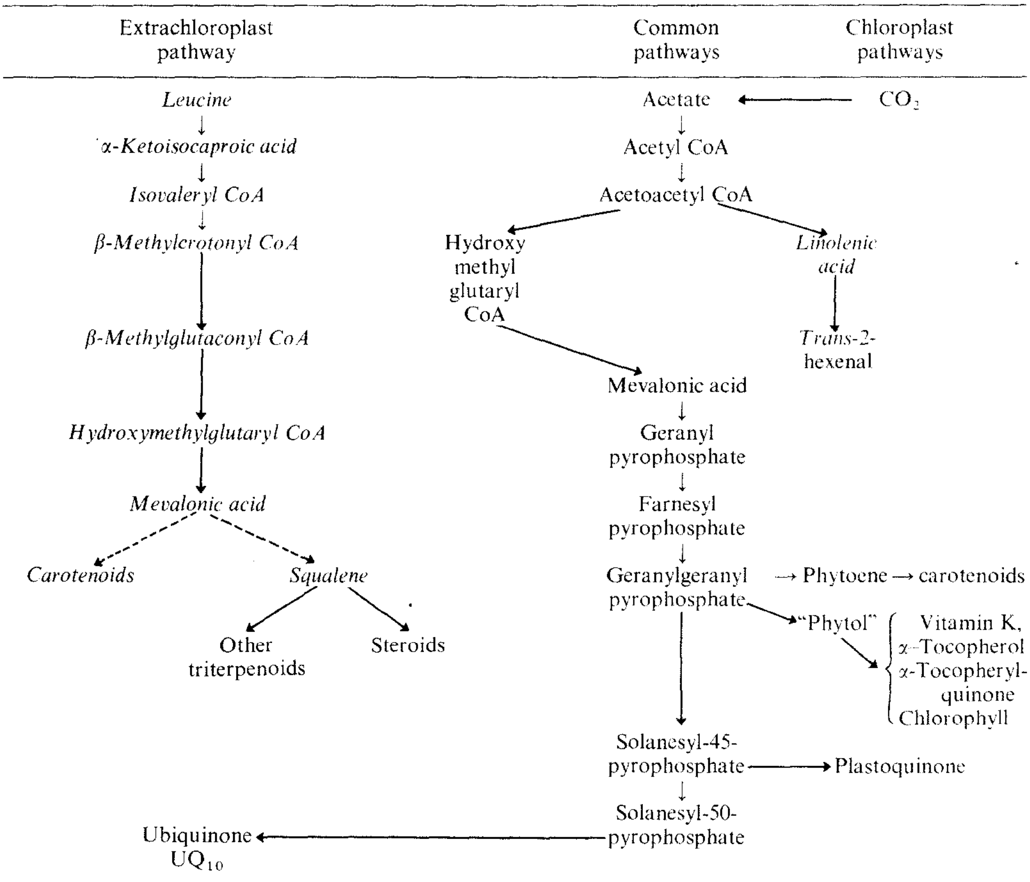
[1] GOODWIN, T. W. (1965) In *Biosynthetic Pathways in Higher Plants* (PRIDHAM, J. B. and SWAIN, T. eds.), p. 57, Academic Press, New York.

proposed as occurring in the tea leaf, are depicted in Table 1, and this extended scheme provides an explanation for the effect of climatic factors on the biogenesis of tea flavour.

TABLE 1. GOODWIN'S SCHEME FOR BIOSYNTHESIS OF TERPENOIDS, SHOWING ADDITIONAL PROPOSED PATHWAYS IN THE TEA LEAF



Proposed additional pathways are italicized.

RESULTS AND DISCUSSION

Before applying the scheme of reactions shown in Table 1 to an explanation of tea flavour, it is convenient to categorize into three groups, some of the observed differences between "flavoury"* and "non-flavoury" teas. Group 1 comprises those observations which are partly responsible for reduced CO_2 fixation by the tea leaf in "flavoury" weather, Group 2 indicates the alternate route of terpenoid biogenesis that comes into operation in these conditions, whilst Group 3 indicates some of the consequences of the reduced CO_2 fixation and alternate route for the biogenesis of terpenoids. These are summarized in Table 2, and the cause or effect of all of these differences fits into the scheme of reactions shown in Table 1 on the basis that tea flavour is due to to a reduction in the amount of CO_2 fixation within

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* Term used in the tea trade to describe black teas with natural tea flavour.

the chloroplast, and the enhanced biogenesis of terpenoid compounds from leucine rather than from acetate.

TABLE 2. DIFFERENCES IN TEA DURING "FLAVOURY" AND "NON-FLAVOURY" SEASONS

	"Flavoury" season	"Non-flavoury" season	Reference
1 Leaf stomata	Closed	Open	2,3
Chlorophyll	Low	High	Table 3
Appearance of processed tea	Brown	Black	5
Mite infestation (Ceylon)	Yes	No	6, 7
Greenfly infestation (Darjeeling)	Yes	No	8
2 Acetate	Low	High	4
Leucine	Low	High	10
α-Ketoisocaproic acid	High	Low	11
α-Alanine	Low	High	12
Carotenoids	Low	High	Table 4
β-Ionone Theaspirone Dihydroactinidiolide	High	Low	17, 18
Rhodoxanthin	High	Low	6, 7
β-Amyrin	+ +	+ +	Table 4
3 Volatile compounds of low boiling point (*e.g.* acetate and *trans*-2-hexenal)	Low	High	4, 19, 20, 21
Phenylacetaldehyde	Low	High	21

Group 1

The bright, dry, cloudless days, desiccating wind and low temperature, necessary for flavour production, cause a reduction in CO_2 assimilation,[2,3] due to the individual and combined effects of (a) closure of stomata, (b) disorganization of the chloroplast and (c) reduction of chlorophyll content (Table 3). In these conditions formation of acetate would be low[4] and there would be only slight terpenoid syntheis via the acetate pathway shown in Table 1. The brown appearance of processed "flavoury" teas is a reflection of the reduced content of chlorophyll, as it has been shown that blackness of tea is dependent on the content of phaeophytin, derived from chlorophyll during the "firing" stage of black tea manufacture.[5]

Tea pests may play a contributory role by damaging the chloroplast, and so further interfering with intra-chloroplastidic CO_2 fixation. In Ceylon, infestation by mites (*Oligonychus coffeae* Nietner—the red spider mite, *Brevipalpus californicus* Banks—the scarlet mite, *Hemitarsonemus latus* Banks—the yellow mite) reaches a peak in the flavour season[6] and there is evidence[6,7] that mites are attracted to tea by the presence of carotenoids, which they suck from the leaf by penetrating the chloroplast. This would injure the chloroplast

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[2] TAYLOR, A. O. and ROWLEY, J. A. (1971) *Plant Physiol.* **47**, 713.

[3] MEIDNER, H. and MANSFIELD, T. A. (1968) In *Physiology of Stomata*, p. 69, McGraw-Hill, New York.

[4] WICKREMASINGHE, R. L., WICK, E. L. and YAMANISHI, T. (1973) *J. Chromatog.* **79**, 75.

[5] WICKREMASINGHE, R. L. and PERERA, V. H. (1966) *Tea Quart.* **37**, 75.

[6] DANTHANARAYANA, W. and RANAWEERA, D. J. W. (1972) *Ann. Appl. Biol.*, **70**, 1.

[7] FERNANDO, E. F. W. (1967) Studies on the aetiology and biochemistry of *Tetranychus coffeae* Nietner (Acarina: Tetranychidae), the red spider mite of tea in Ceylon, M.Sc. Thesis, University of Ceylon.

and so cause its disorganization. In Darjeeling, the counterpart of the Ceylon mite is "greenfly" (*Empoasca flavescens* Fabr.), and it is likely that here too, infestation is accompanied by mechanical injury to the chloroplast. It is believed by some that greenfly attack improves the characteristic Darjeeling flavour.[8]

TABLE 3. RELATIVE CHLOROPHYLL CONTENTS OF CLONAL TEA FLUSH DURING "FLAVOURY" AND "NON-FLAVOURY" SEASONS

Clone	"Flavoury" season	"Non-flavoury" season
DT 1	0·21	0·36
TRI 777	0·28	0·47
TRI 2024	0·28	0·51
TRI 26	0·33	0·50
CH 13	0·17	0·32
CVB1	0·23	0·38
PA 22	0·18	0·39

Values represent absorbance of chloropyll extracts at 665 nm.

Reduced CO_2 fixation in the chloroplast would, therefore, appear to be favourable for the production of tea flavour, and the slow growth which has been observed to accompany flavour production is partly due to a retardation of intrachloroplastidic reactions. It is noteworthy that at least a fortnight of the appropriate weather conditions are necessary before closure of the stomata, decrease of chlorophyll content, and disorganization of the chloroplast combine together to induce flavour, and the immediate abolition of flavour development in the tea leaf by rain may be ascribed to the rapid recovery of the CO_2 fixation mechanism as soon as stress conditions are removed. In Darjeeling, the valuable "first" and "second flush" teas are those obtained at a time when the tea bush is awaking from a winter dormancy, i.e. at a time when the chloroplasts are in an immature state of development, and terpenoids are probably derived by a route which does not involve CO_2 fixation. Here too a complete disappearance of flavour follows the onset of the monsoon rains.

Group 2

In weather conditions favourable for growth, freshly plucked tea flush does not contain detectable free leucine, but the content of this aminoacid increases remarkably during the artificial "withering" stage of manufacture.[9] It was found[10] that the amount of leucine in "flavoury" black tea was relatively less than that in a black tea without flavour, and it was suggested[11] that leucine was converted, during processing, to compounds which contribute to tea flavour. Evidence in support of this suggestion was the identification of a leucine-α-ketoglutarate transaminase in tea leaves,[12] and the detection of labelled mevalonic acid, steam volatile compounds and carotenoids,[11] during the processing to black tea, of tea leaves which had been fed through the stem with ^{14}C leucine. The intermediary

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[8] DAS, G. M. (1957) *Two & A Bud.* **4**, 17.

[9] ROBERTS, G. R. and SANDERSON, G. W. (1966) *J. Sci. Food Agr.* **17**, 182.

[10] WICKREMASINGHE, R. L. and SWAIN, T. (1965) *J. Sci. Food Agr.* **16**, 57.

[11] WICKREMASINGHE, R. L. (1967) *Tea Quart.* **38**, 205.

[12] WICKREMASINGHE, R. L., PERERA, B. P. M. and DE SILVA, U. L. L. (1969) *Tea Quart.* **40**, 26.

compounds shown in Table 1 for the transformation of α-ketoisocaproic acid to mevalonic acid are based on the results of previous investigators[13] on leucine catabolism.

Leucine, as pointed out earlier, is normally not detectable in "unwithered" freshly plucked tea flush, but the dry "flavoury" weather conditions result in what is referred to as "natural wither" of the flush even before it is plucked from the bush, and release of leucine by protein hydrolysis is probably one of the reactions which occur during "natural wither". It was also observed that α-ketoisocaproic acid production was greater during the "flavoury" than "non-flavoury" season, and was especially high in clones with a reputation for flavour[11]—it is noteworthy that the growth rate of these clones was at all seasons much slower than that of other "non-flavour" clones. The observation that the amount of α-alanine during the flavour season is low[12] would favour leucine transamination, by abolishing competition by α-alanine for the α-ketoglutarate available for transamination. The assumption is made that the leucine pathway to terpenoids is extrachloroplastidic, and that it is suppressed, or of secondary importance, in conditions favourable to growth when the intrachloroplastidic acetate pathway is predominant.

TABLE 4. CAROTENOID AND β-AMYRIN CONTENTS OF "FLAVOURY" AND "NON-FLAVOURY" BLACK TEAS

Tea plantation	"Flavoury" black tea Carotenoids	β-Amyrin	"Non-flavoury" black tea Carotenoids	β-Amyrin
1	36	+	54	++
2	26	+	62	++
3	27	++	68	++
4	44	++	67	++
5	40	++	63	++

Black tea samples obtained from five different plantations during and after the flavour season.
Carotenoids determined colorimetrically by reference to a standard curve using β-carotene.
Relative amounts of β-amyrin estimated by visual inspection of thin layer chromatograms.

The amounts of carotenoids and β-amyrin in samples of "flavoury" and "non-flavoury" black teas are compared in Table 4. Three samples of "flavoury" teas were found to contain as much β-amyrin as the corresponding "non-flavoury" teas, but carotenoid contents were invariably much lower in the former. Reference to Goodwin's scheme of reactions in Table 1 indicates that, the formation of triterpenes is extrachloroplastidic, whilst that of carotenoids is intrachloroplastidic. The results shown in Table 4 do, therefore, lend support to the assumption that, in weather conditions conducive to the production of tea with flavour, the route of biosynthesis of terpenoids is extrachloroplastidic. Early investigations with *Phycomyces blakesleeanus* had also indicated that leucine was carotenogenic[14,15] and with regard to the observed similar effect of ^{14}C-leucine in tea leaves it is presumed that this extrachloroplastidic production of carotenoids is quantitatively less than that produced via CO_2 fixation from acetate in normal conditions. Moreover, the high light intensity experienced in conditions of "flavoury" weather could cause an alteration in the composition of carotenoids,[16] e.g. with respect to rhodoxanthin content. This difference in carotenoid composition, apart from attracting mites,[6,7] would also influence tea flavour in

[13] COON, M. J., KUPIECKI, F. P., DECKER, E. E., SCHLESINGER, M. J. and DEL CAMPILLO, A. (1959) In *The Biosynthesis of Terpenes and Sterols*, (WOLSTENHOIME, G. E. W. and O'CONNOR, M. eds.), p. 62, Churchill, London.

[14] GOODWIN, T. W. (1952) *Biochem. J.* **50**, 550.

[15] GOODWIN, T. W. (1955) *Ann. Rev. Biochem.* **24**, 497.

[16] HORVÁTH, G., KISSIMON, J. and FALUDI-DÁNIEL, A. (1972) *Phytochemistry* **11**, 183.

view of the findings[17,18] that β-ionone, dihydroactinidiolide, theaspirone and other volatile compounds, the relative amounts of which are known to affect black tea flavour, are derived by oxidative degradation of the carotenoid compounds present.

Group 3

Predominance of the leucine pathway for mevalonate and terpenoid production over that using acetate would afford an explanation for the well substantiated finding that the proportion of volatile compounds of low boiling point[14,19,20] is greater in "non-flavoury" than in "flavoury" black teas. A high proportion of *trans*-2-hexenal, in particular, has been identified as deleterious to tea flavour.[21] This aldehyde is derived by oxidation of linolenic acid,[22] and it will be seen from Table 1 that the precursor of this fatty acid is acetate. The proportion of trans-2-hexenal will, therefore, be reduced if the leucine pathway for production of terpenoids predominates over the acetate pathway. Furthermore it has been suggested[23] that acetate is significantly utilized for terpenoid biosynthesis by senescing chloroplasts, whilst in healthy chloroplasts, acetate is preferentially used for fatty acid synthesis. This implies that metabolism of acetate inside a subnormal chloroplast will be directed towards increased terpenoid synthesis, and reduced linolenic acid production, both of which would enhance the flavour of the tea.

The observation[21] that "flavoury" black tea contain less phenylacetaldehyde than "non-flavoury" teas also indicates that intrachloroplastidic CO_2 fixation is taking place at a reduced level in the tea leaf in conditions of "flavoury" weather. Phenylacetaldehyde in black tea is derived, by Strecker degradation of phenylalanine,[24] which is itself derived via shikimic acid, from the sugars[25] formed by intrachloroplastidic photosynthesis. Any reduction in the formation of sugars would, therefore, be reflected in a reduction of the amount of phenylacetaldehyde. These are only facets of the accumulating evidence that tea flavour is due to the *relative* proportions of a number of volatile compounds rather than to the absolute level of any particular group of compounds. In this connection it is of interest that on-going experiments in this laboratory indicate that the terpenoids formed on feeding tea leaves with ^{14}C acetate and ^{14}C leucine respectively, are qualitatively similar, but show a disparity in their amounts relative to each other.

EXPERIMENTAL

Comparative amounts of chlorophyll in leaves of different tea clones at different seasons. "Flush"* (10 g sample), plucked during "non-flavoury" weather conditions (July) from separate plots of 7 different tea clones (DT 1, TRI 777, TRI 2024, TRI 26, CH 13, CVB1 and PA22) was extracted with 25 ml 80% aq. acetone by macerating for 3 min in a Waring Blendor. The residue was re-extracted with 3 × 20 ml aliquots of aqueous acetone. Each batch of 4 chlorophyll extracts were pooled, suitably diluted with acetone, and the absorbance read at 665 nm. The procedure was repeated in "flavoury" weather conditions (February of the following year), on "flush" plucked from the identical bushes of the 7 different clones.

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* Terminal bud and two adjacent leaves, with included stem, which is the unit plucked from the tea bush for black tea manufacture.

[17] SANDERSON, G. W., CO, H. and GONZALEZ, J. G. (1971) *J. Food Sci.* **36**, 231.

[18] KAWASHIMA, K. and YAMANISHI, T. (1973) *Nippon Nogei Kagaku Kaishi*, **47**, 79.

[19] YAMANISHI, T. (1967) *Japan Agr. Res. Quart.* **2**, 25.

[20] YAMANISHI, T., WICKREMASINGHE, R. L. and PERERA, K. P. W. C. (1968) *Tea Quart.* **39**, 81.

[21] GIANTURCO, M. A., and YAMANISHI, T. personal communication.

[22] GONZALEZ, J. G., COGGON, P. and SANDERSON, G. W. (1972) *J. Food Sci.* **37**, 797.

[23] SHAH, S. P. J. and ROGERS, L. J. (1969) *Biochem. J.* **114**, 395.

[24] SANDERSON, G. W. and GRAHAM, H. N. (1973) *J. Agr. Food Chem.* **21**, 576.

[25] MEISTER, A. (1965) In *Biochemistry of the Amino Acids*. Vol. II, p. 890, Academic Press, New York.

Estimation of carotenoids. Samples (10 g) of black tea, obtained during the "flavoury" and "non-flavoury" season, from 5 different tea plantations were macerated for 2 mins with 200 ml of $CHCl_3$–MeOH (8:1), and stood overnight at 20°C. After filtration, the residue was washed with 100 ml of the $CHCl_3$–MeOH mixture. The pooled filtrates were evaporated to dryness, and the dry residue extracted successively with EtOH (10, 5 and 5 ml) and finally with 80% aq. EtOH (10 ml). The alcohol extracts were saponified,[26] the ether extracts of the saponified extracts taken to dryness, and the dry residue taken up in $CHCl_3$ (10 ml). The absorbance of this solution was measured, and the carotenoid content determined by reference to a curve, using pure β-carotene as standard.

Semi-quantitative determination of β-amyrin. β-amyrin in the above chloroform extracts was separated by TLC, the spots visualized by chlorosulphonic acid–HOAc spray reagent,[27] located with reference to an authentic marker, and determined semi-quantitatively by visual inspection. 2 μl of extracts were spotted in duplicate on activated silica gel plates, and developed in diisopropyl ether, CH_2Cl_2, and benzene–MeOH (20:1) solvents respectively.

Acknowledgements—The author thanks Mr. K. P. W. C. Perera for excellent technical assistance, Mr. C. E. V. de Silva of George Steuart & Co. Ltd., Colombo, for the samples of black tea, and Professor M. S. Bawa of the University of Sri Lanka, Peradeniya, for the authentic sample of β-amyrin.

[26] GOODWIN, T. W. (1955) In *Modern Methods of Plant Analysis*, (PAECH, K. and TRACEY, M. V., eds.), Vol. 3, p. 282, Springer, Berlin.

[27] KREBS, K. G., HEUSSER, D. and WIMMER, H. (1969) In *Thin Layer Chromatography* (STAHL, E. ed.), p. 863, Springer, Berlin.