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STUDIES ON THE ENZYMIC OXIDATION

OF CATECHINS FROM THE LEAF OF

TEA (CAMELLIA SINENSIS L.)

by

Peter J.R. Hilton.

A thesis submitted in accordance
with the requirements for the degree
of Doctor of Philosophy in the
University of Durham.

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SUMMARY

1. The subject of the study was the morbid enzymic
oxidation of catechins in the leaf of the tea plant :
the "fermentation" process of traditional tea manufacture.

2. A model system was developed, using purified catechins
and a soluble polyphenol-free polyphenol oxidase preparation.
Several forms of theaflavin and theaflavin gallate were
produced by the system, and it was shown that epigallocatechin
was most likely to be limiting with regard to total theaflavin
production. Some, but not all, of the thearubigin complex
typical of a commercial tea liquor was produced using this
model system.

3. Values for the theaflavin content and optical density
of a tea liquor were combined to give a factor which showed
a strong positive correlation to the market value of that
tea.

4. The epigallocatechin content of the fresh leaf showed a
strong positive correlation to the quality of the made tea
liquor, as assessed by the above factor. Thus the
epigallocatechin content of the terminal shoots was used as
a criterion of quality for plant selection and for further
experiments.

5. Certain climatic and cultural factors produced changes
in the catechin composition of the tea shoot, the most
obvious of which were variations in the levels of epigallocatechin
and its gallate, which appeared to be negatively correlated.
Rapid growth during the hot wet season, shading from excess
sunlight, or nitrogen application resulted in a reduction of

the ratio of epigallocatechin to its gallate, and hence
lower quality teas; slow growth during the cold season,
and phosphorus application resulted in raised epigallo-
catechin levels, and higher quality teas.

6. Moderate applications of nitrogen fertilizer resulted
in increased polyphenol oxidase activity, while exposure
of the bush to strong sunlight resulted in a considerable
reduction in activity. However the polyphenol oxidase
activity of the plucked shoot, compared with the catechin
composition, appeared to be of only minor importance with
regard to the quality of the final product.

Contents

INTRODUCTION

The black tea of commerce is a product manufactured from the young vegetative terminal shoots plucked from the tea bush <u>Camellia sinensis</u> L. It differs from green tea in that in the manufacturing process an enzymic oxidation of polyphenols takes place, resulting in a brown colouration which is characteristic of the infusion made with boiling water. The tea industry traditionally refers to the oxidation process as "fermentation" although this is strictly a misnomer, since atmospheric oxygen is used as an electron acceptor in the reaction, and not a carbohydrate source as in fermentation by micro-organisms. Fermentation is prevented in green tea manufacture by steaming the leaf to inactivate the oxidative enzymes.

In the natural state, the polyphenols occur in the vacuoles of the leaf parenchyma cells (Roberts, 1962a, Tambiah et.al., 1966; Shalamberidze et.al., 1969; Forrest and Bendall, 1969a), and are spacially separated from the oxidative enzymes, the localization of which is uncertain, but is certainly extravacuolar (Li and Bonner, 1947; Gregory, 1964; Takeo, 1966; Wickremasinghe et.al., 1967, 1968). Thus fermentation is a morbid process which takes place after disruption of tissue and cellular organisation. Manufacturing processes are designed to bring about cell rupture, and range in severity from simulations of the classical Chinese hand-rolling technique to the more drastic action of CTC[*]

[*]See Appendix for abbreviations.

machines and tobacco cutters (Harler, 1963).

Most black tea for European consumption is sold by auction in London, and the price realized will depend upon market supply and demand, and the quality of the product. This latter is assessed by experienced tasters, whose aim ultimately must be to orientate their criteria of quality to consumer demand. The tea taster has developed a language of his own to describe the various characteristics of a tea liquor, and a glossary of such terms is given by Harler (1963). However, the essential features with regard to a tea's commercial value may be described in only five of these: "briskness", "colour", "strength", "flavour", and "quality"(Roberts, 1962a). Briskness is described as a liveliness on the palate, or the astringency associated with the tea taste. A good colour is of golden hue, with plenty of depth, and will be associated with the strength or "body" of the tea liquor. Flavour, or "aroma", is determined by the low - boiling volatile constituents of the liquor. Quality is a very loosely used term: it may be all embracing, and therefore be equivalent to the tea's valuation on the auction floor; or it may be regarded as a specific attribute, distinct from other tea-taster's criteria, which Sanderson (1965b) defines as " a distinct and desirable effect on palate and nose". Roberts (1962a) refers to quality as "a general integration of desirable characteristics".

Since the term "quality", used in its broadest meaning,

is essentially subjective, what constitutes a quality
tea will vary in different parts of the world where
tea drinking habits and consumer demand differ. The
above tea-tasting terms are relevant to demand in
Western Europe, and thus a quality tea will have an
acceptable combination of colour, briskness, strength,
and flavour. However, a quality green tea or Oolong tea
will be accepted on its flavour alone. Currently, Russian
technologists are exploiting the therapeutic value of the
unoxidized phenolics and vitamin P content in tea; hence
in Russia a quality tea is one in which there is still an
appreciable unoxidized phenolic residue in the finished
product, besides some degree of coloured products
(Bokuchava and Kharebava, 1969; Bokuchava and Skobeleva,
1969).

Teas produced in Malawi are not renowned for
exhibiting much flavour. Nor is the flavour produced by
some Malawi teas at certain times of the year particularly
acceptable to buyers. Furthermore, manufacturing conditions
which produce brisk teas of good colour and strength do not
appear to be compatible with flavour production : the
more drastic cell rupturing techniques of CTC manufacture,
which result in very brisk, strong, coloury teas, are not
used in the manufacture of flavoury teas from Ceylon or
Darjeeling (Harler, 1963). In view of these considerations,
efforts in Malawi are concentrated on improving briskness,
colour, and strength in teas, since these attributes are

immediately acceptable to the market, and little
emphasis is placed on flavour production. Thus the
work conducted in this study is concerned only with
briskness, colour, and strength, and the term "quality"
is used in this limited context to describe the desirable
integration of these factors.

Early investigators of the polyphenolic content of
a tea liquor and its relationship to quality, recognized
that the most important contributors to colour and
briskness could be extracted from an aqueous solution by
ethyl acetate. (Bradfield and Penny, 1944; Mitra and
Chatterji, 1957). Subsequently Roberts and co-workers
showed that the relevant components of the ethyl acetate
extract were a well defined class of compounds, the
theaflavins; all other coloured components were then
grouped together as thearubigins, which was a term of
convenience, not intended to convey any great degree of
homogeneity. Roberts showed that these components of
the tea liquor were produced during the fermentation
process, and that catechins were the principal precursors.
He also developed the ethyl acetate extraction procedure
as an analytical method for objective assessment of
quality, and showed that TF was the major contributor to
briskness and a good colour, while TR contributed to depth
of colour and strength. This work is well reviewed by
Roberts himself (Roberts, 1962a). Other workers have
since used Roberts method for assessing the quality of
tea liquors (Ramaswarmy, 1963; Kharebava and Nikolaishvili,

1964; Bhatia and Ullah, 1965; Wickremasinghe, 1965;
Bokuchava, et.al 1966; Nakagawa, 1970), and an automated
procedure for the analysis has been developed (Casson
and Shenton, 1969).

Besides the fractionation achieved by solvent
extraction, Roberts separated TF from TR by paper
chromatography, and demonstrated that TF was composed of
at least two fractions which he assumed to be theaflavin
and its gallate (Roberts, et.al., 1957). He prepared
TF^1 in a crystalline state (Roberts and Myers, 1959b), and
the chemical structure of TF^1 has since been determined
(Takino et.al., 1965; 1966; Brown et.al., 1966). Paper
chromatography has not proved very useful in fractionating
the TR complex, though various other methods have been used
with varying success, such as chromatography on a cellulose
column (Vuataz and Brandenberger, 1961), Sephadex gel
filtration (Crispen et.al., 1968), and electrofocusing
(Jonsson and Petersson, 1968). Using chemical techniques,
Brown et.al. (1969a, b) identified the thearubigins with
polymeric proanthocyanidins, but such a structure would
not account for the protein amino-acids identified in TR
by other workers (Vuataz and Brandenberger, 1961; Millin
et.al. 1969a), and therefore other substances are likely
to be involved.

The catechins were originally identified as the major
polyphenolic components of the green leaf by Bradfield, who
separated and identified several of the catechins now
recognised (Bradfield, 1946; Bradfield et.al., 1947;

Bradfield and Penny, 1948). Roberts subsequently used paper chromatography to separate the six major catechins occurring in tea shoots, and identified them as EGCG, ECG, EGC, EC, GC, and C, which occur approximately in that order of concentration (Roberts and Wood, 1951, 1953), and his work has since been confirmed by other workers using paper chromatography (Nakagwa and Torii, 1964[a]; Oragvelidze et.al., 1966; Bhatia and Ullah, 1968), thin-layer chromatography, (Forrest and Bendall, 1969a) and gas chromatography (Pierce et.al., 1969).

Quantities of purified catechins have been prepared by preparative paper chromatography (Buzun, 1962), partition chromatography on a cellulose column (Vuataz, et.al., 1959), counter-current distribution (Roberts and Myers, 1960a), and fractional precipitation with lead acetate (Takino and Imagawa, 1963a). Various workers have attempted to produce TF and TR by incubating combinations of the six catechins described by Roberts with a washed acetone-dried powder of fresh tea shoots, though their results do not agree in all respects. Thus while Roberts obtained TF[1] and TFG from an incubation of EGC and EGCG together (Roberts and Myers, 1959a), Takino claimed that theaflavin required EC as a precursor (Takino and Imagawa, 1963b). The preparation of TFG that Roberts obtained from black tea was chromatographically homogeneous, and although he failed to crystallise it he considered that his preparation was a single substance (Roberts and Myers, 1959b). Yet Nakagawa

and Torii (1965) reported the production of several heterogeneous, forms of theaflavin gallate in incubations of individual catechins. Nor was Roberts able to produce TR in his incubations, though Nakagawa and Torii could in theirs. However, in all incubations using a washed acetone-dried powder, the products of oxidation became strongly adsorbed to the powder, staining it heavily, and one suspects therefore, that some products may have been difficult to detect in the supernatent.

An understanding of the oxidizing enzymes concerned in fermentation was complicated by the observation that peroxidase activity was considerably greater than the oxidase activity of the shoot (Roberts and Sarma, 1938). However, Lamb and Sreerangacher (1940 a,b) demonstrated that fermentation was dependent upon atmospheric oxygen, the system being completely inhibited by 0.01 M KCN, and now Sreerangacher's view that the tea oxidase is a polyphenol oxidase of the copper-protein type are generally accepted. Gregory and Bendall (1966) obtained a pure preparation which confirms this view. While the tea oxidase has the necessary specificity to be classed as an o-diphenol: oxygen oxidoreductase (1.10.3.1) this group represents a heterogeneous class of enzymes, none of which appear to have exactly the same specificity as the tea oxidase (Gregory, 1964). Hence the term polyphenol oxidase as used in this work refers only to that enzyme system in the tea plant which uses oxygen and an o-diphenol or vic-triphenol as substrates.

Any plant breeding programme designed to select
bushes which will produce high quality teas is ultimately
dependent upon the tea-taster to assess the quality of the
final product. This is highly unsatisfactory, for his
comments will obviously be influenced by market fluctuations,
and he will have no objective scale of values to which he
can refer in suceeding years. Furthermore, assessment of
the final product is in itself undesirable, as it will
introduce variations in quality due to variations in
plucking standards and manufacturing procedure. Also,
it takes a tea bush several years to mature to the stage
when enough shoots can be plucked for a sample to be
manufactured. A knowledge of which components of the
green leaf were responsible for the quality of the final
product would enable the plant breeder to make an objective
assessment of bushes at the seedling stage.

The same knowledge would also be useful in assessing
the effects of various cultural factors upon quality. It
was noticed that shading, which was known to reduce quality
in East and Central Africa (McCulloch et.al., 1966)
reduced the catechin content of the shoot (Eden, 1958).
It has long been suspected that nitrogen application, while
increasing yield, is deterimental to quality (Eden, 1958),
which may be due to high nitrogen applications producing
lower levels of extractable phenolics in the shoot
(Mirzoyan, 1963; Guseinov and Mirzoyan, 1967 b). There
have been claims that this loss in quality may be counteracted

by phosphorus application (Shavishvili, 1966; Willson
and Choudhury, 1968), though other workers have found
that high rates of phosphorus application lowered the
level of phenolics in the green leaf and resulted in
a loss in quality (Ishigaki and Takayanagi, 1966;
Guseinov and Mirzoyan, 1967a). This discrepancy needs
clarifying, though it may prove that the tea plant shows
different fertilizer responses when grown under different
soil conditions.

Various attempts have been made to correlate chemical
components of the green leaf with the quality of the made
tea. Whilst caffeine is an essential component of "cream",
which is the coloured precipitate which forms when a tea
liquor cools, and is considered a desirable attribute, no
correlation has been found between the caffeine content
of the green leaf and the overall quality of the made tea
liquor (Torii and Ota, 1960; Doolidge, 1966; Fecak and
Struhav, 1970). Copper and boron are considered desirable
elements, while calcium is deterimental to quality (Kajita,1963;
Nakagawa and Yokoyama, 1968). The presence of free amino-
acids to their corresponding aldehydes, which may make some
contribution to flavour and aroma (Bokuchava and Popov,
1954; Popov 1956, 1966a, b; Wickremasinghe and Swain, 1964
1965). Also, there is a possibility of the amino-acids
being incorporated into the TR complex: James et.al. (1948)
reported a coloured complex formed from the action of
catechol oxidase upon catechol and amino-acids in Belladonna,
and while this is not compatible with the structure for

TR proposed by Brown et.al. (1969a,b), there has been a report of a dicatechin-amino-acid complex occurring as an impurity in the extraction of caffeine from waste tea material (Vachnadze, 1966).

But from Roberts work described above, it is clear that the most important components in the green leaf with regard to TF and TR production are the catechins. Except when teas are being produced exclusively for flavour, when high catechin levels may be deterimental (Hon-Kai Ho et.al., 1969), bushes with a high catechin content produce quality teas having high levels of TF and TR (Roberts, 1962b; Bhatia and Ullah, 1965; Ullah, 1967; Millin and Rustidge, 1967).

It was against this background that the tea research organisations of Central and East Africa contributed towards a research programme, conducted at Cambridge University, into the biochemistry of the fermentation process. Initial research was directed towards conducting fermentation under controlled conditions, in which the importance of polyphenol oxidase was established, and the development of TF and TR studied (Bendall, 1960). Routine analytical methods were devised for assaying polyphenol oxidase activity, and facilities for analysing tea liquors by Roberts' method were made available in Malawi. Concurrently, developments in horticultural technique made propagation of large clonal populations possible, and the available techniques were immediately exploited in the plant selection programme. Doubts on the nature of

the oxidizing system were dispelled when polyphenol

oxidase was purified from tea shoots and shown to have

oxidase activity towards all the six catechins isolated

from tea (Gregory, 1964; Gregory and Bendall, 1966).

Forrest and Bendall (1969a) subsequently developed

techniques for assaying the six catechins after

separation on cellulose thin layers. These techniques

were considerably more accurate than those used

previously (Roberts and Wood, 1951; Nakagawa and Torii,

1964^{a}; Bhatia and Ullah, 1968), both from the point of

view of separation and quantitative estimation. This

enabled accurate determination of the catechin composition

of a large number of bushes. From his work, Forrest

concluded that the catechin composition of a clonal

population was a very stable entity, not much affected

by seasonal changes, and where differences were observed

between samples of the same clone grown under different

agronomic conditions, they were small in comparison to

the differences encountered between genetically different

material (Forrest, 1967). However, stability under

seasonal trends in quality, and concurrent work in India

showed that there were considerable changes in the

proportions of the six catechins throughout the season

(Bhatia and Ullah, 1968). From an investigation of the

catechin composition, polyphenol oxidase activity, and

fermentation behaviour of a large sample of different clones,

Forrest concluded that the levels of EGC and EC were most

likely to be limiting in TF production, and that only

moderately low enzyme activities were necessary to ensure

the best use was made of the available catechins.

At the time of the author's entry into this work, there were strong indications that the world tea markets were over supplied, and negotiations were under way to adopt a controlled international quota system of production, such as had been operating in the coffee producing countries for some years. This quota system came into voluntary operation amongst most of the tea producing countries during 1969. With the emphasis therefore removed from quantity production, it became of paramount importance for producers to increase the quality of their product. For plant selection, therefore, it was increasingly important to know the optimum proportions of catechins for quality production. Also, where estates in Malawi had plantings of established tea of widely different quality, it was recommended that they orientated their fertilizer application towards getting the highest yield from the best tea; information on the effects of heavy fertilizer applications on quality was thus urgently required.

Hence it was projected to work backwards from the auction floor to the green leaf: to relate selling price directly to some character in Roberts TF/TR analysis; to investigate the production of TF&TR in a model system using purified catechins and purified polyphenol oxidase; to investigate how many species of TF or TFG were formed, and from which catechins; to determine which catechins were limiting in terms of the character selected to represent

quality; to investigate ways in which the catechin
composition might vary with seasonal and cultural factors,
and therefore to relate such variations to changes in
the quality of the marketed product.

The developments in the field since Roberts'
investigations suggested that this programme had a good
chance of achieving its aims. Following directly from
the work of Bendall, Gregory, and Forrest, an in vitro
system could be developed in which all components were
soluble, and therefore no assumptions need to be made
with the regard to the physical availability of the
enzyme in the preparation or the location of the products.
(In fact, complete purification of polyphenol oxidase
was not achieved due to the difficulty in transporting
suitable material from Malawi to England, but the soluble
preparation eventually used was probably more illustrative
of the commercial fermentation process than a pure
homogeneous preparation would have been.) Better methods
of fractionating the oxidized components were available,
and therefore more information could be obtained on the
nature of TR. Well established clonal material was
available in Malawi to study environmental effects, and
this, together with Forrest and Bendall's more accurate
method for catechin analysis, meant that sampling and
experimental errors were considerably reduced compared
with previous work.

The work concerning the in vitro incubations and

preparation of components for these was conducted in
the Botany Department at Durham University. All other
work was conducted on the research stations of the Tea
Research Foundation of Central Africa during two visits
thêre in 1968 and 1970. Whilst the material for this
thesis was intended as an excercise in pure research,
the direction of investigation was determined not only
by philosophy, but also by the immediate needs of the
tea industry in Malawi, and was to some extent limited
by the availability of equipment in Malawi.

MATERIALS AND METHODS

1. ### Biological Materials

Tea leaf was grown on the research stations of the Tea Research Foundation of Central Africa, in Mlanje Malawi, and unless otherwise stated was harvested as the first two leaves and the bud of young vegetative shoots.

Clonal material had the following origins:

MT 12 : Selected broad-leafed Assam, Tingamera variety.

SFS 371 : Hybrid Indian, selected at Swazi Research Station.

SFS 204 : "   "   "   " "   "   "

PC 1 : Selected progeny of cross between a China hybrid type clone (CL 17) and a clone selected from Betjan variety of Assam type (M 7).

2. ### Chemicals and Reagents

With the exceptions listed below, chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, and were of analytical reagent grade when available. In Malawi, supplies of BDH chemicals were occasionally supplimented with Baker Analysed Reagent, supplied by Miller and Wixley, Blantyre, Malawi.

Pyrogallol

Vanillin

were obtained from Hopkin and Williams Ltd.,
  Chadwell Heath, Essex.

Polyvinylpyrrolidone   (M.W. 24,000)

(-) Epicatechin

Benzidine

were obtained from Koch-Light Laboratories Ltd.,
  Colnbrook, Bucks.

Cellulose powder (MN 300)

was obtained from Machery-Nagel Co., Düren, W. Germany

Cellulose powder (CC 31)

DEAE - cellulose (DE 52)

were obtained as Whatman products through Reeve Angel Ltd.

Sephadex G25

Sephadex LH - 20

were obtained from Pharmacia Ltd., Uppsala, Sweden

Ethyl acetate

was obtained from May and Baker Ltd., Dagenham, Essex.

Ascorbic acid

was obtained from Roche Products (U.K.) Ltd.,
    Manchester Street, London.

(+) Catechin

was supplied by Sigma Chemical Co. Ltd. London

Polyclar AT

was supplied by General Aniline and Film Corp. (U.K.)
    Manchester

Flavognost

was supplied by Heyl & Co., Berlin

Nonidet P 40

Acetone

Methyl iso-Butyl Ketone (MIBK)

were obtained from Shell Chemical Co., either in U.K.
    or Malawi.

Ethanol

was obtained from the Distillers Company (Rhodesia) Ltd.

Oxygen

Nitrogen

were obtained from the Rhodesia Oxygen Company Ltd.,
    Salisbury.

Solid carbon dioxide

was obtained from National Chemical Products
    (Rhodesia) Pty., Salisbury.

All chemicals were used as supplied, except for
acetone, MIBK, butan-1-ol , and acetic acid, which were
redistilled once before use.

3. Other Materials

Visking dialysis tubing was obtained from the Scientific
Instrument Centre Ltd., Leeke Street, London W.C.1.

4. Field Histories

(a) Mimosa Research Station Field 3 - Nitrogen variation

seedling tea planted 1951

Nitrogen (as ammonium sulphate) was increased

progressively until 1956, when following rates

were reached and maintained:-

No	N_1	N_2	N_3	
40	80	160	240	lbs/acre/annum.

P and K fertilization constant

(b) Swazi Research Station Field 13 - 3^3 NPK variation.

Seedling tea planted 1934, and established under

uniform fertilizer treatment

1952 - 1961, maintained at following N levels:-

> No N_1 N_2
>
> 40 80 120 lbs/acre/annum

Since 1961, maintained at following N levels:-

> No N_1 N_2
>
> 40 120 200 lbs/acre/annum

P and K maintained since 1952 at

0, 30, and 60 lbs P_2O_5 and K_2O respectively

(c) Swazi Research Station - Field 9 - nitrogen and clone variation.

Seedling tea planted 1934

1934 - 1947 - organic and inorganic manures experiment

1947 - 1962 - various experiments

1962 - 1968 - uniform fertilizer treatment

1968 - uprooted

> replanted under irrigation with clones
>
> SFS 204, MT 12, SFS 371, MFS 76

Nitrogen rates 1969-70 were as follows:

> N_o N_1 N_2 N_3
>
> 120 180 240 300 lb/acre/annum

5. **Preparation of Solutions**

(i) **Extraction solution for polyphenol oxidase**

5% (w/v) Polyvinylpyrrolidone - 50 ml

0.05 M phosphate buffer pH 7.2 - 50 ml

(KH_2PO_4, Na_2HPO_4)

(ii) <u>Reaction medium for polyphenol oxidase assay</u>

    20 ml 0.05 M sodium citrate buffer pH 5-6.

    6 ml water

    4 ml M pyrogallol solution

(iii) <u>Acid acetone for polyphenol oxidase assay</u>

    97 ml acetone

    3 ml conc. HCl

(iv) <u>Acid acetone for catechin extraction</u>

    97 ml 80% (v/v) aqueous acetone

    3 ml conc. HCl.

(v) <u>Ammonium Molybdate reagent for phosphorus assay</u>

    15g $(NH_4)_6Mo_7O_{24}.4H_2O$ dissolved in 300 ml water.

    350 ml conc. HCl added, and volume made up to

    1 litre with water. Stored in brown glass

    bottle and renewed every 2 months.

(vi) <u>Stannous chloride reagent for phosphorus assay</u>

    1.25g $SnCl_2. 2H_2O$ dissolved in 5.6 ml conc. HCl.

    volume made up to 50 ml with water. Prepared

    fresh each day.

6. STANDARDIZATION OF SHOOT SIZE

Shoots of two leaves and a bud were sampled for most experiments, but there was a considerable variation of size within this category. The chemical constituents of a leaf change with age; thus a large shoot, which will contain a larger proportion of older leaf material, will have a different chemical composition from a small one. In long-term analysis experiments, therefore, an attempt was

made to eliminate this variable by sampling shoots at exactly the same stage in development.

For each clone under long term observation, a large sample of two-and-a-bud shoots was weighed, and the mean shoot weight determined. 6 shoots having exactly this mean weight were then selected, and their silhouette reproduced on map copying paper. Subsequently, all experiments on these clones were conducted on shoots which had been matched to one of the corresponding silhouettes.

7. Assay of Polyphenol Oxidase

(a) Colourimetric

All measurements of PPO activity conducted in Malawi were colourimetric.

Two and-a-bud shoots were stood in water under polythene bags for 2 hours to become fully turgid. 5 shoots were weighed and ground with half their weight of fine acid-washed sand in a chilled mortar in a deep-freeze cabinet. When thoroughly homogenized, 8.88 ml cold grinding solution per gm leaf was added, making $\frac{1}{10}$ homogenate. One ml of this was added to 14 ml iced water, making $\frac{1}{150}$ homogenate. 1ml of $\frac{1}{150}$ homogenate was added to 3 ml reaction medium and shaken at 30° for $\frac{1}{2}$ hour, when the reaction was stopped with 4 ml acid acetone. The sample was centrifuged in an MSE bench centrifuge and the extinction read against acid acetone in a Unicam SP.600 spectrophotometer. The following blanks were subtracted

from the reading:

   (a)   Incubation with no enzyme (i.e. 3ml reaction mix incubated with 1 ml water.

   (b)   Incubation with no substrate (i.e. incubation of 1ml enzyme with 3mls buffer without pyrogallol).

Calculation:-

The reaction involves the oxidation of two molecules of pyrogallol to one molecule of purpurogallin (mol.wt.220) using 3 atoms of oxygen.

A value of E_{460} = 1.000 is given by a solution of purpurogallin in acid acetone containing 0.1019 mg/ml.

i.e. 8ml reaction medium contains 0.815 mg purpurogallin, produced in 30 min.

Theoretically 1 mol purpurogallin $\equiv$ 3 gram atoms O_2

$$\therefore \; 1 \; \mu g \text{ purpurogallin} \equiv \frac{3}{2 \times 220} \; \mu mol \; O_2$$

$$\text{or} \quad 1 \text{ mg purpurogallin} = \frac{3 \times 1000}{2 \times 220} = 6.82 \; \mu mol \; O_2$$

$\therefore \; E_{460}$ = 1.000 is equivalent to an O_2 uptake of

$$\frac{0.815 \times 6.82}{30} = 0.185 \; \mu mol \; O_2 \; / \; min$$

$$= 0.185 \; IU$$

8 ml reaction medium contains 1 ml homogenate at $\frac{1}{150}$ dilution.

$\therefore$ Activity of leaf = E_{460} x 0.185 x 150

$$= E_{460} \text{ x } 27.75 \text{ IU/g fresh weight.}$$

The E_{460} value was proportional to the enzyme concentration up to E_{460} = 0.7 (Figure 1) and above this value the enzyme preparation was diluted further and reread.

Reproducibility of the analysis was not good (coefficient of variation was approximately 10%). Further investigation
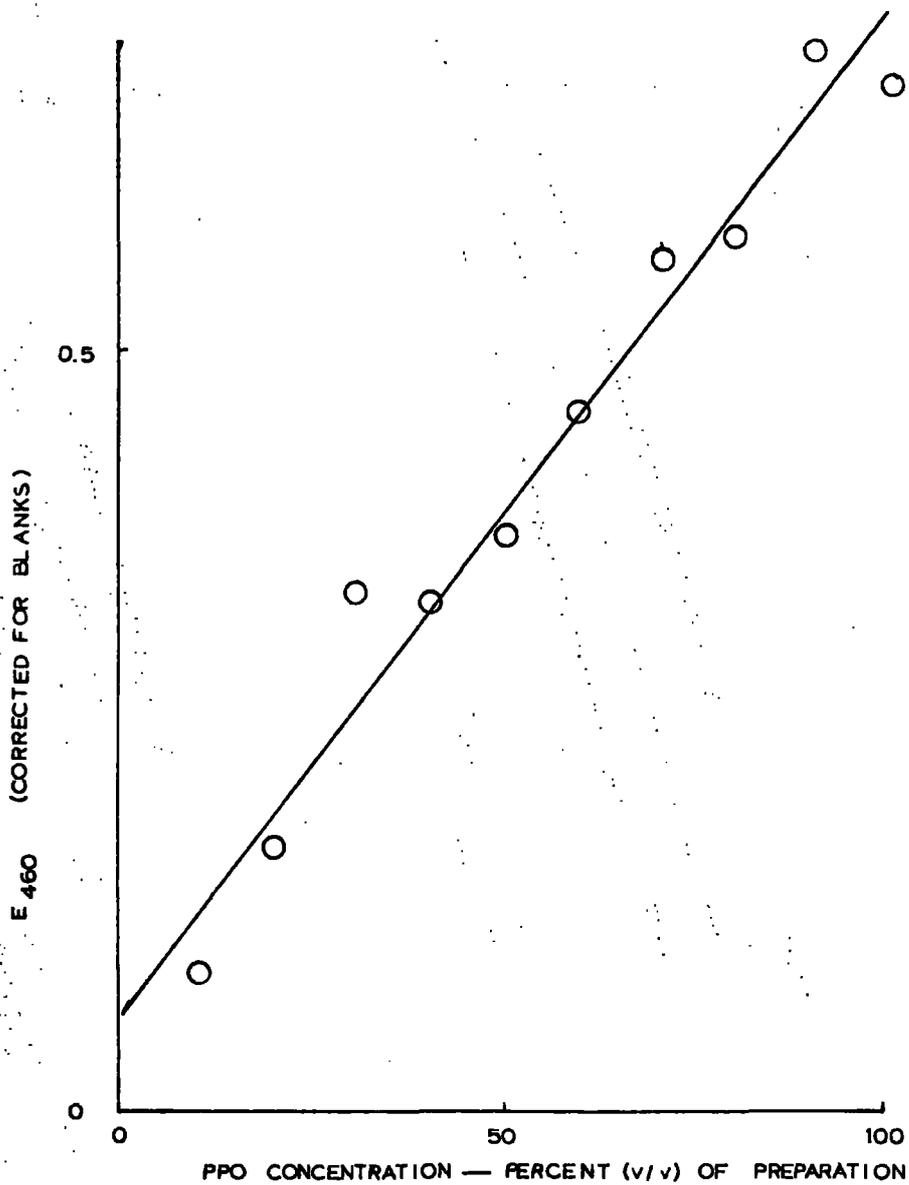
FIGURE 1

DEPENDENCE OF COLOUR RRODUCTION UPON
CONCENTRATION OF ENZYME PREPARATION.

Preparation: $^1/_{150}$ homogenate of fresh shoots
of clone SFS 371. For details of assay procedure
see text.

showed that most of the error was introduced during the dilution of the homogenate : a series of analyses from the same $\frac{1}{10}$ homogenate showed a coefficient of variation of 11.0%, while a series conducted from the same $\frac{1}{150}$ homogenate showed a coefficient of variation of only 4.1%. Assays quoted in Tables 4 and 15 , and Figure 4 are means of three assays from the $\frac{1}{150}$ homogenate. Greater accuracy was sometimes desireable, and for Tables 5 and 6 figures are means of twelve readings : four replications of the $\frac{1}{150}$ dilution, with three assays from each. Statistical significance between means was then assessed.

(b) **Polarographic**

All measurements of polyphenol oxidase activity conducted in Durham were performed by directly measuring oxygen uptake in a Clark oxygen electrode (Rank Bros., Bottisham, Cambs.) coupled to a Servoscribe or Vitatron chart recorder, following the method of Gregory and Bendall (1966).

The reaction medium was 0.01 M pyrogallol in 0.05 M citrate buffer pH 5.6, and each assay was performed in a total volume of 3 ml.

The chamber of the electrode was kept at 30° with a water-jacket, and the required volume of reaction medium introduced. After temperature equilibration, the rate of background oxidation at approximately the air value was noted, after which the oxygen concentration of the medium

was raised by bubbling oxygen. The enzyme preparation was introduced by means of a constriction micro-pipette (the balance of the 3ml volume), and the rate of oxygen uptake noted at the air value.

Calculation:-

The solubility of O_2 in a medium of approximately the same ionic strength as that used here is 0.445 µg - atoms O/ml at 30° (Chappell, 1964). Equilibration of the buffer with air by standing overnight in an open bottle at 30° resulted in a chart reading of 37 units (100 units = full scale deflection), and this was taken as the air value, both for calibration of the scale and for the O_2 concentration at which rates were measured. Activities were expressed in O_2 uptake (µmol O_2 / min).

8. **Assay of Catechins**

Catechins were separated by chromatography on cellulose thin layers, eluting in the first dimension with water, and in the second with butan-1-ol-acetic acid-water (4 : 1 : 5 by volume top phase), following the method of Forrest and Bendall (1969a). The six catechins under investigation separated well, but initial washing of the plates in the organic solvent prevented ECG from becoming contaminated with impurities from the cellulose eluted with the organic solvent front, and also revealed a further spot (Spot V, Figure 18   ).

Catechins were located by spraying with bis-diazotized benzidine solution (Roux and Maihs, 1960),

except where quantitative assessment followed, when
location was either by fluorescence under ultra violet
light after fuming with ammonia, or by fuming in iodine
vapour. The latter method was found more convenient
for routine analysis.

Quantitative estimation was by the vanillin
reaction (Forrest and Bendall, 1969a), which involves the
production of a pink colouration by the reaction. This
is directly proportional to the concentration of catechin
present below E_{500} = 0.9 after reagent blank correction
(Figure 2   ), and all estimations were within this
range. In the absence of sufficiently pure standards,
the factors for converting extinction values into
weight of catechin were taken from the literature (Forest
and Bendall, 1969a).

Reagent blanks for this reaction were normally within
the range described by Forrest and Bendall, i.e.
E_{500} = 0.05 - 0.07, but towards the conclusion of work in
Malawi a fault developed in the spectrophotometer (a
Unicam SP 600) which lead to reagent blanks reading as
high as E_{500} = 0.3. Results seemed to be consistent with
previous experience, however, although accuracy was
obviously reduced.

Forrest and Bendall claim cellulose blanks to be
negligable in this reaction, but with the materials used
here this was far from true (Figure 3   ). Ideally, a
cellulose correction should have been applied according
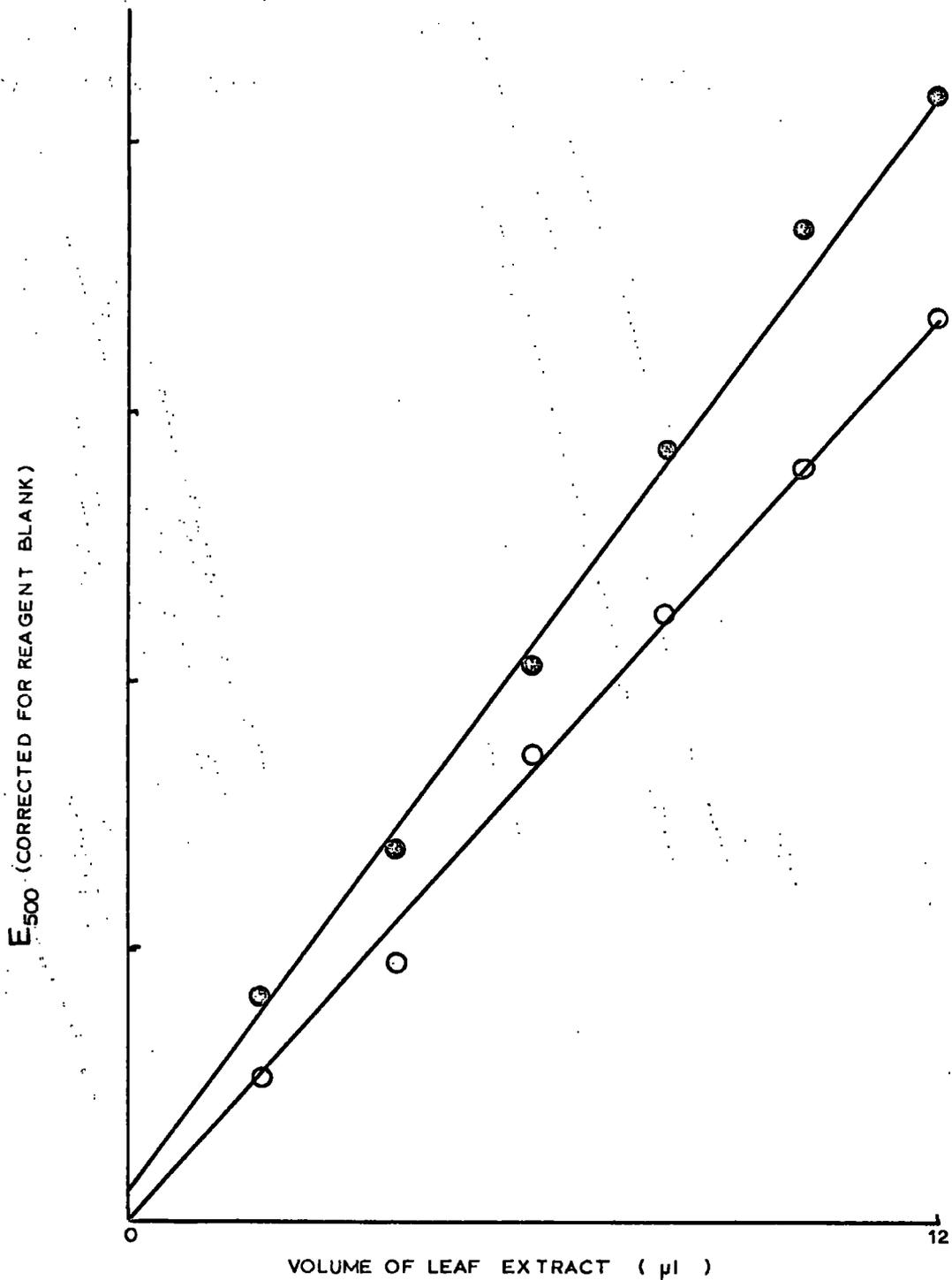
FIGURE 2

Vanillin reaction : dependence of colour production upon concentration of substrate.

O—O EGC   ●—● EGCG

1g dried leaf from clone MT 12 was extracted with 10ml 50% (v/v) aqueous ethanol. Varying volumes of extract were subjected to t.l.c., the resulting catechin spots removed, and assayed by the vanillin reaction as described in the Methods chapter.
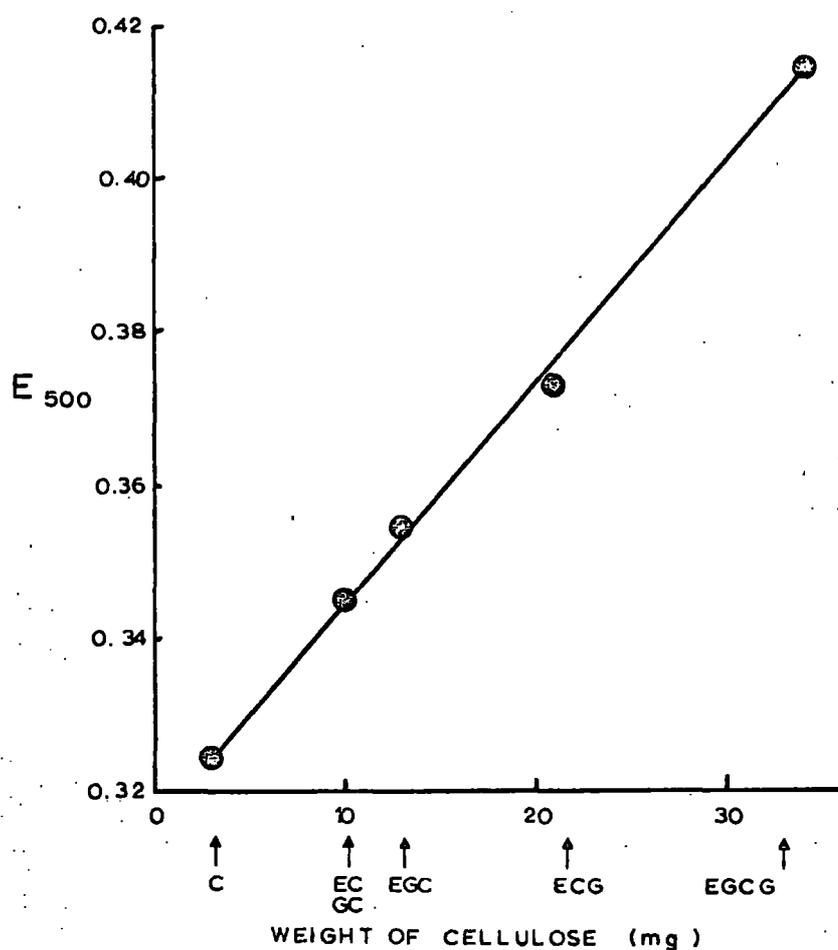
FIGURE 3

Cellulose blanks in vanillin reaction

Spots of varying size were scraped from a cellulose
thin layer which had been developed in solvents as
for catechin separation, and used as blanks in the
vanillin reaction (see Methods chapter). The
approximate weights of spots corresponding to the
six catechins as normally experienced during routine
analysis are indicated on the abscissa.

to the weight of each spot, but no balance of
sufficient accuracy was available in Malawi even if
the extra effort involved were justified by the
increase in accuracy.   In routine work, however, an
attempt was made to remove the same area of cellulose
when removing spots of the same catechin for analysis.

Even with these inaccuracies prevailing, the
following degree of accuracy was achieved by the method
during an experiment in which 8 thin layer plates were
loaded from the same extract of clone PC 1 :

Catechin	Coefficient of Variation (σ %)
EGC	3.3
EGCG	2.1
ECG	3.0
GC	15.0
EC	5.1
C	20.0
Σ	3.8

Shoots for catechin estimation were dried for
2 hr at 95°, homogenized in a Wareing Blendor or a
Moulinex domestic coffee grinder, and stored under
vacuum in a Dessicator containing self-indicating silica
gel. 1g of the dry powder was then shaken for 30 min
with 10 ml 50% (v/v) ethanol at 30°, centrifuged, and
10 μl loads applied to the thin-layers with a Shandon
"Terumo" microsyringe.

Occasionally fresh material was analysed, either whole shoots or minced leaf undergoing fermentation. In such cases approximately 10g material was weighed accurately, ground with about 40 ml acid 80% (v/v) acetone, and shaken for 15 min. The volume was adjusted to 50 ml with acid acetone, the sample centrifuged, and 20μl loads applied to thin-layers. At the same time a weighed sample of leaf was dried to constant weight to determine the dry matter content. Catechins were then expressed as μmol/g dry wt.

9. Analysis of fermentation Products

(i) Fractionation with organic solvents - Roberts' Method

Use of different solubilities in organic solvents was made by Roberts to separate and estimate TF and TR components (Roberts and Smith, 1963), and the following modification of his method was used.

9 g black tea was weighed into a vacuum flask, and 375 g boiling distilled water run in from an overhead boiler. The flask was shaken in a rotary shaker at a fixed speed for twenty minutes. The liquor was filtered rapidly through cotton wool, and immediately 5 ml removed and added to 45 ml cold water. At this concentration the liquor does not "cream down". For the TC and $E \frac{380}{460}$ readings the extinction of this solution after suitable dilution with ethanol (generally 2 mls liquor + 4 mls ethanol) was determined. (TC is the total colour of the solution expressed as the E_{460} value of the original

liquor. $E \frac{380}{460}$ is the ratio of extinction at these two wavelengths.) For the TF determination, 5 ml of the diluted liquor were shaken vigorously for 5 min with 5 ml MIBK in a stoppered centrifuge tube. The layers were separated by centrifugation in an MSE bench centrifuge for 5 min, and the aqueous layer removed and discarded by means of a pipette attached to a filter pump. The MIBK layer was then washed vigorously for 5 mins with 5 ml 0.2 M tris buffer pH 8.0, the layers separated, and the extinction at 380 and 460 nm read in the spectrophotometer. According to Roberts $E \frac{0.02\%}{1 \text{ cm}}$ at 380 nm for TFG dihydrate is 2.225. In the absence of any pure standard, therefore, this figure was assumed to be correct, and used to find the amount of theaflavin in the solution. TF% is thus a w/w percentage of total theaflavins, expressed in terms of anhydrous TFG, in the original black tea material. B% is the "brightness" of the liquor, and is derived as described by Roberts.

In an experiment in which a series of dilutions of a tea liquor were analysed for TF by the above method, the response of the E_{380} or E_{460} reading of the final TF solution to the dilution of the original solution was found to be linear (Figure 4).

2.5% (w/v) $NaHCO_3$ solution was tried in place of the pH8 buffer, but this compound tended to oxidise the
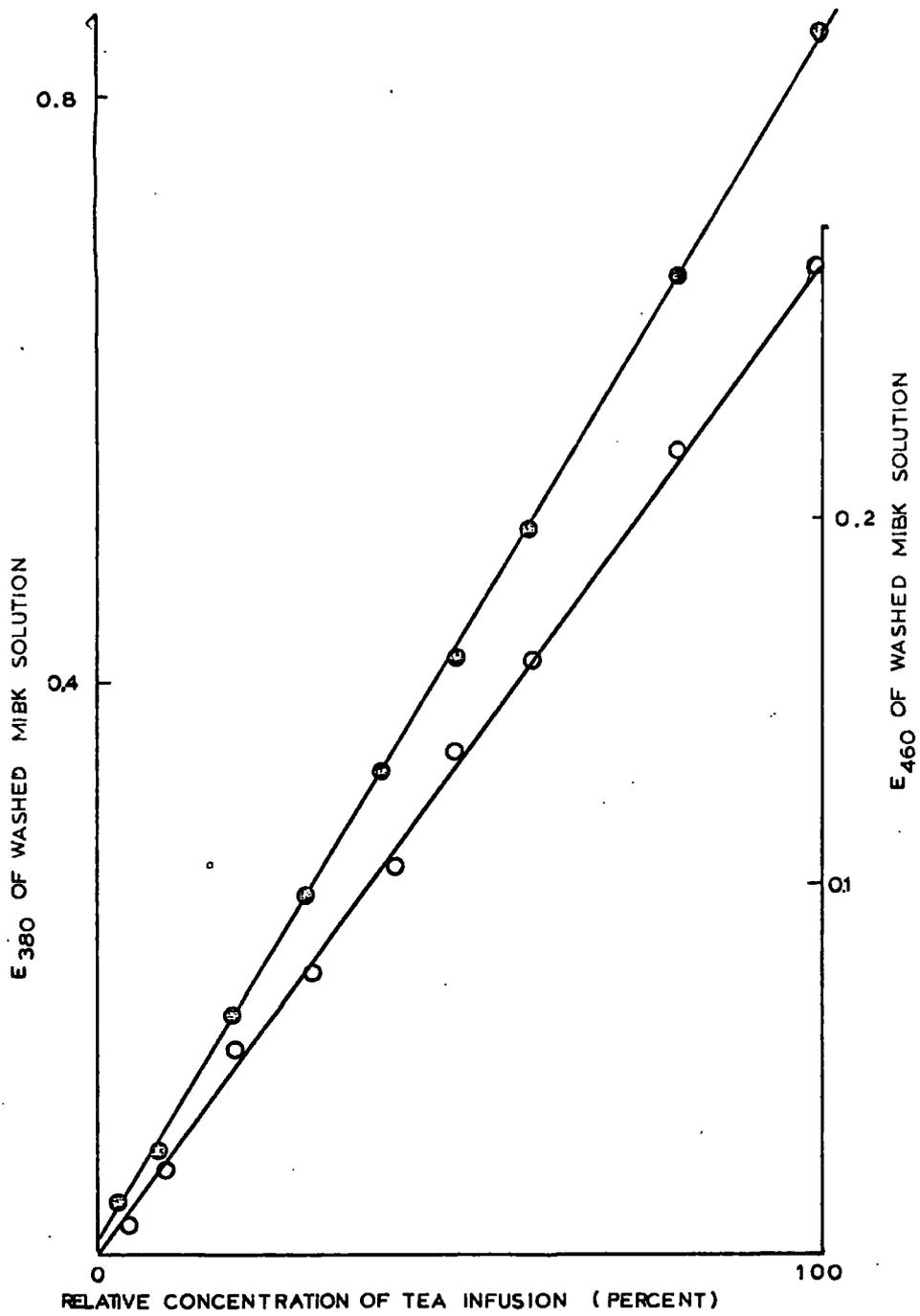
FIGURE 4

Estimation of TF: dependence of colour of a
washed MIBK extract of a tea infusion upon
the concentration of that infusion.

E_{380}

E_{460}

theaflavin complex and resulted in poor reproducibility. In an experiment in which the same diluted tea liquor was analysed for TF using either 0.2M tris buffer pH8 or 2.5% (w/v) $NaHCO_3$ for washing the MIBK extract, with 12 replications of each method, the coefficient of variation for each series was found to be 1.45% and 5.94% respectively, and the mean value of the $NaHCO_3$ washed samples was reduced by 20% with respect to the buffer washed samples.

In an experiment in which ten samples of the same black tea were each separately infused and analysed, the coefficient of variation of the TF determination was 2.47%, and that for the TC determination 1.87%. A comparison of this method with that originally published by Roberts (Roberts and Smith,1963) is shown in Figure 5 The correlation is linear, and in absolute terms the TF values in the modified method are 18% higher than in the original method.

(ii) <u>Reaction of organic solvent extract with Flavognost reagent.</u>

This method involves the production of a green colouration by the action of the Flavognost reagent upon TF, and is a modification of the method published by Nestle's Products Ltd. (1966).

5 ml of the tea infusion (9g in 375 g boiling distilled water) are shaken with 10ml MIBK in a mechanical shaker for 15 min. After separation of the
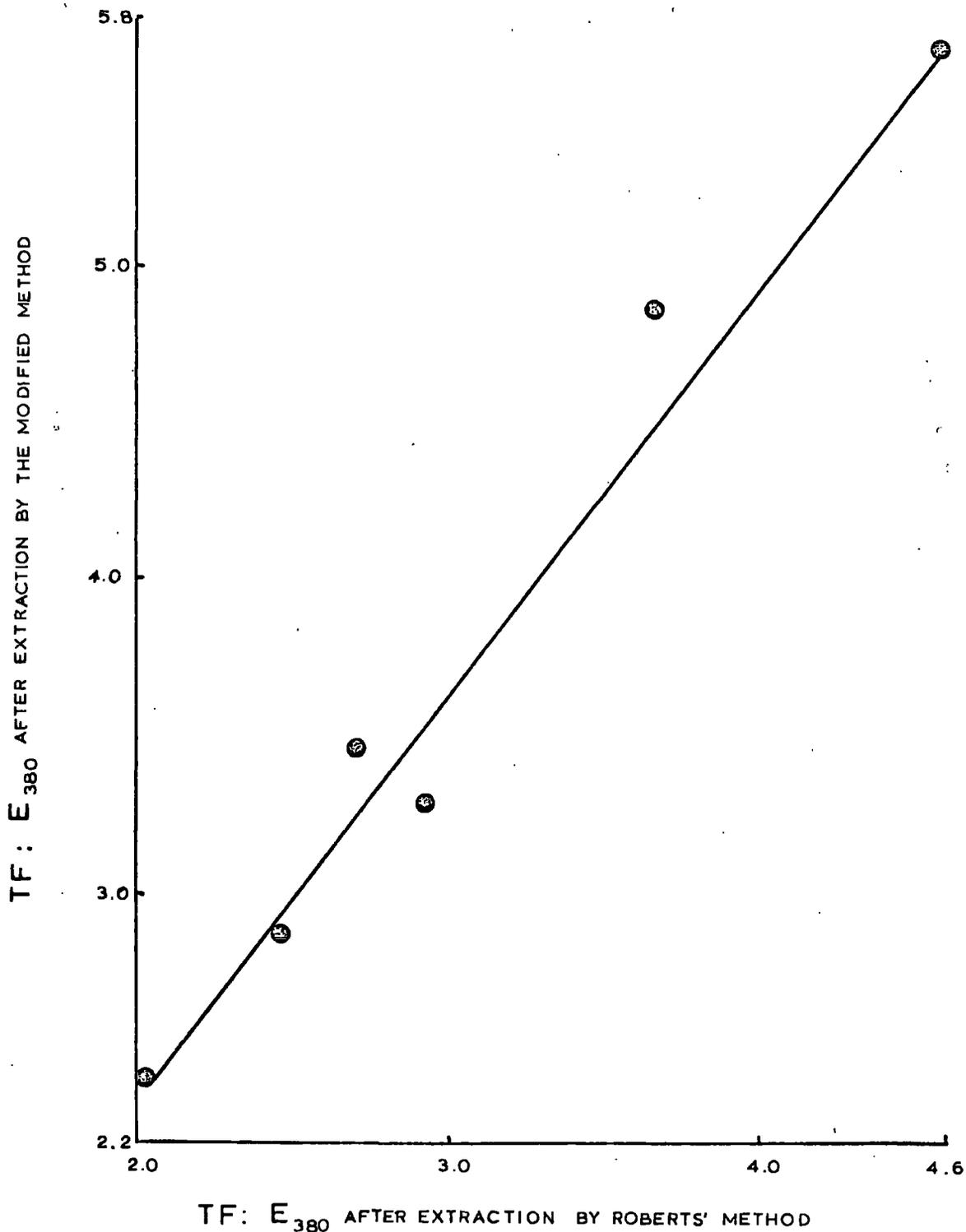
FIGURE 5

Estimation of TF: comparison of modified method with original method as published by Roberts.

E_{380} readings of a washed MIBK extract of a tea liquor after correction for dilution.

layers, 2 ml of the upper layer were added to 2ml

ethanol and 2 ml Flavognost reagent (2% (w/v)

Flavognost in ethanol) and shaken. A blank was

prepared by adding 2 ml of the upper layer to 4 ml

ethanol. After standing for 15 min the extinction of

the test sample was measured in a Gallenkamp colourimeter,

using a filter of 600 nm optimum light pass, adjusting

the meter zero to the blank in each case.

The method was highly reproducible (coefficient of

variation was 0.92% over 12 replications of analysis from

the same infusion), and showed good linear correlation

(coefficient of correlation = 0.976) with the modified

Roberts' method described above.(Figure 6).

(iii) <u>Chromatography on LH-20 Sephadex</u>

This method was taken from that of Crispen et.al.

(1968). All work reported here was performed on the

same chromatographic column of the gel under the following

conditions.

The sample for analysis, contained in 2ml 60%

(v/v) aqueous acetone, was pumped onto the top of a

precision chromatography column (Whatman Ltd) containing

a bed of LH-20 Sephadex gel of effective length 30 cm and

2.5 cm diameter, equilibrated in 60% (v/v) aqueous acetone.

The pump was adjusted to give a flow rate through the whole

system of about 100 ml / hour, and the sample eluted with

60% (v/v) acetone. The eluate was monitored at 380 nm in

a Unicam SP 800 spectrophotometer, using a flow-cell of

2mm path length and X5 scale expansion, coupled to a
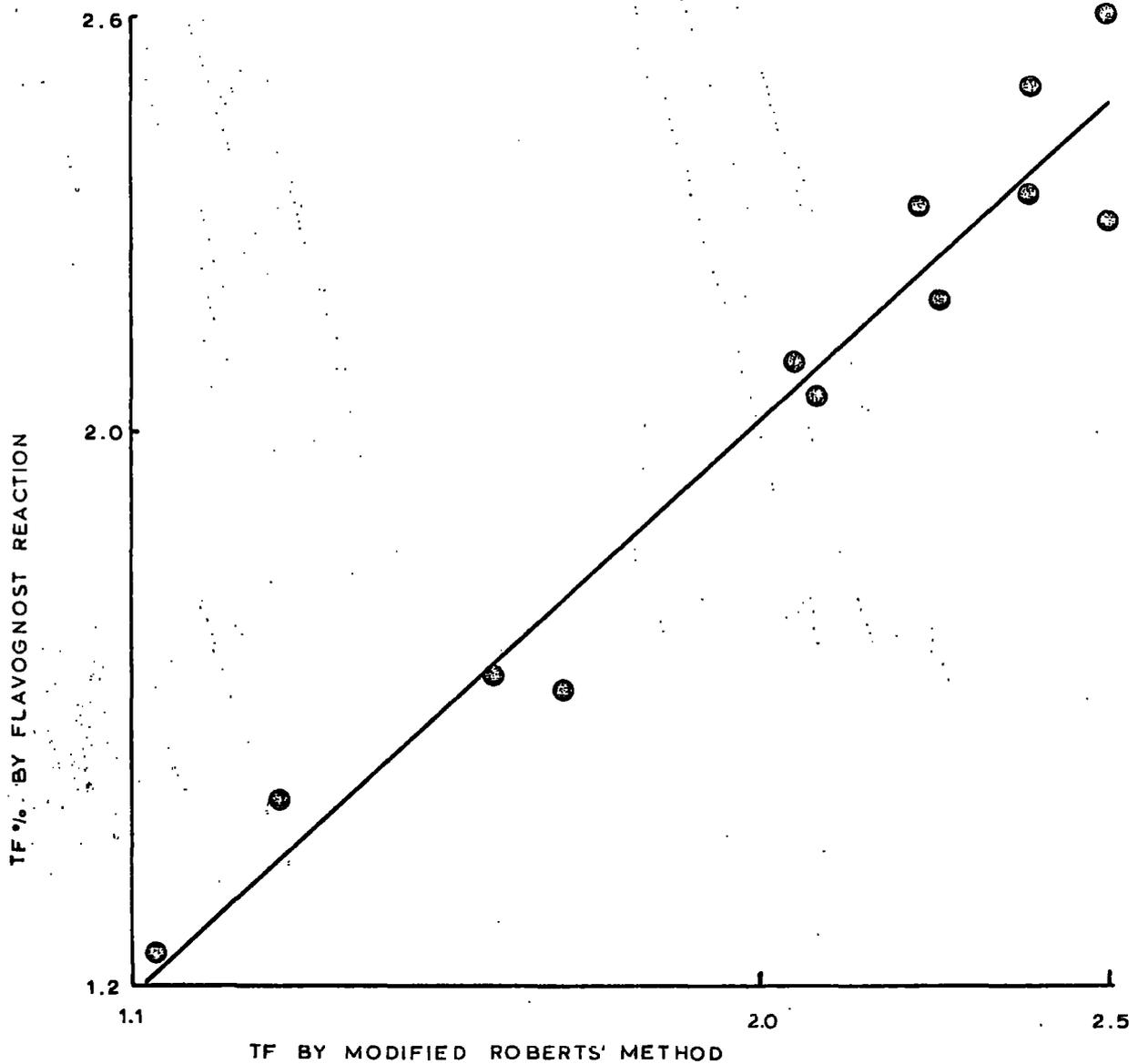
FIGURE 6

Estimation of TF : comparison of flavognost
technique and modified Roberts' method.

8 clonal teas manufactured in the miniature factory
and 4 commercial teas were analysed by both methods.
For flavognost technique TF = 6.6 x E_{600} (see text),
where the factor 6.6 is obtained from the literature
(Nestles Products Ltd.,1966). The line drawn represents
the regression of y upon x, and has the formula:

$$TF\%_{(flavognost)} = 0.91 \times TF\%_{(Roberts)} + 0.19$$

Vitatron or Servoscribe chart recorder set to record in linear mode at 10mv full scale deflection. Under these conditions the chart reads from 0 - 2E.

For analysing made tea liquors, the infusion made from 9g tea in 375g boiling distilled water was freeze-dried or evaporated to dryness under vacuum, and 25 - 100 mg soluble solids applied to the column. (Some of the larger peaks from the profile are not completely recorded with more than 30 mg. sample) A typical elution profile is shown in the Results chapter (Figure 3 9)

Recorder traces for components 6, 7, and 8 were extrapolated from the component peak to the zero line, and the area under each component estimated with a Technicon integrator, set in linear mode. After correction of these figures for variations in flow rate (see following), the component 8 could be very accurately assessed (coefficient of variation = 2.8%), and component 6 and 7 assessed reasonably well (coefficient of variation 11% and 5% resp). Heights of components 4 + 5, 6, and 7 were also fairly reproducible (coefficient of variation approximately 5%)

Having established the identity of component 8 as TF (see Results chapter, section 8), the quantity of TF in component 8 was calculated as follows:

Using Technicon integrator

736 integrator units = 4 cm chart run at E_{380} = 1.000

When 4 cm chart run corresponds to 100 ml eluate,

736 integrator units = 100 ml at E_{380} = 1.000

According to Roberts, $E^{0.02\%}_{1\ cm}$ at 380 nm for TFG = 2.225

(Roberts and Smith,1961)

i.e. E_{380} = 1 is given by $\frac{0.02}{2.225}$ g TFG per 100 ml

or 8.9 mg TFG per 100 ml

736 integrator units $\equiv$ 8.9 mg TFG

Hence if x is number of integrator units of component 8

y is distance in cm of chart run equivalent to 100ml

$$TF = 8.9 \times \frac{x}{736} \times \frac{4}{y}$$

$$= 0.0484 \frac{x}{y} \text{ mg (as anhydrous TFG)}$$

(Where pure TF^1 as opposed to a mixture of theaflavins was being measured, the above procedure was followed, using $E^{0.02\%}_{1\ cm}$ at 380 nm = 3.400 for TF^1)

(iv) Thin-Layer Chromatography

Oxidation products can be separated to some extent by t.l.c. as previously described, and TF^1 separates well from TFG (cf Figures 13 and 19). Where only TF^1, TFG_1, or TFG_2 were being produced in an incubation, the resulting spots were removed and assayed with the vanillin reagent, calibrating the E_{500} values in each experiment from an analysis on LH-20 Sephadex at the conclusion of the experiment. However, when mixtures of TF components were present, it was not possible to achieve a good enough separation by t.l.c. for quantitative assay to be possible.

10. FOLIAR MINERAL ANALYSIS

When plots were small (i.e. less than 100 bushes) one shoot per bush was sampled for foliar analysis. Otherwise

one shoot was selected from each of 100 bushes chosen
at random within the plot. Shoots were dried at 95°
homogenized in a Wareing blendor, and the powder stored
under vacuum over self-indicating silica gel until
analysed.

(I) Nitrogen Analysis

Nitrogen analysis was carried out by the Kjeldhal
method. 1g material was digested in a 500 flask with 5g
catalyst ($CuSO_4$ - K_2SO_4, 1 : 3 by weight) 5 ml distilled
water, and 20 ml conc. H_2SO_4 (N - free grade). 150 ml
water was added, followed by 50 ml 50% (w/v) NaOH
solution, and the mixture distilled into 50 ml 4% (w/v)
H_3BO_3 solution. When about 100 ml had distilled across,
the distillate was titrated against standard 0.1 N HCl.

then if t ml is titre of 0.1 N HCl

$$\frac{t}{10} \times 17 \text{ mg } NH_3 \text{ are released}$$

$$= \frac{t}{10} \times 17 \times \frac{14}{17} \text{ mg N per 1g leaf sample}$$

$$\% \text{ N in leaf} = \frac{t}{10} \times 17 \times \frac{14}{17} \times \frac{100}{1000} = t \times 0.14.$$

(ii) Phosphate analysis

0.25g material were ignited in a muffle furnace for
3 hours at 550°. The ash was dissolved in 2 ml 4N HCl,
transfered to a 100 ml volumetric flask, and the volume
made up to 100 ml with water. 2ml of this preparation
was pipetted into a second volumetric flask, and two
drops 4N ammonia added. 10 ml ammonium molybdate reagent
were added and the volume made up to 100 ml. After

shaking 0.25 ml stannous chloride reagent was added,

the flask shaken again, and after standing for 5 min

E_{660} determined on a Unicam SP 600 spectrophotometer.

A standard curve was prepared using suitable

concentrations of KH_2PO_4 solution.

(iii) Potassium analysis

0.25 g material were shaken with 25 ml 0.1 N HCl

for 1 hour in a mechanical shaker. The solution was

filtered through Whatman No. 42 filter paper, and the

emission of the solution measured in an EEL flame

photometer (Evans Electroselemium Ltd., Halstead, Essex).

A standard curve was prepared using suitable

concentrations of KCl.

Analytical methods for foliar N, P, K, analysis were

part of the Tea Research Foundation's routine Laboratory

programme, and no further attempt was made to assess their

validity.

11. POLYPHENOL OXIDASE PREPARATION

5 kg acetone powder were initially prepared in Malawi

during June - July by the method outlined below. This was

flown to Durham, packed with solid CO_2. Early pilot

attempts to purify polyphenol oxidase from this material

were successful, but later it became impossible to extract

more than 5% of the activity, and the powder was abandoned.

A further supply of fresh leaf was flown from Malawi,

and acetone powder made from the undamaged leaf as follows.

150 g batches of two-and-a-bud shoots were blended with

700 ml 80% (v/v) aqueous acetone. Two further blendings

produced an almost white powder, which was spread
thinly on blotting paper to dry at room temperature.
As soon as the acetone had evaporated, the powder was
sealed in polythene bags and stored at 40°.

Batches of 20g powder were extracted twice with
500 ml portions of the pH 10.2 buffer (Gregory and
Bendall,1966), pressed through terylene mesh, and
clarified by centrifuging for 30 min at 2.300x$\underline{g}$ (MSE
Mistral 4L centrifuge). About 500 g were extracted this
way, the extracts combined, and dialysed overnight
against running tap water at 15°. After centrifugation
as before, the supernatent was discarded, and the
precipitate extracted twice with 0.1M citrate buffer
pH 5.6. The extracts were combined, and the precipitate
discarded. After degassing the extract, 390g $(NH_4)_2SO_4$
per litre was added with stirring at 4°, and the stirring
continued for 3 hours. The solution was centrifuged for
15 min at 23,000 x $\underline{g}$ (MSE High speed 18 Centrifuge) and
the precipitate discarded. A further 190 g $(NH_4)_2SO_4$
per litre was added while stirring and the solution
equilibrated at room temperature. The resulting
precipitate was collected by centrifuging at 23,000 x $\underline{g}$
for 15 mins, and the supernatent discarded. The precipitate
was dissolved in the minimum quantity of water, and
dialysed overnight against 0.05 M sodium acetate buffer
pH 5.4 at 4°C. A brown precipitate was separated by
centrifugation at 23,000$\underline{g}$ for 15 min, and this contained
some activity. Most of the activity was contained in the

straw-coloured supernatent, and the precipitate was discarded. The supernatent was applied to a column of DEAE cellulose (length 15 cm, diameter 3.5cm) and eluted with 0.05 M sodium acetate buffer pH 5.4. Some of the activity passed through the column and was almost free from coloured matter. The remainder was retained on the top of the column in association with a dark brown band.

The clear unretanded fraction was concentrated by selective filtration ('Dynaflow' pressure filter) using a membrane excluding molecules above a mol. wt. of 10,000. It was frozen in 2ml aliquots and stored at -40^{0}. 20 ml of a preparation of activity 240 U/ml was obtained. The activity was constant over three months.

Progress of the purification is shown in Table 1.

Unsuccessful attempts to extract PPO from acetone powder.

It proved impossible to extract more than 5% of the activity of either acetone powder which had spent up to 24 hr at room temperature or acetohe powder made from damaged leaf using the pH 10.2 buffer described above. The addition of the non-ionic detergent "Nonidet P40", sodium lauryl sulphate, EDTA, or urea also failed to increase the solubility of the enzyme.

Polyvinylpyrrolidone, in an insoluble form (Polyclar AT) was used in an attempt to adsorb phenolic material which may have been interfering with the enzyme extraction process. When used in the extraction (the same weight as the acetone

STAGE	TOTAL ACTIVITY (U)	YIELD FROM LAST STAGE %
I Fresh tea shoots (20-30 U/g fresh wt)		
II Acetone powder (80-120 U/g dry wt)		c80
III Soluble extract	180,000	c20
IV Dialysis precipitate	70,000	39
supernatent (discarded)	9,000	
V 60-80% saturated $(NH_4)_2SO_4$ fraction	46,000	67
VI Dialysis against 0.05 M acetate pH 5.4		
supernatent	34,600	74
precipitate (discarded)	5,600	
VII After chromatography on DEAE-cellulose		
and concentration	4,800	14

TABLE 1

PROGRESS OF PURIFICATION OF POLYPHENOL OXIDASE

Stage III soluble extract: activity = 20 U/ml
$$E_{275} = 520$$

Stage VII final preparation: activity = 240 U/ml
$$E_{275} = 4$$

powder) and subsequently removed by centrifugation,
no increase in extracted PPO was noticed, but there
was amarked decrease in the amount of coloured material
in the supernatent.

To investigate the effect of soluble polyvinylpyrrolidone
(mol. wt. 24,000), 2.5g acetone powder was blended with
40 ml McIlvaine buffer pH 7.0 (McIlvaine, 1921) containing
1.2g cystein per l, 40 mg Tween 80 and 180 mg
polyvinylpyrrolidone. The last two additives in combination
resulted in a five-fold increase in the activity of the
clarified extract. Dialysis of the extract against running
tap-water for 24 hours failed to produce a precipitate, and
since without this step the volume of extract would be too
great for ammonium sulphate fractionation, the method was
not used.

12.   PURIFICATION OF CATECHINS

EGC., EGCG, GC, and ECG were prepared from green leaf
of a mixture of clones from Swazi Research Station, dried
rapidly in a blast of air at 95°, and transported to Durham
in sealed polythene bags.

A preparation of the six catechins present in the leaf
was prepared by the method of Vuataz (Vuataz et.al., 1959),
modified by extracting the ethyl acetate - soluble fraction
with wet ether (Gregory and Bendall, 1966). T.l.c. of this
preparation showed it to be almost free from contaminants
(Figure 7   ).

Initial separation of the catechins was performed by
partition chromatography on a column of cellulose, using

a modification of Vuataz's method (Vuataz et.al., 1959).
350g cellulose (Whatman CC 31) was suspended in light
petroleum (boiling range 60° - 80°) and 150 ml water
added while stirring. This was packed in a column
(80cm x 3.5 cm diameter) with a ramrod to give a bed
about 70cm long, and equilibrated with wet light
petroleum. A 4g sample was applied and eluted as described
by the above authors. 10ml fractions were collected, and
their E_{275} determined. A typical elution profile is
enclosed (Figure 8). ECG elutes first, and is coloured
yellow by an impurity which elutes slightly earlier.
EGCG and C then appear together, followed by EC. GC and
EGC are eluted much later, and may immediately be collected
in a chromatographically pure state (Figures 34 and 9).

ECG was further purified by adsorption chromatography
on a cellulose column (Whatman C.C. 31, 26cm x 1.5cm diameter)
equilibrated and eluted with water. Complete separation
of ECG from the contaminant was achieved, and a chromatograph-
ically pure sample obtained (Figure 9).

EGCG and EC were separated a column of LH-20 Sephadex
(30cm x 2.5 cm diameter) equilibrated and eluted with
40% aqueous acetone. Complete separation was achieved,
C being eluted before EGCG. The EGCG fraction was collected
to give a chromatographically pure sample that was
recrystallised once from water (Figure 9).

All samples were freeze-dried and stored at -40° in an
evacuated dessicator over silica gel. ECG had a tendancy to
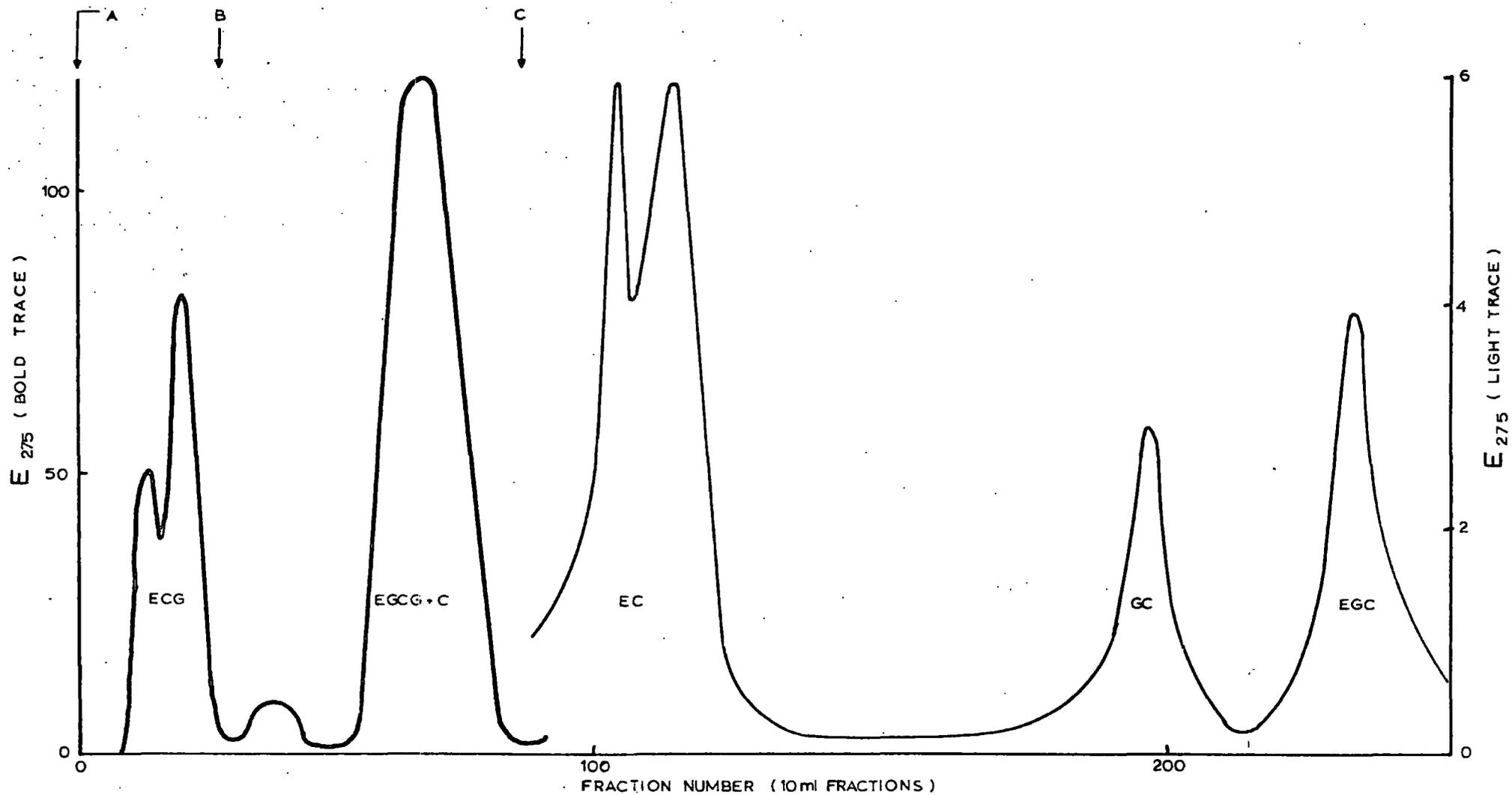
FIGURE 8.   Elution of a mixture of six catechins from cellulose.

Solvents changed as follows: A: ethyl propionate/light petroleum (9:1 v/v); B: ethyl propionate;
C: ethyl acetate. Catechins identified by t.l.c.
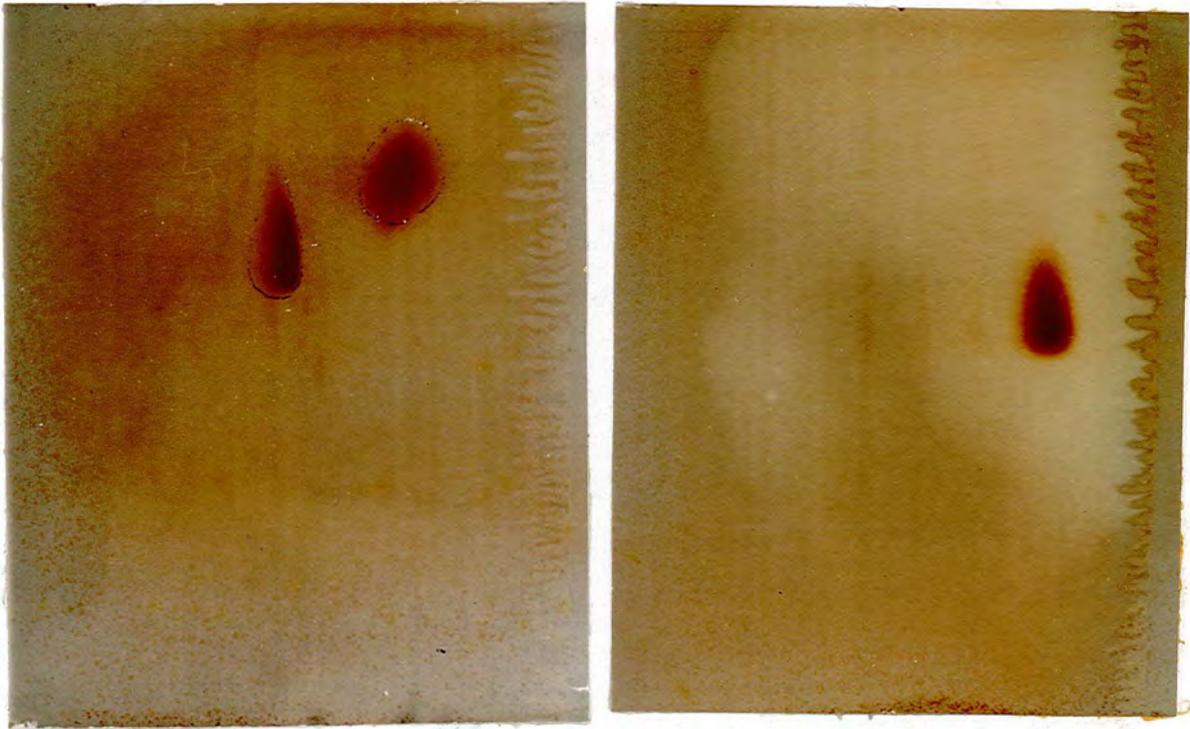
(a)   (b)

FIGURE 9

Thin layer chromatograms of pure
catechins.

For details of chromatography see text. Plates
sprayed with bisdiazotized benzidine. Preparation
(b) was overrun in the first dimension (downwards).

(a) Separate loadings of EGC and EGCG preparations.
(b) ECG preparation.

turn pink upon exposure to air and sunlight but the oxidation product responsible for the colouration was not detected by t.l.c. Otherwise, all catechins were of a greyish white appearance, and remainēd free from oxidation contaminants for at least three months.

13 MODEL FERMENTATION SYSTEMS

(i) Pilot-scale manufacture

Swazi Tea Research Station was equipped with a miniature factory to perform CTC manufacturing methods on 100g samples. Fermenting leaf at various stages was analysed for catechins and fermentation products by the above methods.

(ii) Fermentation initiated by sand-grinding

To investigate the variation in TF and TC production over a long period of time, a controlled fermentation system was required. Shoots of standard size were ground with twice their weight of acid-washed sand in a deep-freeze. The mixture was then transfered to a flask kept at 30° in a waterbath, and warm moist O_2 blown gently through it. Periodically samples were taken out weighed, infused with boiling water, and analysed for oxidation products by the modified Roberts' method.

(iii) Uncharacterized in vitro system

5g acetone powder was blended with 300 ml cold 0.05 M phosphate buffer pH 10.0 containing 25g/litre NaCl. The extract filtered through Whatman No. 1 paper, and adjusted to pH 5.6 with HCl. This was used as the PPO component.

6g air-dried leaf of clone MT 12 was shaken with
300 ml 0.05 M phosphate buffer pH 5.6 for 30 min at
30°, and filtered through Whatman No. 1 paper. This
was used as the catechin preparation.

Equal quantities of the components were incubated
together in a water bath at 30°, bubbling air, oxygen
or other gases through if required. 5ml samples were
withdrawn at intervals and analysed for TF and TC by
the modified Roberts' method, expressing TF production
as a percentage of the dry weight of material originally
extracted for the catechin preparation.

(iv) In vitro incubation using pure catechins

This system utilized the pure catechins and partially
purified PPO preparation described above.

The catechins under investigation were dissolved in
2.5 ml 0.05 M citrate buffer pH 5.6 and equilibrated in the
oxygen electrode chamber at 30°. The reaction was started
by adding 0.5ml of the enzyme preparation. The level of
O_2 was kept at an arbitrary value as measured on the chart
recorder (the same value for all incubations) by bubbling O_2
slowly through the medium. Periodically the rate of oxygen
uptake was measured by cutting off the O_2 supply and closing
the electrode chamber.

The reaction was monitored by t.l.c. at intervals,
using 5 μl samples, and stopped by heating when the rate
of O_2 uptake was 10-15% of its initial value unless
otherwise stated in the Results Chapter. The reaction

mixture was divided into two equal quantities, and
one half evaporated to dryness under vacuum. The other
half was exhaustively extracted with ethyl acetate and
an absorption spectrum determined, occasionally after
washing the organic extract with 2.5% (w/v) $NaHCO_3$
solution. The ethyl acetate extract and the aqueous
residue were then evaporated to dryness. Either the
total incubation mix, the ethyl acetate extract, the
aqueous residue, or two or three of these extracts
was taken up in 60% aqueous acetone (2ml), centrifuged,
and subjected to chromatography on LH-20 Sephadex gel.

RESULTS

1. CORRELATION OF MADE TEA ANALYSIS PARAMETERS WITH MARKET VALUE.

This series of experiments was designed to ascertain which of the parameters, or combination of parameters, of the Roberts' made tea liquor analysis showed the closest correlation to the market value of a particular sample of black tea.

24 teas were selected by a London firm of tea brokers, and analysed by the modification of the Roberts' procedure outlined in the Methods chapter. The teas were all of the same leaf grade (Pekoe Fannings), from C.T.C. manufacture, offered for auction on the same day, in the same auction room. According to the brokers' tea taster, none of the teas exhibited "flavour" characteristics. Results are presented in table 2.

A regression analysis on this data indicated a positive relationship between TF, TC, and the selling price (SP) as expressed in the multiple regression equation

$$SP = 24.8 \ (TF) + 7.17 \ (TC) - 23.7 \text{ pence per pound}$$

in which the partial regression coefficients for TF and TC are both significant ($P < 0.01$). Use of this formula to predict the selling prices of this series of teas results in a standard error of estimate of 3.8 d / lb.

A linear regression of selling price on the product TF x TC was also performed, resulting in the regression

REF. No.	MARK	COUNTRY OF ORIGIN	ANALYSIS					Selling Price	Predicted Selling Price
			TF	TC	E $\frac{380}{460}$	B%	TFxTC		
1	METILILE	MOZAMBIQUE	1.13	4.75	3.59	15.1	5.36	41	43.2
2	MANCOTIA	ASSAM	1.40	5.41	2.94	19.5	7.58	43	50.1
3	MAFISI	MALAWI	1.14	5.02	3.25	15.6	5.72	44	44.3
4	LOONG SOONG	ASSAM	1.30	5.44	2.88	18.1	7.08	45¼	48.5
5	BLOOMFIELD	MALAWI	1.18	4.62	3.31	17.2	5.45	45½	43.5
6	MAJULIGHUR	ASSAM	1.41	5.35	3.10	18.9	7.55	46	50.0
7	LA FLORA	MAURITIUS	1.20	5.48	3.53	14.1	6.58	46¼	47.0
8	BALANGAI	TANZANIA	1.52	5.51	3.42	18.4	8.38	48	52.5
9	KAPKOROS	KENYA	1.32	4.90	3.64	18.5	6.46	48½	46.6
10	NYANKOBA	"	1.39	5.65	3.41	16.3	7.86	49	50.9
11	ACHABAM	ASSAM	1.52	5.75	2.85	19.9	8.75	49¼	53.1
12	THOWRA	"	1.31	5.42	2.92	18.2	7.10	51	48.6
13	RAJMAI	"	1.44	5.42	2.89	20.3	7.80	51¼	50.7
14	DIMBOLIL	KENYA	1.42	5.18	3.64	17.3	7.36	51¾	49.4
15	AMBANGULU	TANZANIA	1.45	5.69	3.11	18.5	8.25	52	52.1
16	MUSEKERA	"	1.63	6.09	3.06	19.5	9.92	52¾	57.3
17	KAIMOSI	KENYA	1.43	5.13	3.48	18.3	7.35	53	49.4
18	KIGANGA	TANZANIA	1.57	6.10	3.06	18.4	9.58	53¼	56.3
19	CHIVANJEE	"	1.42	5.78	2.96	18.4	8.22	54	52.0
20	KUNGA	"	1.55	5.80	3.11	19.2	9.00	55½	54.5
21	CHINGA	KENYA	1.56	6.06	3.44	17.1	9.45	56	55.9
22	TINDERET	"	1.59	6.12	3.26	18.2	9.82	66½	57.0
23	KANGAITA	"	1.64	5.77	3.62	18.2	9.46	64¼	55.9
24	KARIRANA	"	1.58	6.22	3.33	17.6	9.82	65¼	57.0
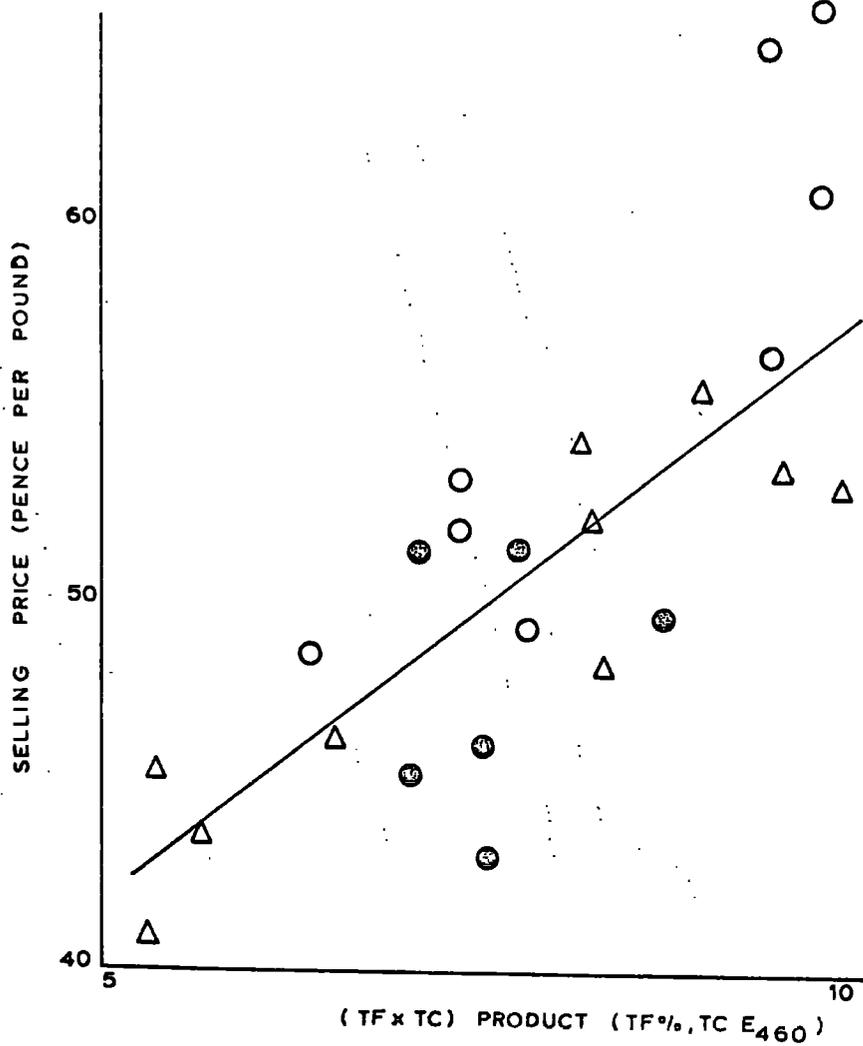
FIGURE 10

Relation between (TFxTC) Product and Selling Price

O	KENYA TEAS
⊕	ASSAM TEAS
△	OTHER TEAS

Regression equation

Selling Price = 3.09(TF x TC) + 26.6
        (units as above)

                        is significant at P< 0.001

equation

SP = 3.09 (TF x TC) + 26.6 pence per pound,

in which the regression coefficient is highly significant
(P < 0.001). Use of this formula to predict the
selling price of this sample of teas results in a
standard error of estimate of 3.7 d / lb.

Figure 10 illustrates the regression of selling
price upon the product TF x TC, the plot of each sample
being coded according to country of origin. It can be
seen that 7 out of 8 teas from Kenya realised prices
higher than would be predicted by the regression line,
while 4 out of the 6 teas from Assam realised lower
prices.

2. PRELIMINARY INCUBATIONS

(i) Production of theaflavin by an uncharacterized
     in vitro system

This experiment was the basis for several others
conducted in Malawi to investigate the time-course of
catechin disappearance and pigment production under
various conditions of leaf rupture. Figure 12 illustrates
the development of TF and TC in a completely soluble
system. The production and decay curve for TF, and the
slightly later peak value for the product TF x TC was
typical of all such incubations.
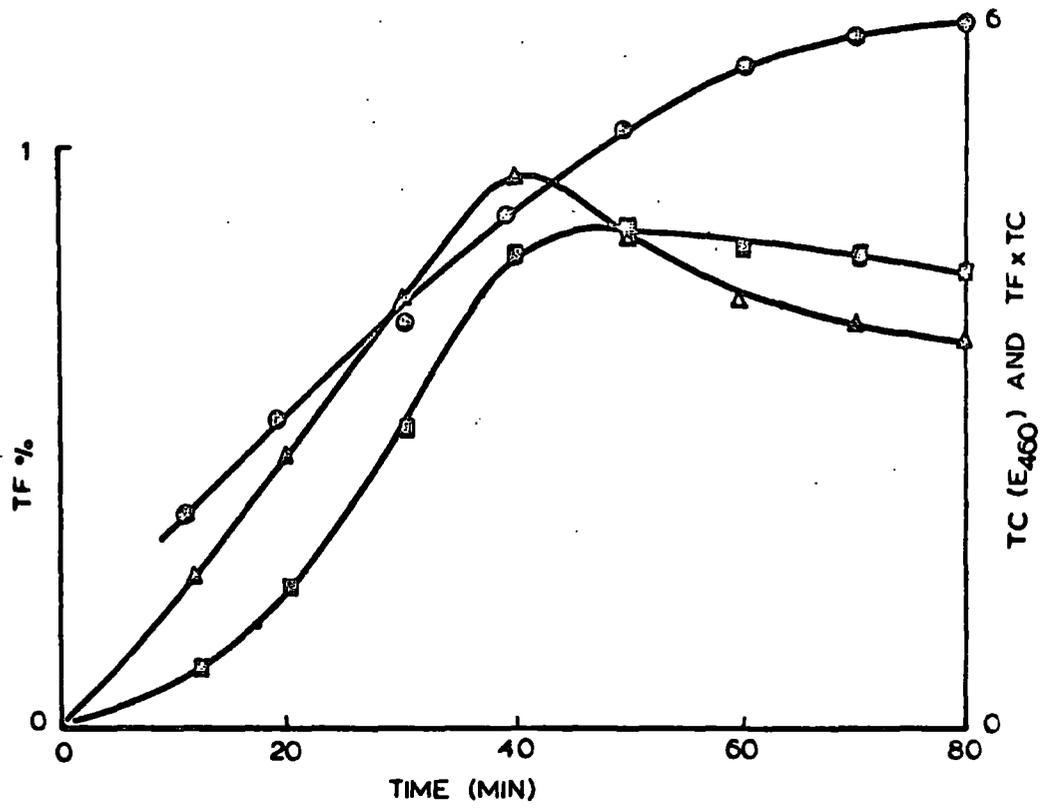
FIGURE 11

Production of TF and TC by an uncharacterised *in vitro* system

△—△ TF
⊕—⊕ TC
▣—▣ TF x TC

(ii) <u>Production of theaflavin in vivo after cell</u>
<u>rupture</u>

Numerous analyses by the modified Roberts' method
indicated that C.T.C. manufacturing techniques,
conducted either on an analytical scale on the Research
Station, or full scale on a commercial estate, or
controlled fermentation initiated by sand-grinding
(see methods chapter) produced similar curves for TF
and TC development as in the cell-free system described
above. TF values produced by the sand-grinding
technique tended to be 10 - 30 % higher than in
analytical C.T.C. manufacture, while the TF produced
in the <u>in vitro</u> technique, when expressed as a percentage
(w/w) of the total extracted solids in the medium,
was considerably lower.

A series of experiments conducted with the tea-
maker at Bloomfield Factory of Lujeri Tea Estates Ltd.,
indicated that the optimum firing time, as assessed by
the tea-maker, was approximately ten minutes after
peak TF production, i.e. at the peak of the TF x TC
curve.

(iii) <u>Disappearance of catechins during in vivo</u>
<u>fermentation</u>

Figure 12 illustrates the time-course of catechin
disappearance and concurrent TF production in leaf
undergoing normal manufacture. All the catechins, except
perhaps catechin itself, were consumed during the
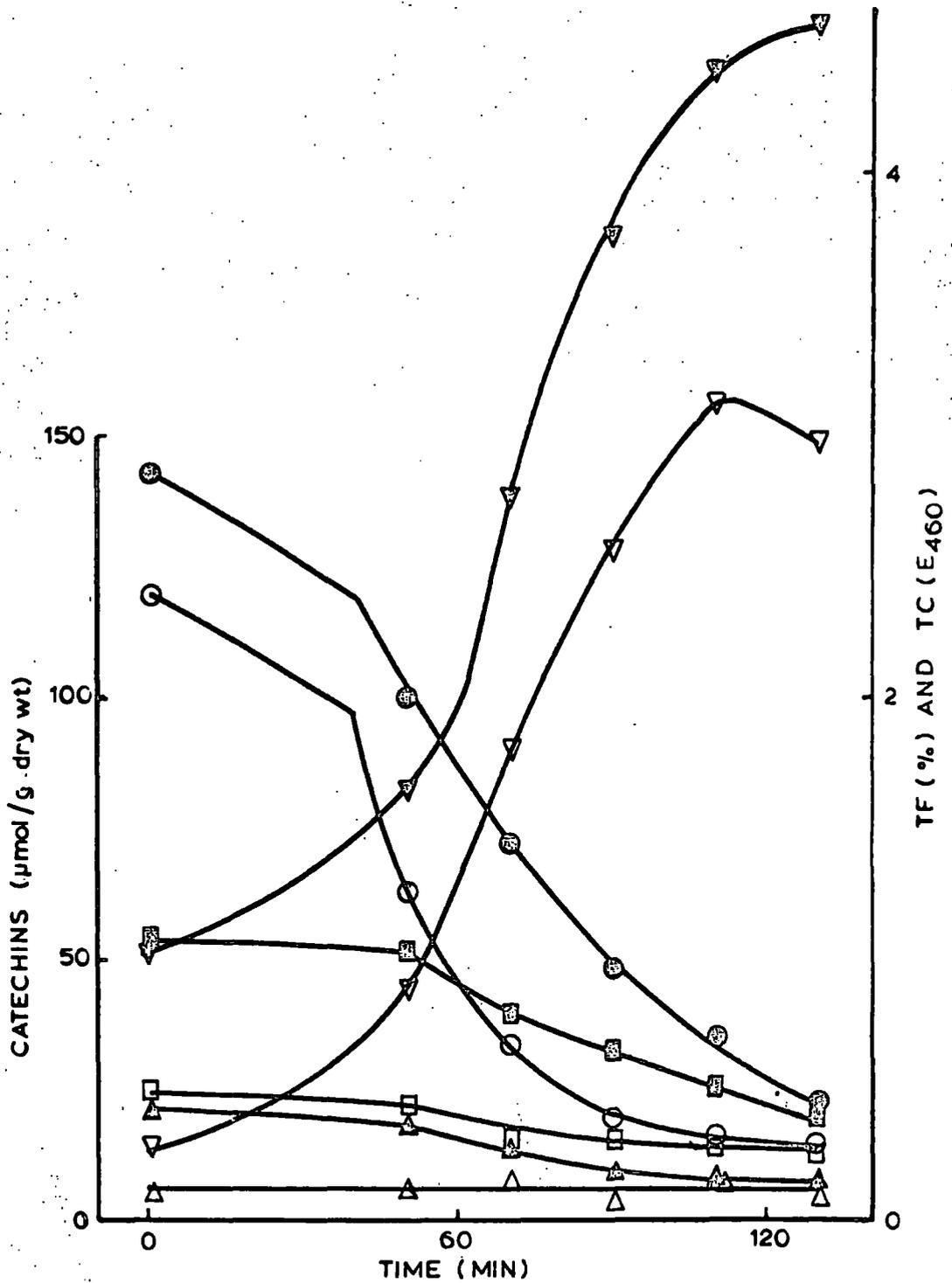
FIGURE 12

Disappearance of catechins during manufacture

⊕——⊕ EGCG   ○——○ EGC   ▽——▽ TF
⊟——⊟ ECG   ☐——☐ EC   ▼——▼ TC
▲——▲ GC   △——△ C

Shoots of clone SFS 371 were rolled for 40 min.
before passing through CTC machine. From this
figure, half-lives of EGC, EGCG, and ECG were
10, 40, and 60 min. respectively.

fermentation process, though EGC and EGCG were most
prominent, the former being oxidised the fastest.
Little TF was produced after exhaustion of EGC, though
at this point some EGCG remained, and there was still
appreciable TC production.

3. EFFECT OF ENZYME CONCENTRATION UPON THE RATE OF
   APPEARANCE OF PRODUCTS

A series of in vitro incubations were performed,
using a soluble extract of acetone-dried leaf as the
enzyme source, and a soluble extract of dried green leaf
as the catechin source. Different concentrations of the
same enzyme extract were used in each incubation.

Initial rates of TF and TC production (measured as
soon as possible after the commencement of the incubation)
were directly proportional to the enzyme concentration,
and doubling the enzyme concentration doubled the rate
of development of either parameter (Figure 16).

Maximum TF production was adversely affected by
either too high or too low enzyme concentrations, but at
the two dilutions studied between these limits, there was
little change in maximum TF production (Figure 13). (Due
to a laboratory power failure, the monitoring of the $\frac{1}{2}$
dilution was not completed). Two further enzyme dilutions
were subsequently incubated, both initiating TF and TC
production rates between those of the $\frac{1}{4}$ and $\frac{1}{8}$ dilutions.
TF peaks at 40 and 60 minutes were at 1.04% and 1.06%
respectively.
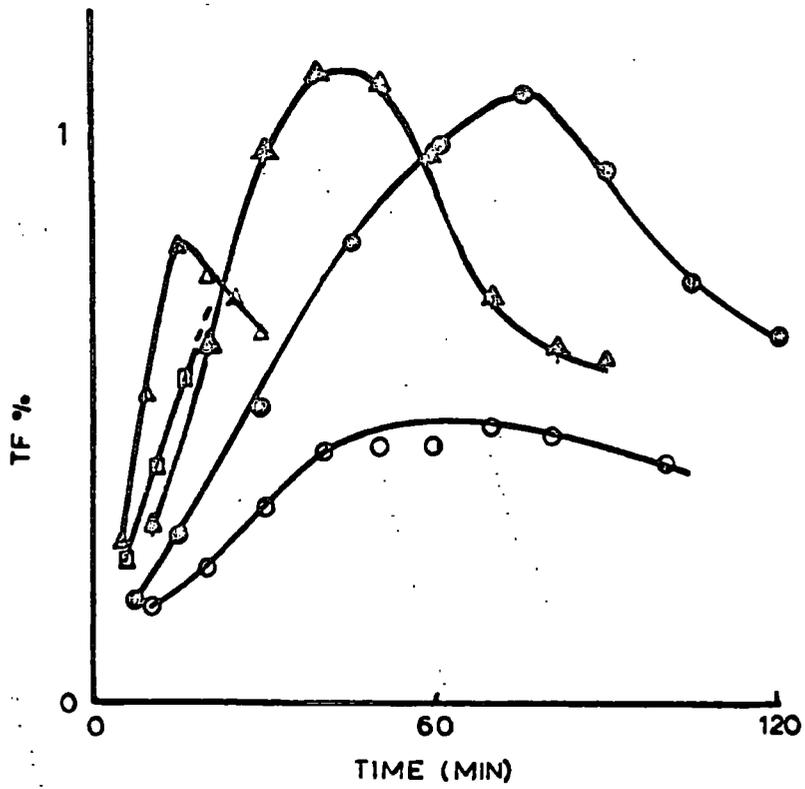
FIGURE 13

EFFECT OF ENZYME CONCENTRATION ON TF PRODUCTION
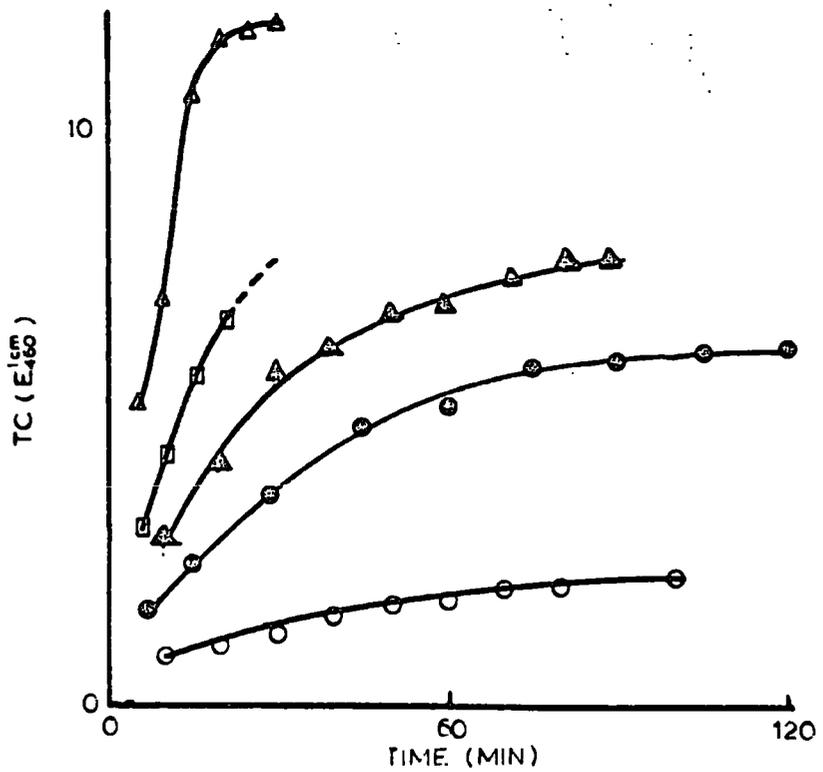
FIGURE 14

EFFECT OF ENZYME CONCENTRATION ON TC PRODUCTION

Enzyme dilution for figures 13,14 & 15

△—△ × 1   ⊕—⊕ × ⅛
☐—☐ × ½   ○—○ × ⅒
▲—▲ × ¼

FIGURE 15

Effect of enzyme concentration on
quality as assessed by the product TF×TC.
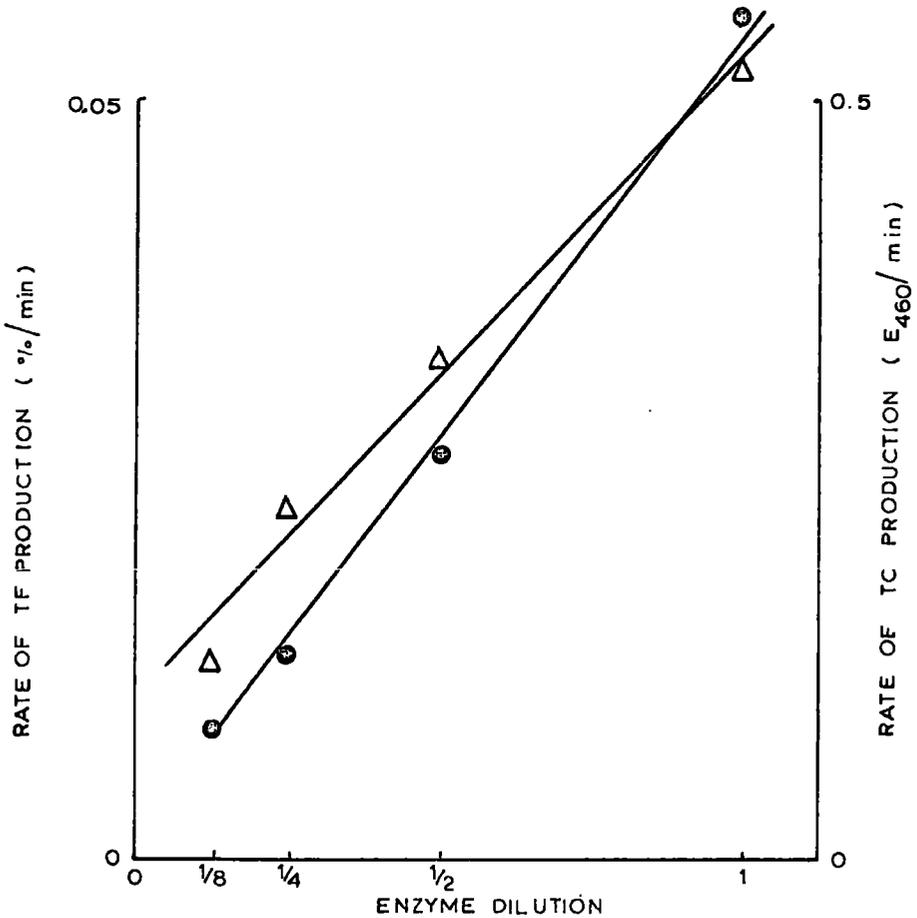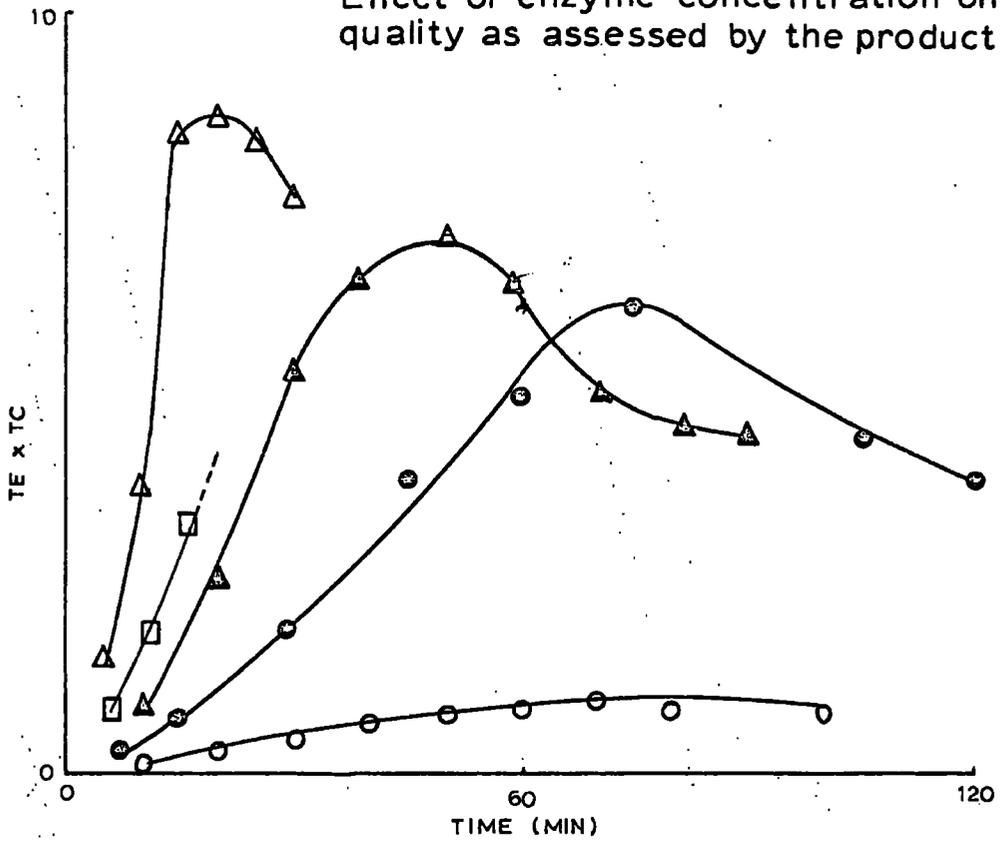
FIGURE 16

Effect of enzyme concentration on initial rate
of TF and TC production.

△—△ TF   ⊕—⊕ TC

Maximum TC development was progressively higher
in higher concentrations of enzyme (Figure 14). Thus
higher enzyme concentrations produced progressively
higher quality tea liquors as assessed by the product
TF x TC plotted from the same results (Figure 15).

4. EFFECT OF OXYGEN CONCENTRATION UPON THE RATE OF
   APPEARANCE OF PRODUCTS

In order to ascertain whether oxygen is likely to
be a limiting factor in the production of TF, separate
incubations similar to those described above were performed
whilst bubbling either oxygen, air, or nitrogen through
the medium.

TF development was faster under oxygen than under
air, and a much higher peak was reached (Figure 17a).
TC development was more rapid under oxygen than under air,
but similar levels were ultimately reached (Figure 17b).
No significant production of TF or TC was noticed when
nitrogen was bubbled through the medium.

Thus it appears that oxygen is likely to be a
limiting factor in maximum TF production.

5. ENZYMIC OXIDATION OF PAIRS OF CATECHINS

In this section the results of incubations of pure
catechins with a partially purified polyphenol oxidase
preparation are reported.

Figure 18 illustrates a summary of all the major
spots produced on thin-layer cellulose chromatograms
during this series of incubations, and, for comparison,
Figure 19 shows a chromatogram of a hot aqueous infusion
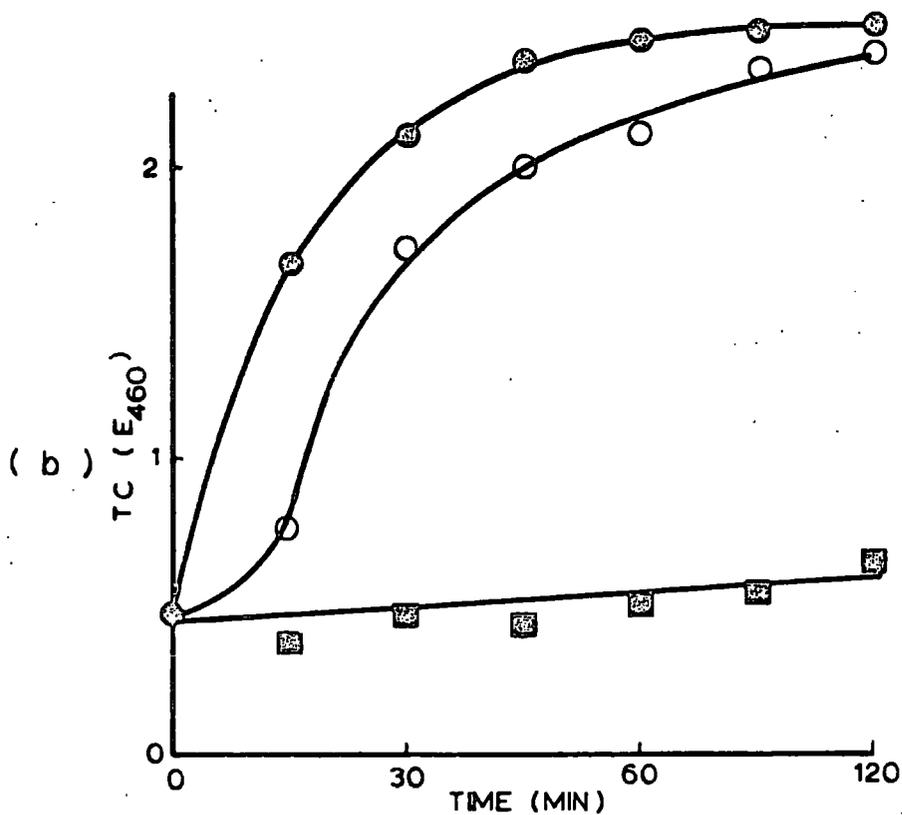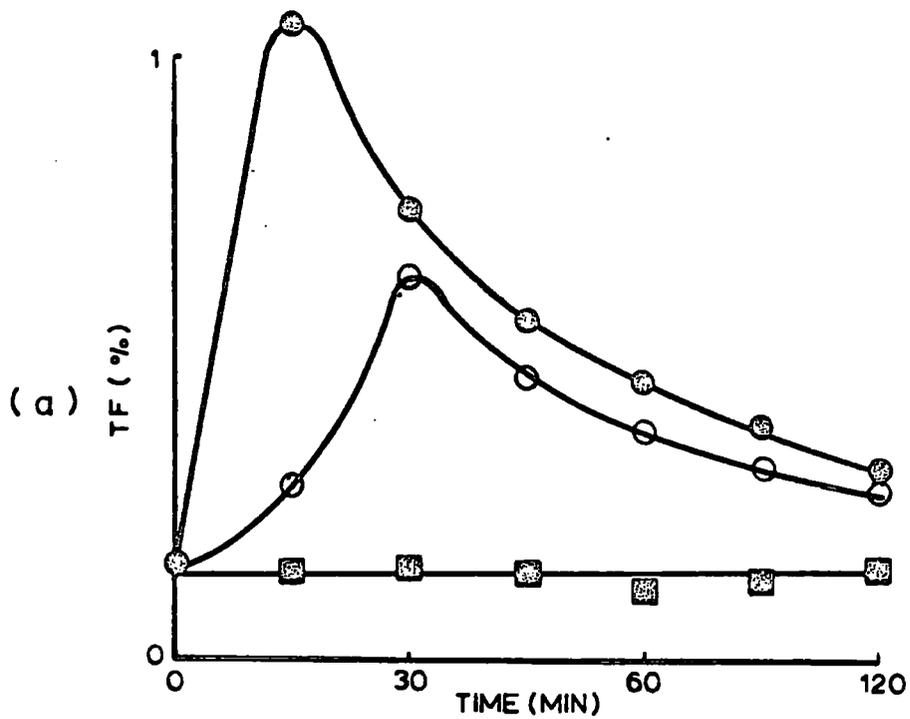
FIGURE 17

Effect of oxygen concentration in a fermenting system
upon production of TF and TC.

⊕—⊕   OXYGEN
O—O   AIR
▣—▣   NITROGEN

Oxygen, air, or nitrogen were bubbled through the
cell-free system described in the methods chapter.

of black tea.

Initial control incubations indicated that

(a) none of the compounds responsible for any of
the spots in Figures 18 and 19 could be demonstrated
on control chromatograms from the polyphenol oxidase
preparation at the concentrations used during incubations;

(b) no compounds, other than the catechins themselves,
could be demonstrated by chromatography as appearing as a
result of an incubation of any catechin, or combination
of catchins, with a polyphenol oxidase preparation which
had been immersed in a boiling waterbath for 20 minutes.

(i) <u>Identification of spots on chromatograms</u>

EGC, EGCG, ECG, GC, EC, C, GA, CA, QA and TG were all
identified by their behaviour under identical chromatographic
conditions in other investigations (Forrest and Bendall
1969a) and by similar positions being reported by other
workers using paper chromatography and similar solvent
systems (Roberts 1962a; Vuataz et.al. 1959). TF^1 and TFG
were identified from their absorption spectrum and their
R_F values being similar to those reported by other workers
using paper chromatography (Roberts 1962a; Vuataz and
Brandenburger 1961; Takino et.al. 1964). Spots A, B, and
C^1, were identified by their R_F values, solubility in
ethyl acetate, and their origins from pure catechins, in
incubations reported later, corresponding to information
from other sources (Roberts 1962a; Ferretti et.al. 1968).

Spots S_I and S_{II} are the thearubigin fraction as
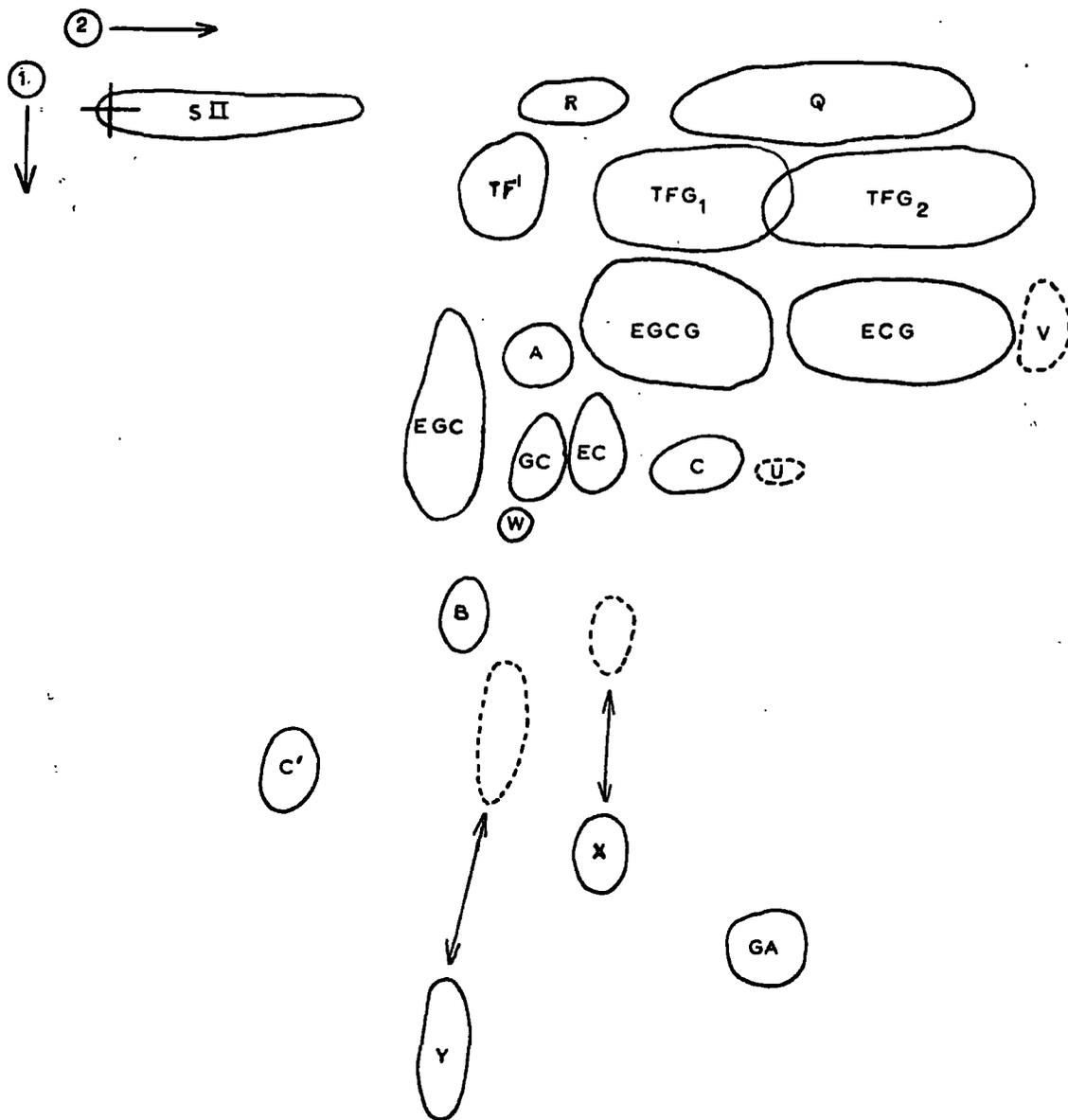
FIGURE 18

Composite Diagram of Components produced

during Enzymic Oxidation of Catechin

Mixtures

For details of chromatography see Methods chapter.
Spots X and Y had variable R_f in first dimension.
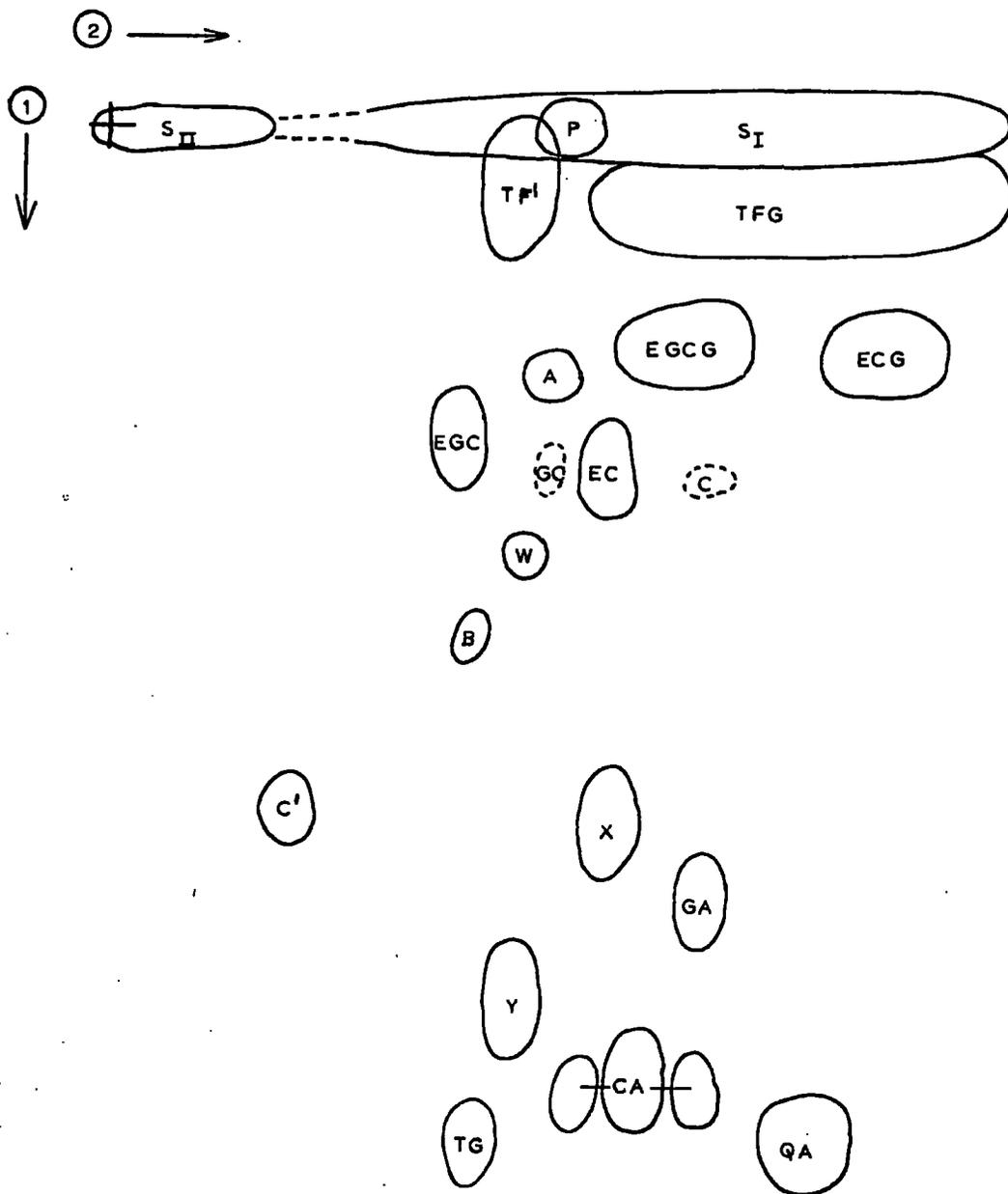
FIGURE 19

Thin-Layer Cellulose Chromatogram of a Hot
Aqueous Infusion of Black Tea

For details of chromatography see Methods chapter. Spots
due to kaempferol, quercetin, myricetin, and their
respective glucosides and rhamnoglucosides are omitted
for clarity.

described by Roberts (Roberts et.al.,1957), and the spot Q, from its absorption spectrum and R$_F$ values, is identical to the compound refered to by Roberts as substance Q (Roberts et.al., 1957), which Roberts tentatively identified as a chromatographically inseparable mixture of purpurogallin carboxylic acid, a flavanotropolone gallate derived from EGCG oxidation, and the main oxidation product of ECG (Roberts,1961). By the same reasoning, spot P is identified with Roberts substance P, which he identified with a 3' : 4': 5:5':7 - pentahydroxyflavylium salt (Roberts and Williams,1958), while spot R is probably Roberts uncharacterised substance R by nature of their similar origins in incubations of pure catechins.

Spots X and Y are yellow coloured streaks, of variable R$_F$ in water. Connected with the thearubigin fraction, their identity is obscure, and no previous reference has been made to them in the literature. Spots U and V are of unknown identity, but may be further catechins : they are colourless, and stain red-brown with the bisdiazotized benzidine reagent.

Spot W is colourless, reactive to benzidine, but of unknown identity.

(ii) Enzymic oxidation of EGC + EC

Following the procedure outlined in the methods chapter, EGC and EC were incubated with a phenol-free polyphenol oxidase preparation.

The almost colourless preparation turned bright

orange within a few minutes, and the reaction was stopped by heating when the rate of oxygen uptake was approximately half its initial level. The compound responsible for the orange colouration transfered easily to ethyl acetate.

Control oxidations of EGC and EC alone produced dark brown solutions, and very little of this colour would transfer to ethyl acetate.

The only visible spots on thin-layer chromatograms were the dark streak S_{II}, a faint yellow spot Y, and the bright orange TF^1 spot. Spraying with biediazotized benzidine revealed EGC, EC, some C (present as an impurity in the EC preparation), and C^1, in addition to S_{II}, Y, and TF^1(Figure 20   ). Control incubations of EC alone produced a strong brown S_{II} streak, and a number of minor spots not corresponding to any of the above spots. Oxidation of EGC alone produced spots C^1 and Y, and other minor spots. The TF^1 spots was only produced during the incubation of the mixture. No GA spots was noticed.

Quantitative analysis showed that EGC disappeared much faster than EC, though the rate of disappearance of EC increased once the EGC had become exhausted. The TF^1 content fell abruptly upon exhaustion of EGC (Figure 21   )

Chromatography of the final solutions after incubation on a column of LH-20 Sephadex showed that only

FIGURE 20

Thin Layer Chromatogram taken during
Oxidation of EGC + EC

For details of chromatography see Methods chapter.

FIGURE 21

ENZYMIC OXIDATION OF EGC + EC : Production of
TF and disappearance of catechins.

○——○   EGC
□——□   EC
△——△   TF[1]

FIGURE 22

Oxidation of EGC+EC: elution of products from
LH-20 Sephadex.

For details of chromatography see methods section.
For numbering of components see Figure 39.

      (a)   EGC oxidation.

      (b)   EC oxidation.

      (c)   EGC + EC oxidation.

FIGURE 7

Wet ether extract of ethyl acetate-soluble polyphenols.

For details of chromatography see methods section.
Chromatogram sprayed with bisdiazotized benzidine
solution. For identification of spots see Figure 18.
(This preparation was used as the source for the
purification of individual catechins, and for the
control mixture in incubations of the six catechins
together (see Results chapter).

the EGC + EC incubation produced component 8

(Figure 22). Integration of the area under

component 8, and following the procedure outlined

in the methods chapter, indicated that 1.19 mg TF^1

were produced by the incubation after 40 mins. Thus,

using the information in Figure 21 , 2.10 mg (i.e.

3.73 umd) TF^1 were present at 35 min, produced from

27.4 umd EGC and 31.6 umd EC ; i.e. 0.063 moles per

mole of catechin substrate.

(iii) <u>Enzymic oxidation of EGC + ECG</u>

Using the same procedure, an incubation of

approximately equal weights of EGC and ECG together

produced a bright orange colouration, for which the

compound responsible could be transfered easily to

ethyl acetate. The absorption spectrum of this compound

was measured after washing the solution with 2.5% (w/v)

sodium bicarbonate solution and after elution from

LH-20 Sephadex (Figure 26). Apart from the absorption

maximum at approximately 275 nm, maxima were noticed at

378 and 458 nm, and a slight shoulder at approximately

300 nm. The ratio of absorption at 378 to that at 458 nm

fell between 2.8 and 3.0, but the variation between

different repetitions of this experiment made more

accurate estimations meaning less.

Thin-layer chromatography demonstrated the

appearance of traces of TF^1, GA, and Q, with larger

amounts of R, TFG_2, C^1, and Y (Figure 23). The precise

position of the TFG_2 spot was difficult to determine, as its R_{EGC} or R_{ECG} value varied during the course of the incubation (cf. Figure 40).

Quantitative analysis indicated that EGC was consumed much faster than ECG, and that there was a continuous increase in the amount of TFG_2 produced (Figure 24).

Chromatography on LH-20 sephadex demonstrated a prominent component 8, which was the sole feature of the profile from an ethyl acetate extract (Figure 25). By integrating the area under the peak it was determined that 5.17 mg TFG_2 were produced by the incubation, which started with 55μmol EGC and 39μmolECG; thus 0.072 moles TFG_2 were produced per mole of catechin substrate.

A control incubation of ECG alone produced a light orange colouration, for which the compound responsible was extracted easily by ethyl acetate. Thin-layer chromatography failed to detect any TF^1 or TFG, but there was a pronounced spot Q, which was also demonstrated as component Q in an LH-20 sephadex chromatogram (Figure 25). The absorption spectrum for this component had maxima at 276 and 395 nm, with a shoulder at approximately 475 nm (Figure 26).

(iv) <u>Enzymic oxidation of EGCG + EC</u>

Using the same procedure, an incubation of approximately equal weights of EGCG and EC produced an orange colouration,
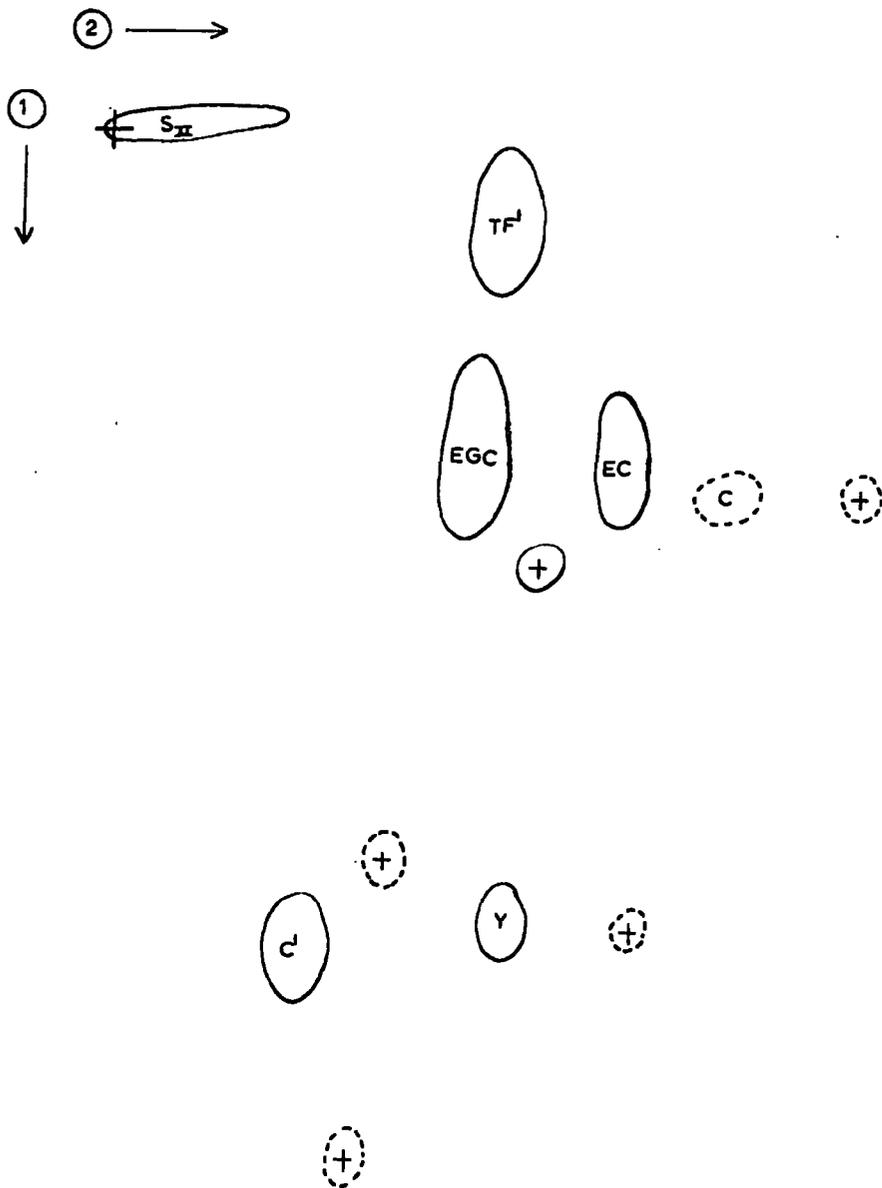
FIGURE 23

Thin Layer Chromatogram taken during
Oxidation of EGC+ECG

For details of chromatography see Methods chapter.

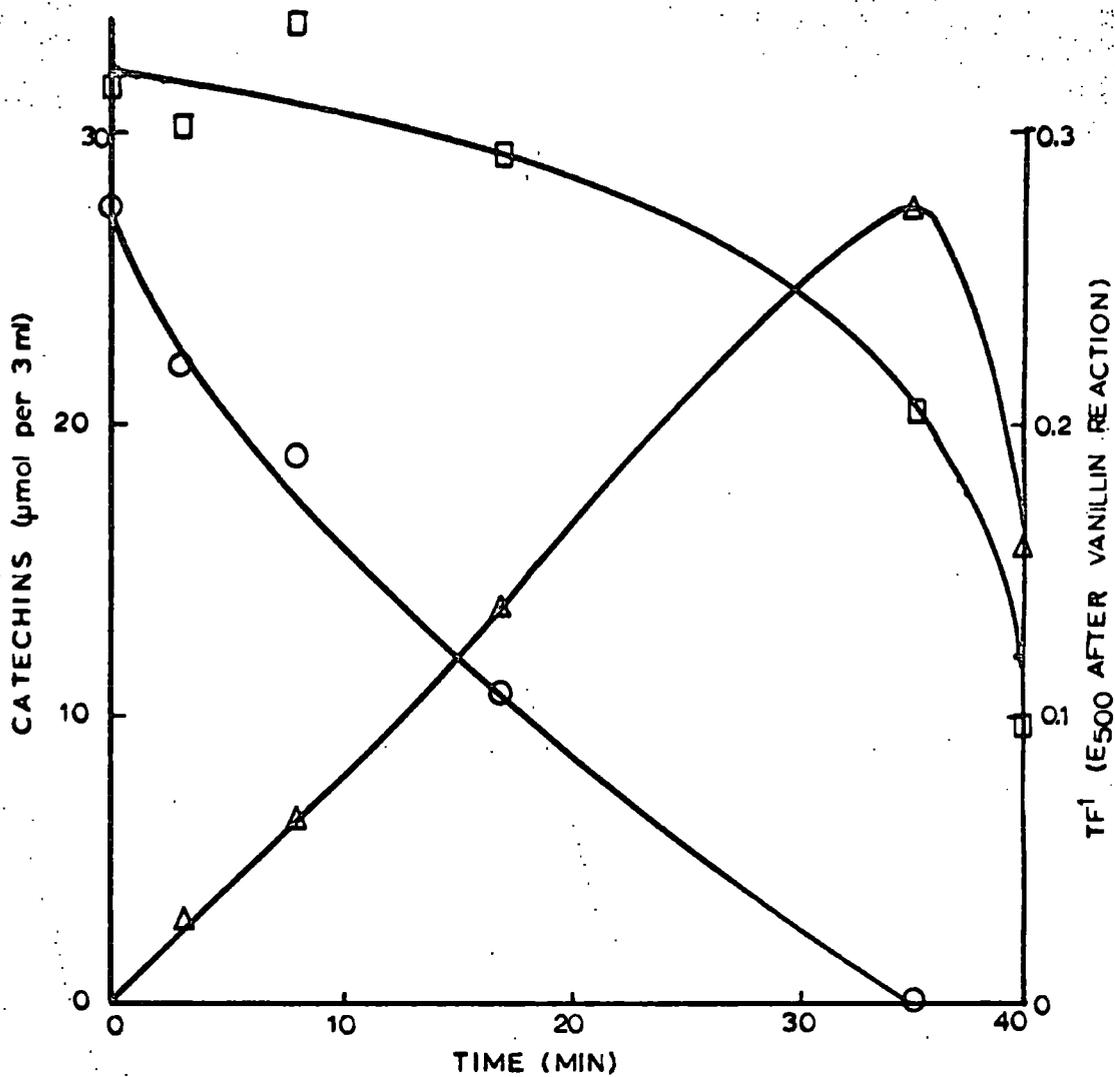FIGURE 24. Enzymic oxidation of EGC+ECG: production of TFG$_2$ and disappearance of catechins.

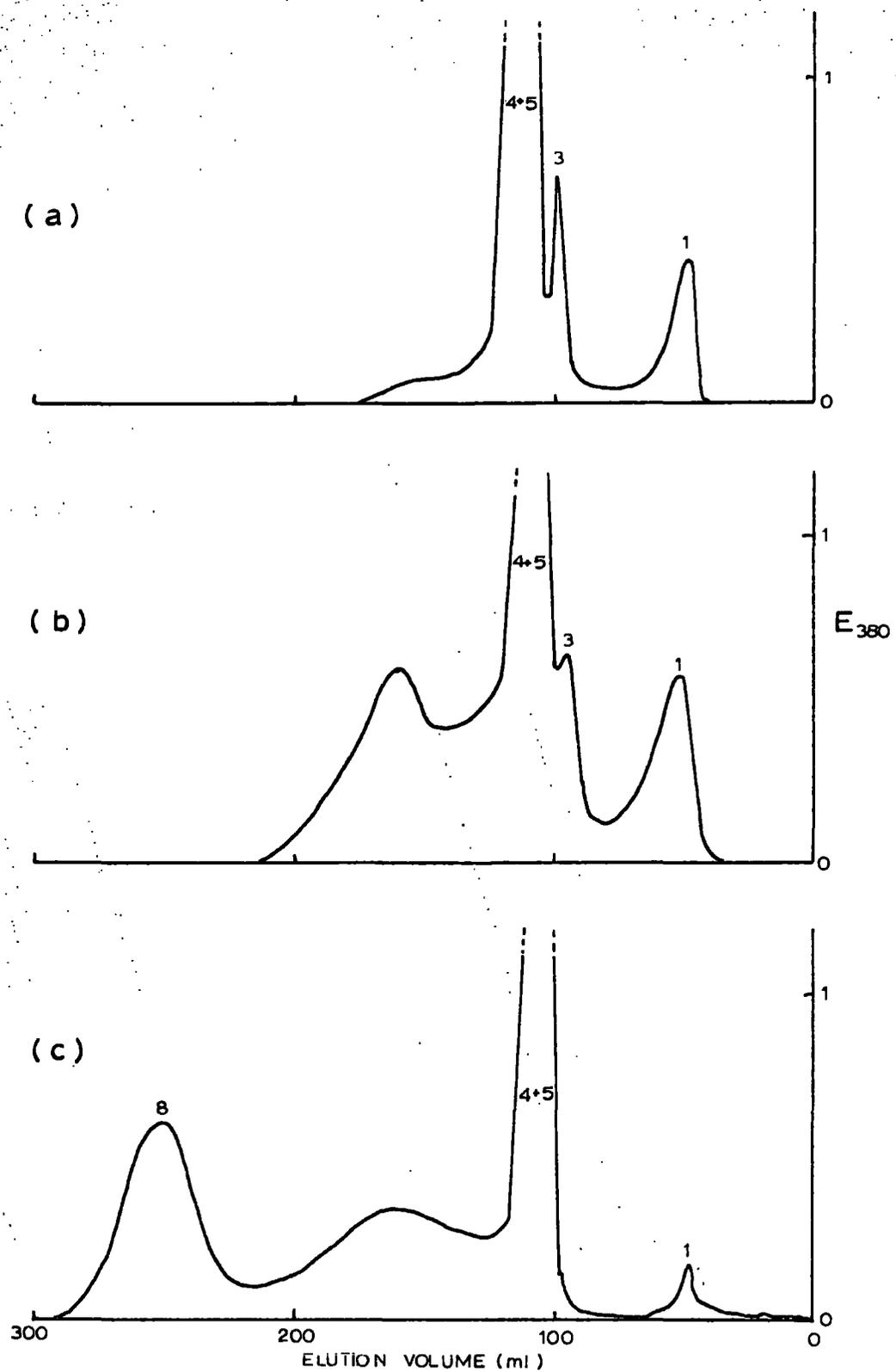O—O EGC   ■—■ ECG   △—△ TFG$_2$

FIGURE 25

Oxidation of EGC + ECG; elution of products from LH-20 Sephadex.

For details of chromatography see methods section.
For numbering of components see Figure 39.

(a)   EGC + ECG oxidation.
(b)   ECG oxidation.

for which the compound responsible could be easily

transfered to ethyl acetate. Thin-layer chromatography

demonstrated spot Q, a strong spot for TFG_1, spots A,

SII, X, Y, R, and GA. Chromatography of an ethyl

acetate extract at the end of the incubation showed

that spots R, Q, TFG_1 and A were all extracted by the

organic solvent, while spots S_{II}, X, Y, and GA remained

in the aqueous layer (Figure 27). From inspection of

plates it was observed that both spots Q and TFG,

reached a maximum during the incubation, and then became

weaker. Quantitative analysis confirmed this for TFG_1,

and also showed the rate of oxidation of EGCG to be very

much faster than that of EC (Figure 28). (It was also

evident that the EGCG preparation contained more water

than the EC preparation, possibly due to incomplete

removal of water of crystallisation during freeze-drying,

as the difference in the number of moles of each catechin

detected in the initial incubation mix was greater than

can be accounted for by differences in molecular weight).

The absorption spectrum for the orange product

had maxima in the same positions as for TFG_2 produced

from EGC + ECG (Figure 26), and the ratio of extinction

at 380 and 460 nm fell within the same range.

Chromatography on LH-20 Sephadex demonstrated

component 8, plus a considerable component Q (Figure 29).

By integration of area under this peak, together with a

knowledge of the decay curve for TFG_1 (Figure 28), the
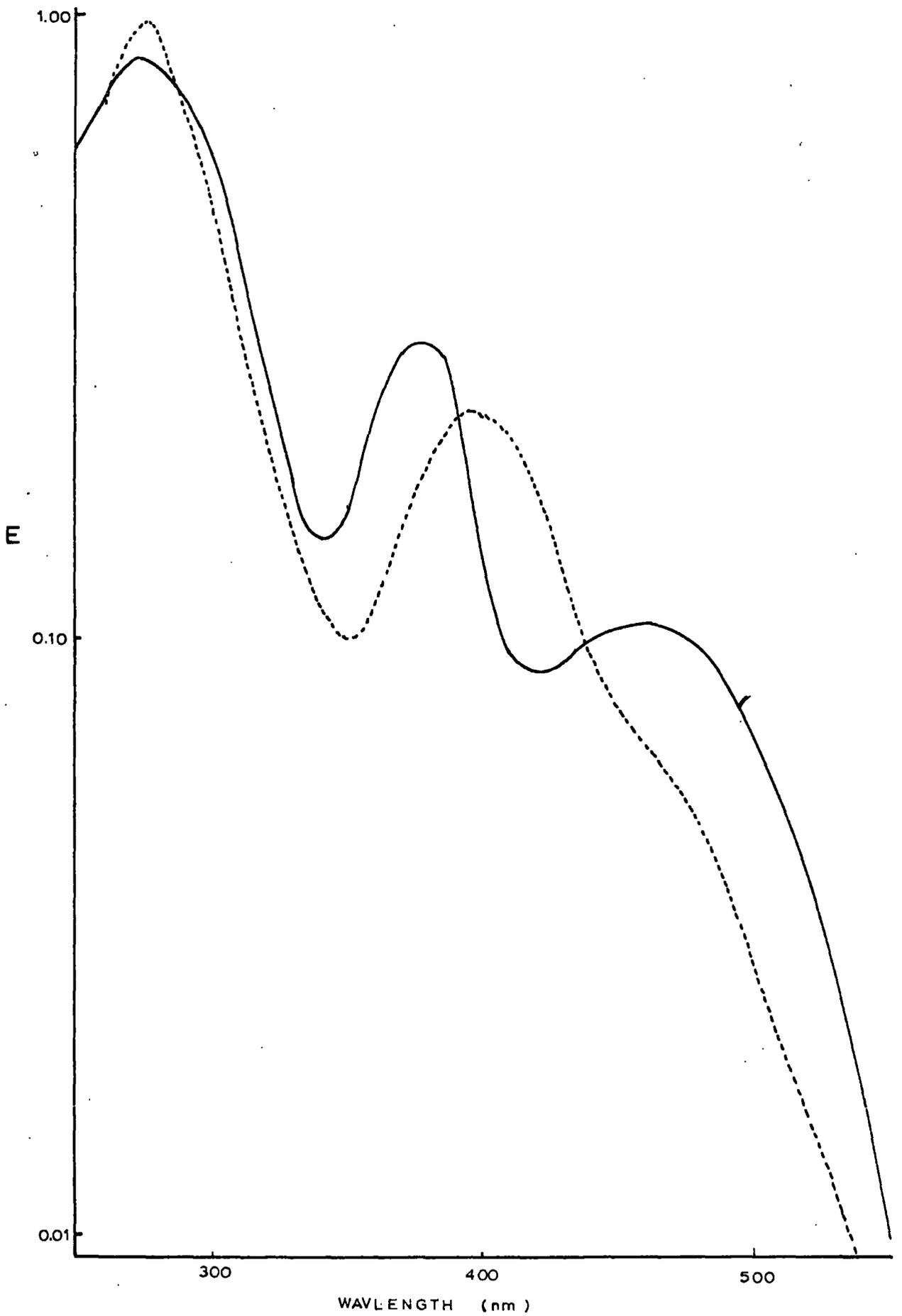
FIGURE 26

Absorption spectrum of TFG$_2$ (——) and substance Q (- - - -)
in ethanol and after elution from LH - 20 Sephadex.
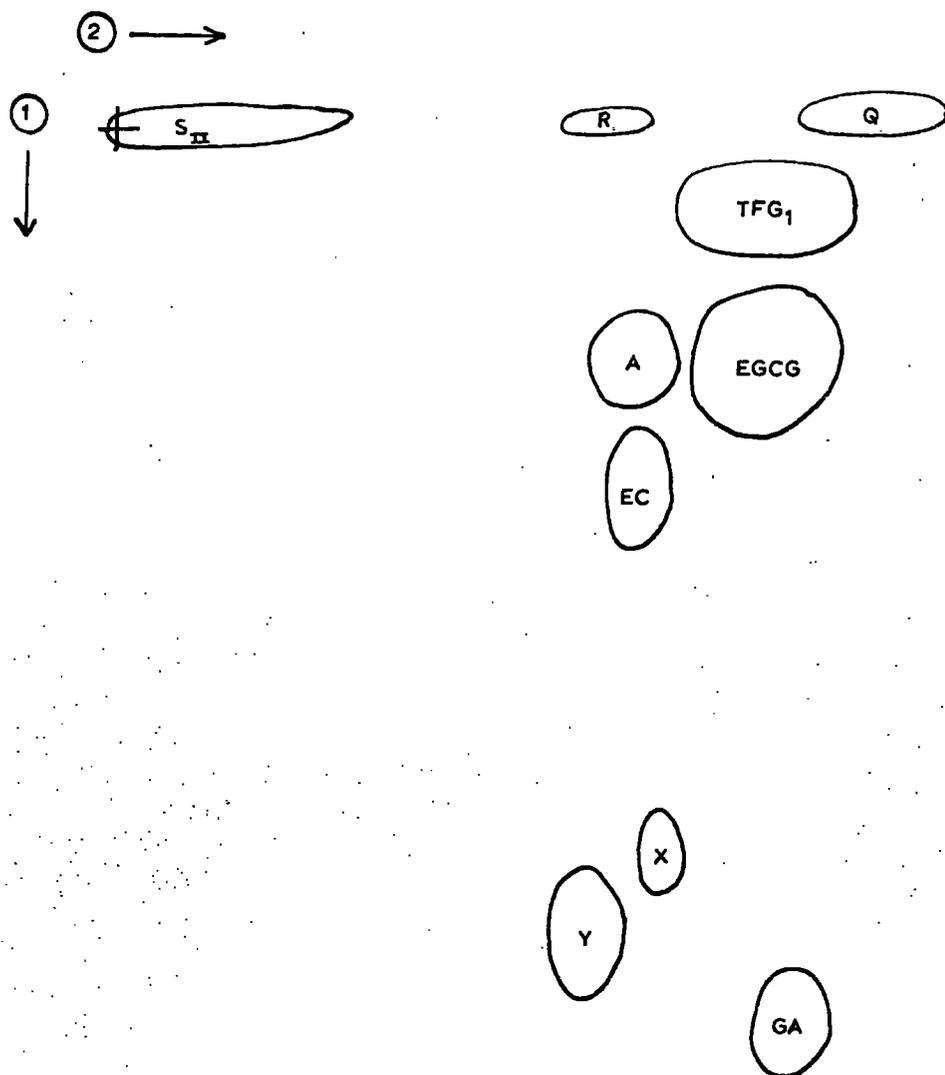
FIGURE 27

Thin Layer Chromatogram taken during

Oxidation of EGCG + EC

For details of chromatography see methods chapter.

CATECHINS (umol / 3ml)

60

40

20

0

TFG$_1$ (E$_{500}$ AFTER VANILLIN REACTION)

0.08

0.04

0

0   20   40   60   80

TIME (MIN)
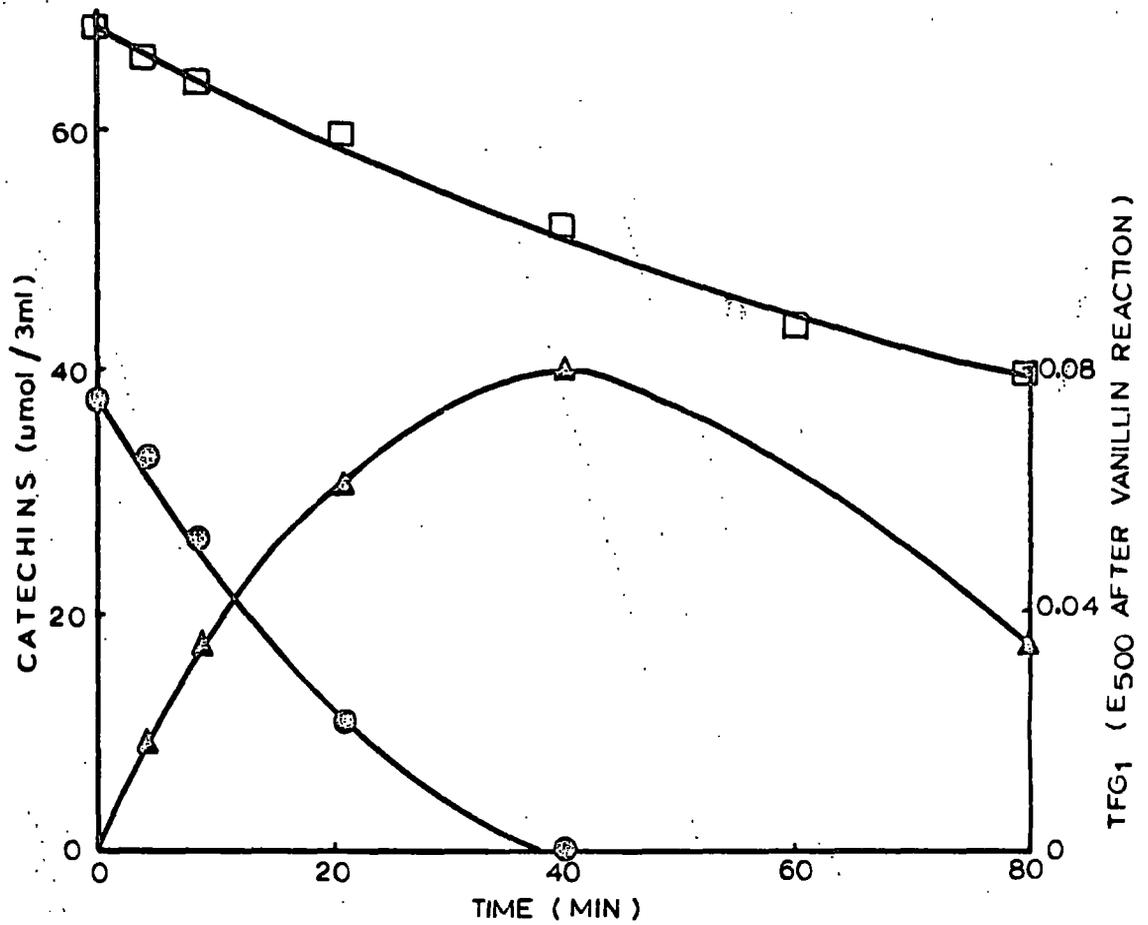
FIGURE 28

Enzymic oxidation of EGCG + EC: disappearance of
catechins and production of TFG$_1$.

☐—☐ EC
⊕—⊕ EGCG
△—△ TFG$_1$
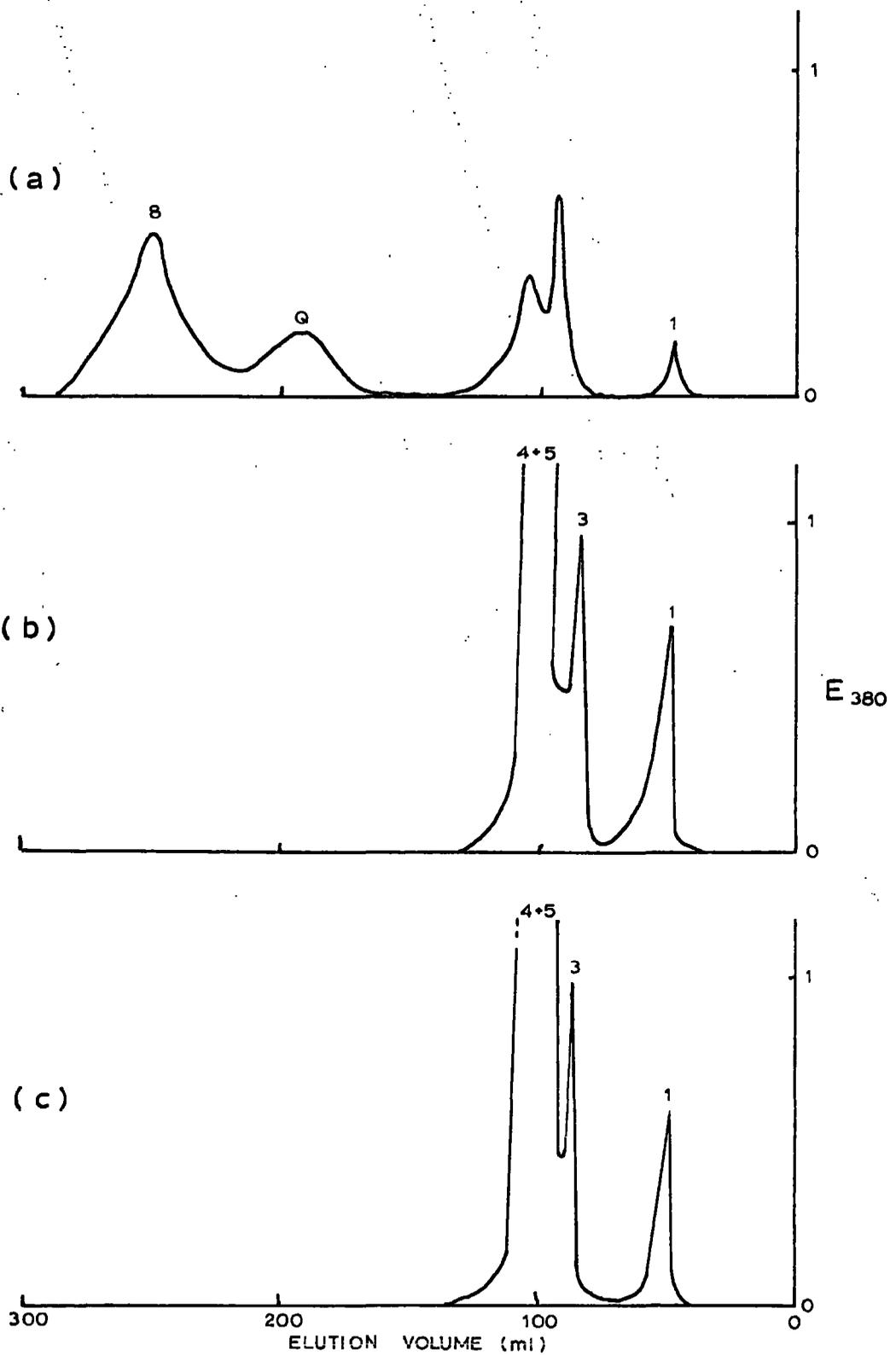
FIGURE 29

Oxidation of EGCG+EC: elution of products from
LH-20 Sephadex.

For details of chromatography see Methods chapter.
For numbering of components see Figure 39.

(a)   EGCG + EC oxidation (ethyl acetate
        extract).

(b)   EGCG + EC oxidation (aqueous residue).

(c)   EGCG oxidation.

maximum quantity of TFG_1 produced in this experiment was estimated at 2.62 µmol, i.e. 0.025 moles per mole of catechin substrate.

A control incubation of EGCG alone produced spots R,A,X,Y, and GA, but no TFG or Q. On LH-20 Sephadex, strong components 1, 3, 4 and 5 were evident, but not components 8 or Q (Figure 29).

(v) <u>Enzymic Oxidation of EGCG + ECG</u>

Following the same procedure, incubating approximately equal weights of EGCG and ECG resulted in a muddy brown colouration; ethyl acetate extraction removed some orange coloured compounds from this. Traces of TF, TFG, and TFG_2 could be seen on chromatograms, though it was difficult to differentiate between TFG_1 and TFG_2. Spots A, X, Y, and GA were in evidence, as was a trace of spot Q (Figure 30). Quantitative measurement showed that EGCG was consumed much faster than ECG. Spot A increased until EGCG was exhausted, and then decayed very rapidly (Figure 31). Unfortunately, the diffuse nature of the TFG spots prevented a quantitative estimate of these compounds.

The absorption spectrum of the ethyl acetate extract was identical to that of TFG_1 and TFG_2 (Figure 26). Portions of the incubation mixture were subjected to chromatography on LH-20 Sephadex (Figure 32). Large components 1, 3, 4 & 5 were in evidence in the aqueous residue, and the ethylacetate extract exhibited component 8,
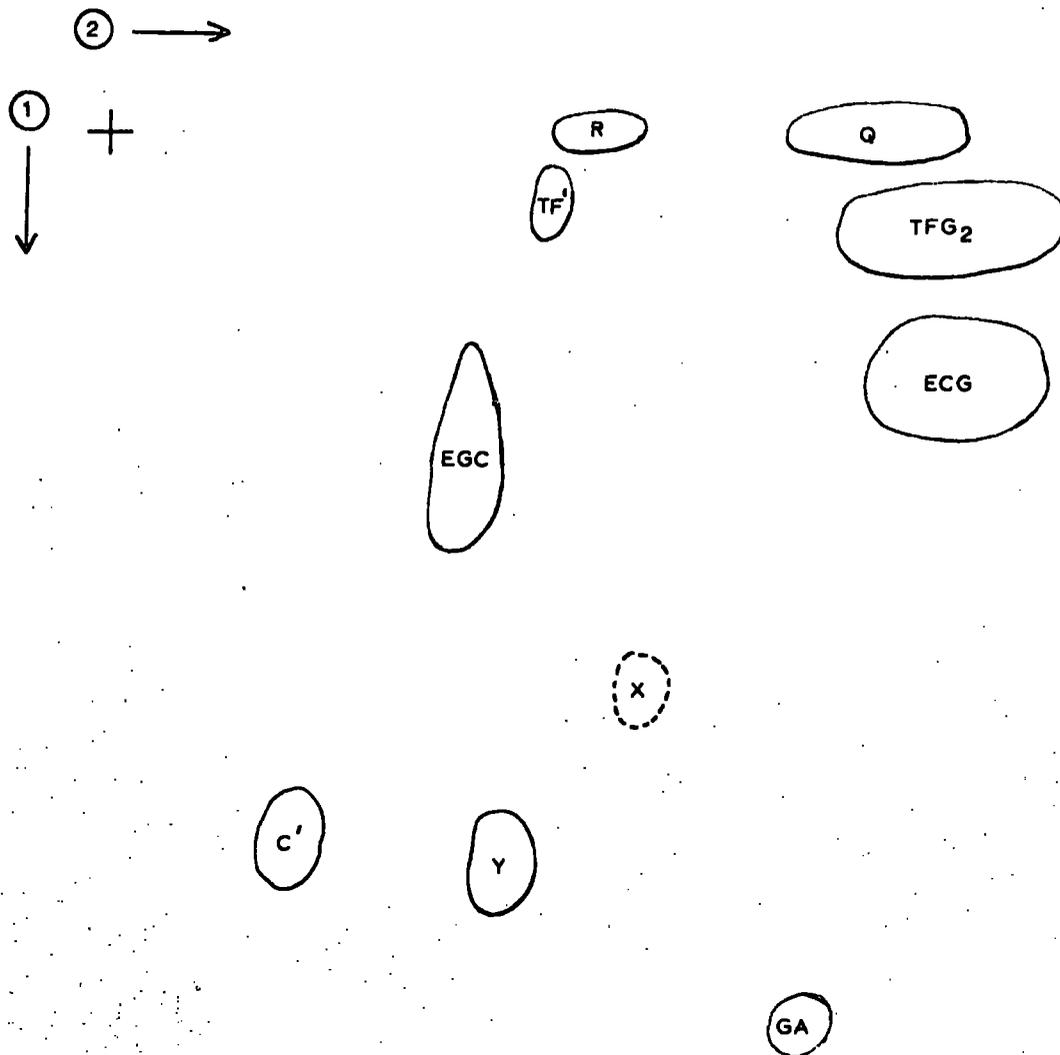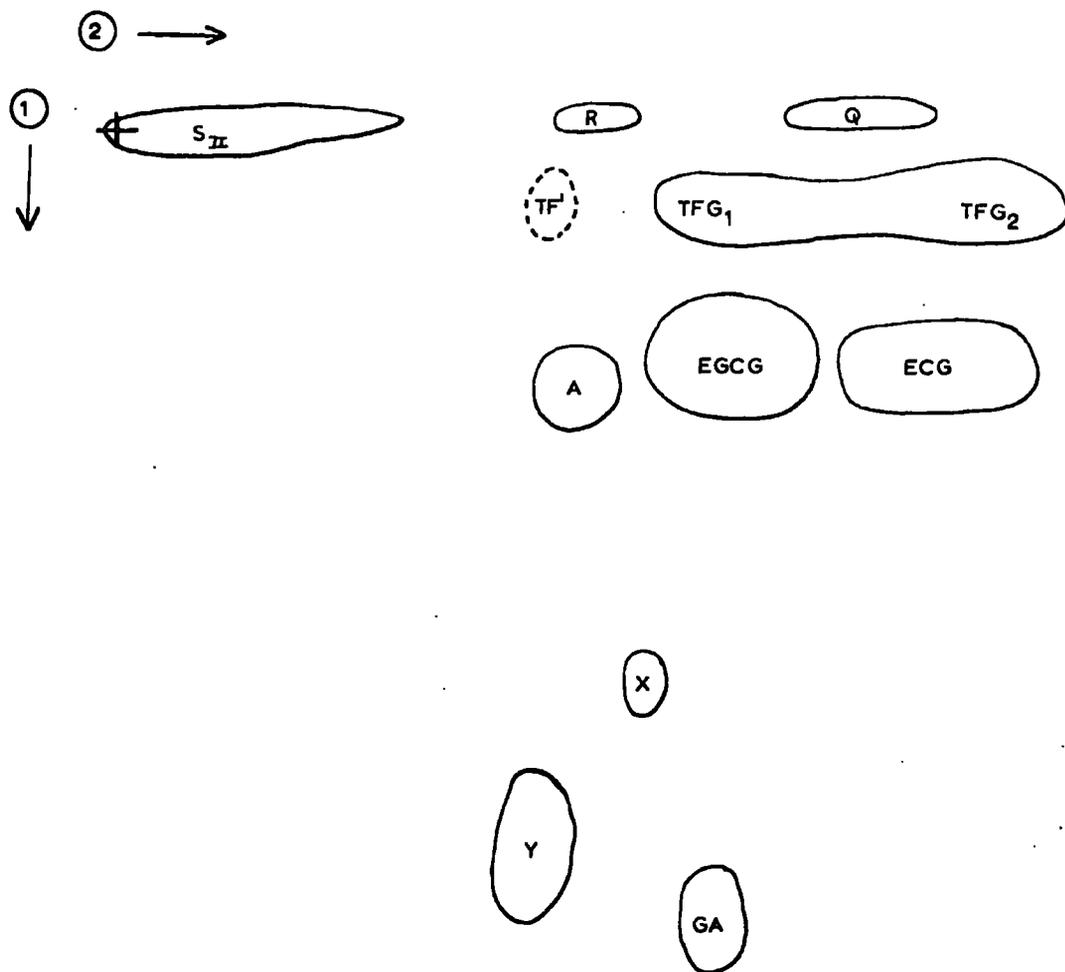
FIGURE 30

Thin Layer Chromatogram taken during Oxidation of EGCG + ECG

For details of chromatography see methods chapter.
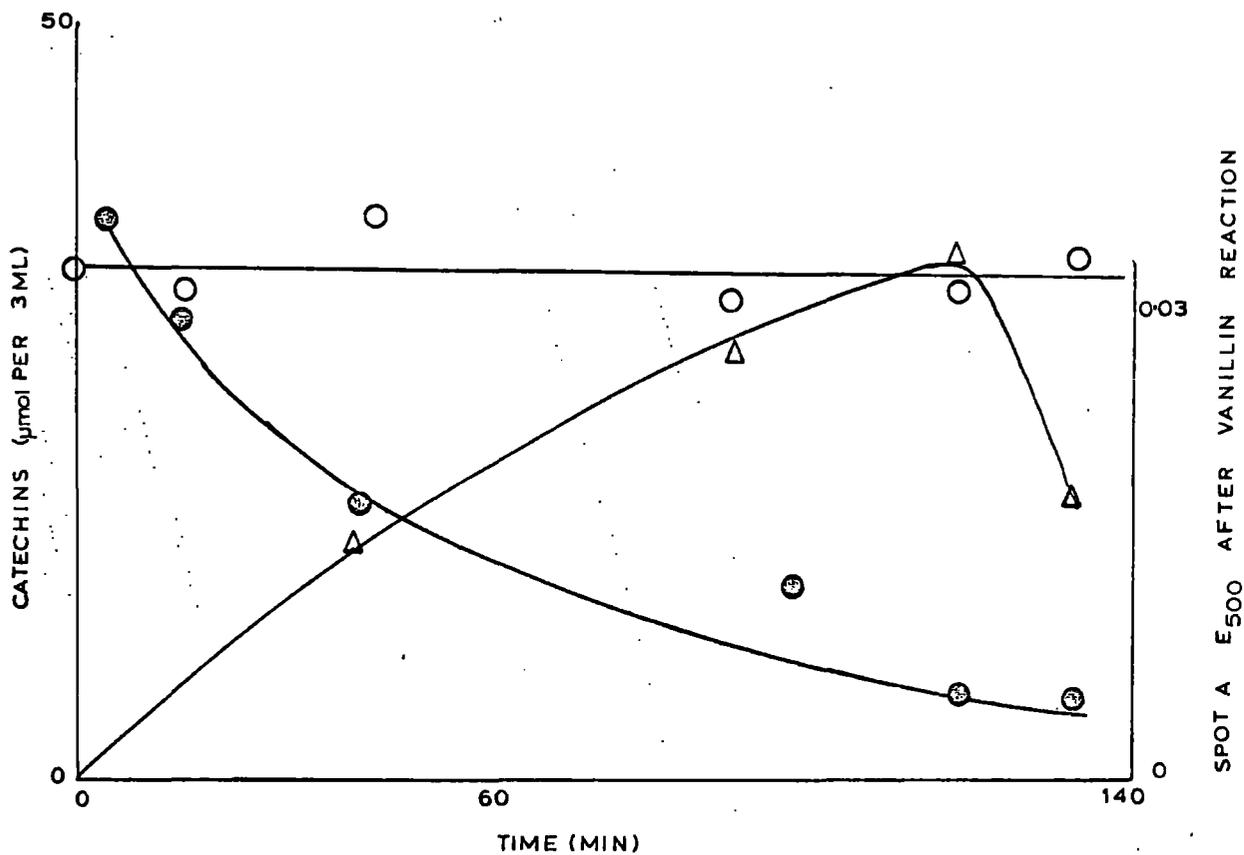
FIGURE 31

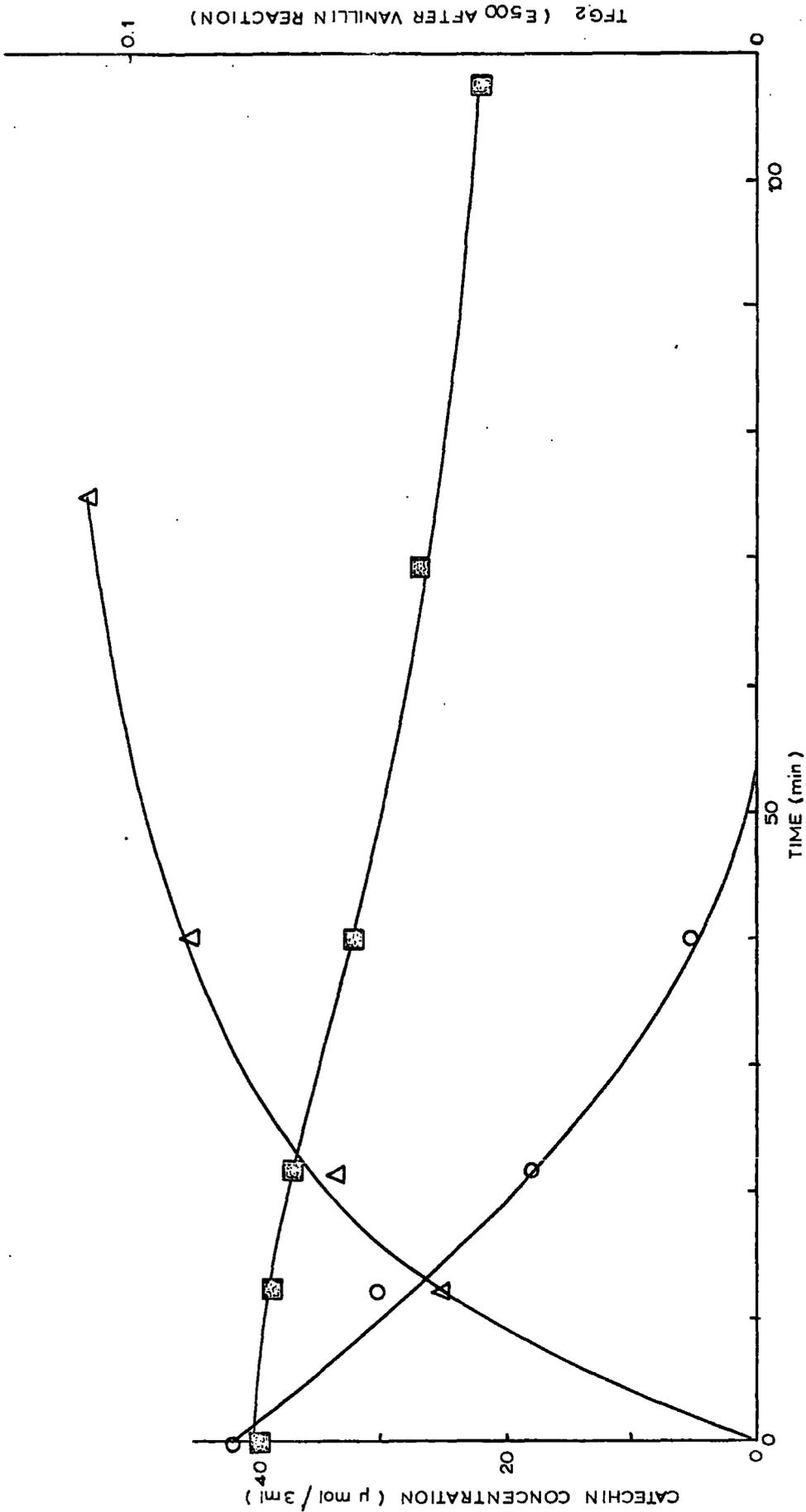Enzymic Oxidation of EGCG+ECG:
Disappearance of Catechins and Appearance
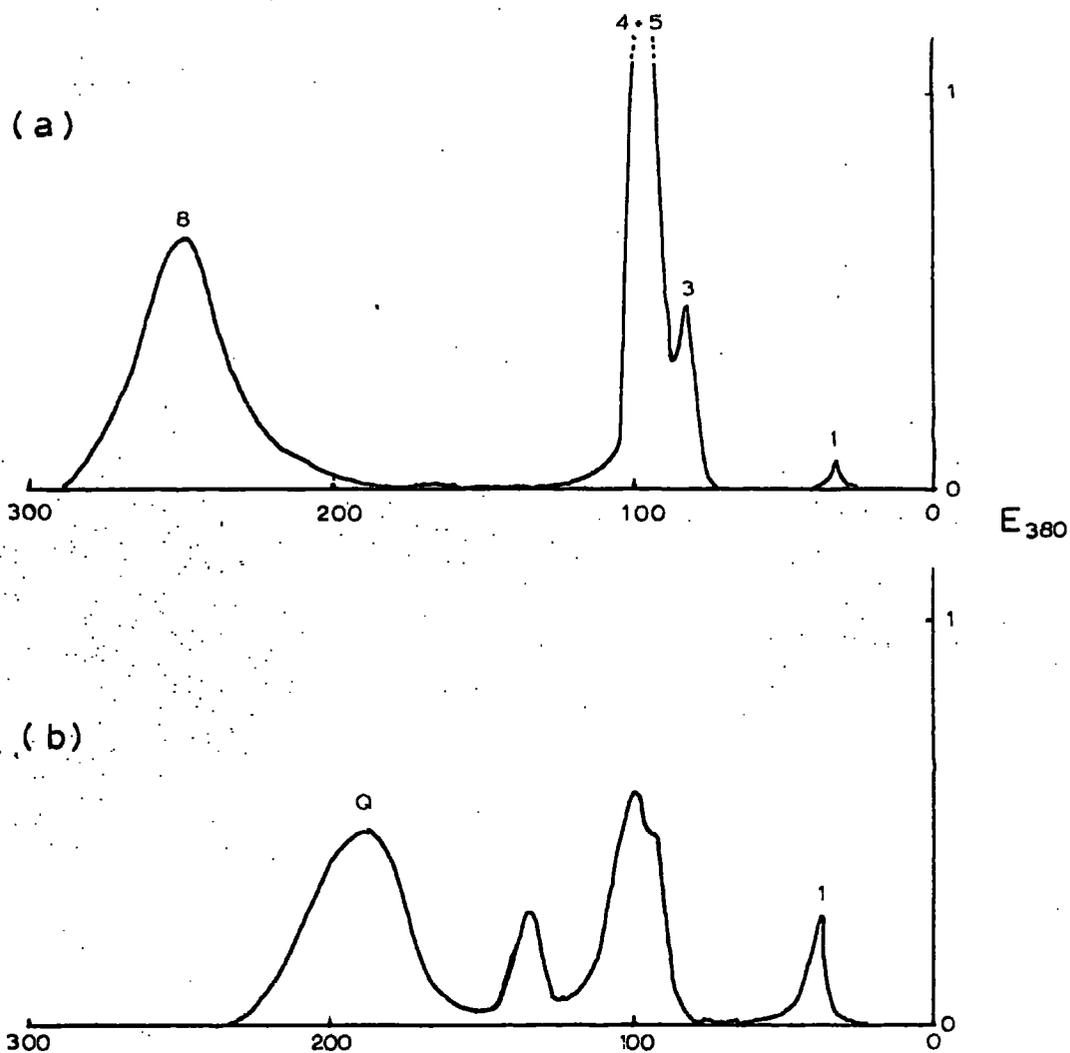of Spot A .

⊕—⊕ EGCG

O—O ECG

△—△ SPOT A
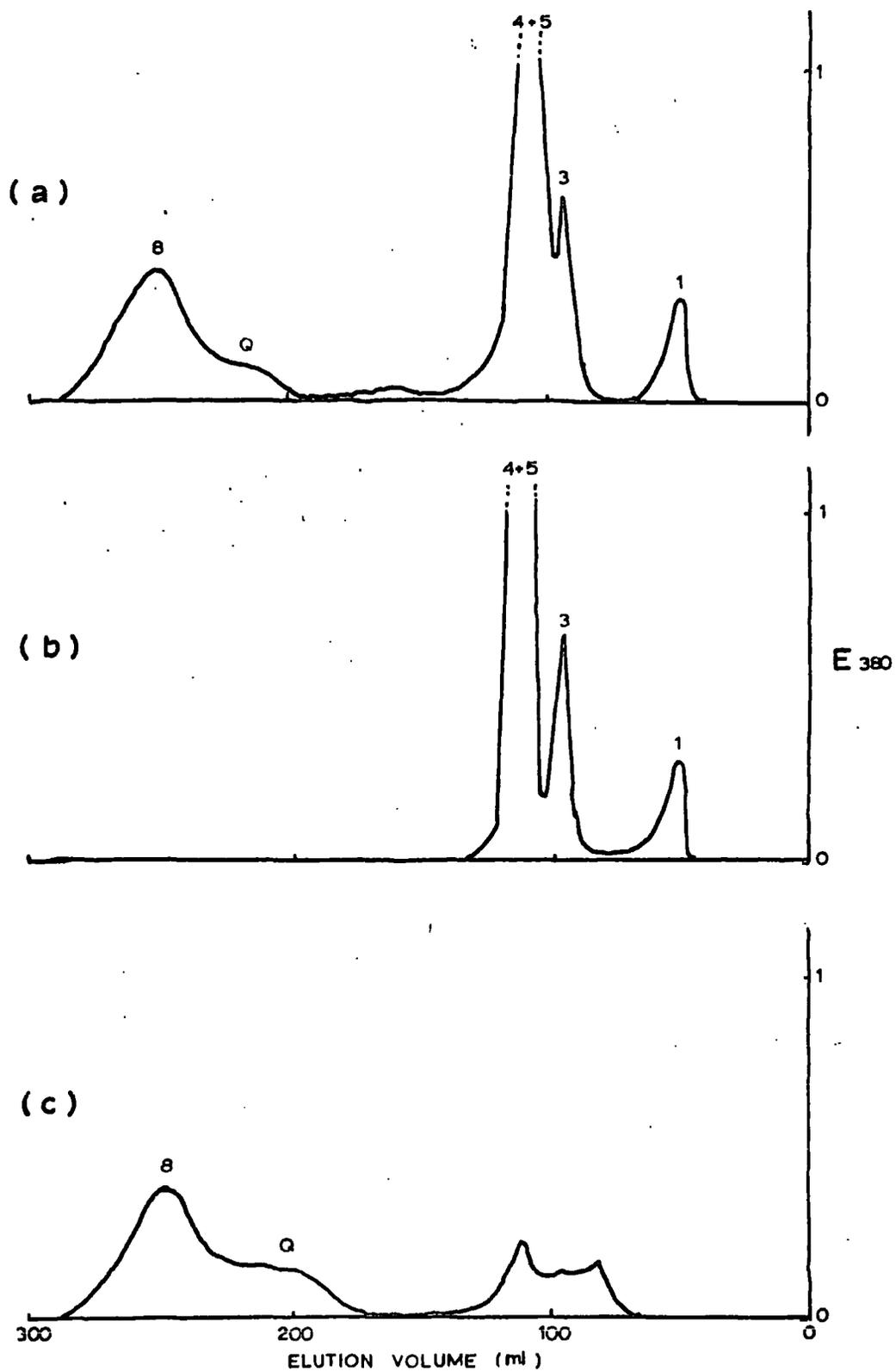
FIGURE 32

Oxidation of EGCG + ECG : elution of products
from LH-20 Sephadex.

For details of chromatography see Methods chapter.
For numbering of components see Figure 39.

     (a)   complete extract.

     (b)   aqueous residue after ethyl acetate
               extraction.

     (c)   ethyl acetate extract.

and assuming that the incubation was stopped near the peak of TFG production, an estimate of 0.038 moles TFG per mole of catechin substrate was arrived at.

(vi) Enzymic Oxidation of GC + EC and GC + ECG

These two incubations were performed to see whether GC could replace EGC in the incubations (ii) and (iii) above. The incubation procedure was the same.

Thin layer plates run druing incubations of GC + ECG produced traces of TF^1 and TFG, but not in sufficient quantity to remove from the plates for assay. Control incubations of GC and GC + EGC also produced some TF^1 and TFG spots. LH-20 Sephadex chromatography of these incubations indicated the following quantities of component 8 (estimated as mg TFG) to be formed:

$$9.1 \text{ mg GC} \qquad 0.29 \text{ mg TFG}$$
$$10.2 \text{ mg GC+10.9mg ECG } 1.29 \text{ mg TFG}$$
$$9.5 \text{ mg GC+10.9mg EC } 1.20 \text{ " "}$$
$$9.8 \text{ mg GC+9.7 mg EGC } 0.43 \text{ " "}$$

The incubation of GC + EC produced a strong spot having an R_F in the organic solvent intermediate between TF^1 and TFG_1 as determined by its relation to the GC and EC spots. (Figure 34).

To check the purity of the GC preparation, a thin layer chromatogram was loaded with ten times the normal quantity. There was still no evidence of catechin impurities. (Figure 34).
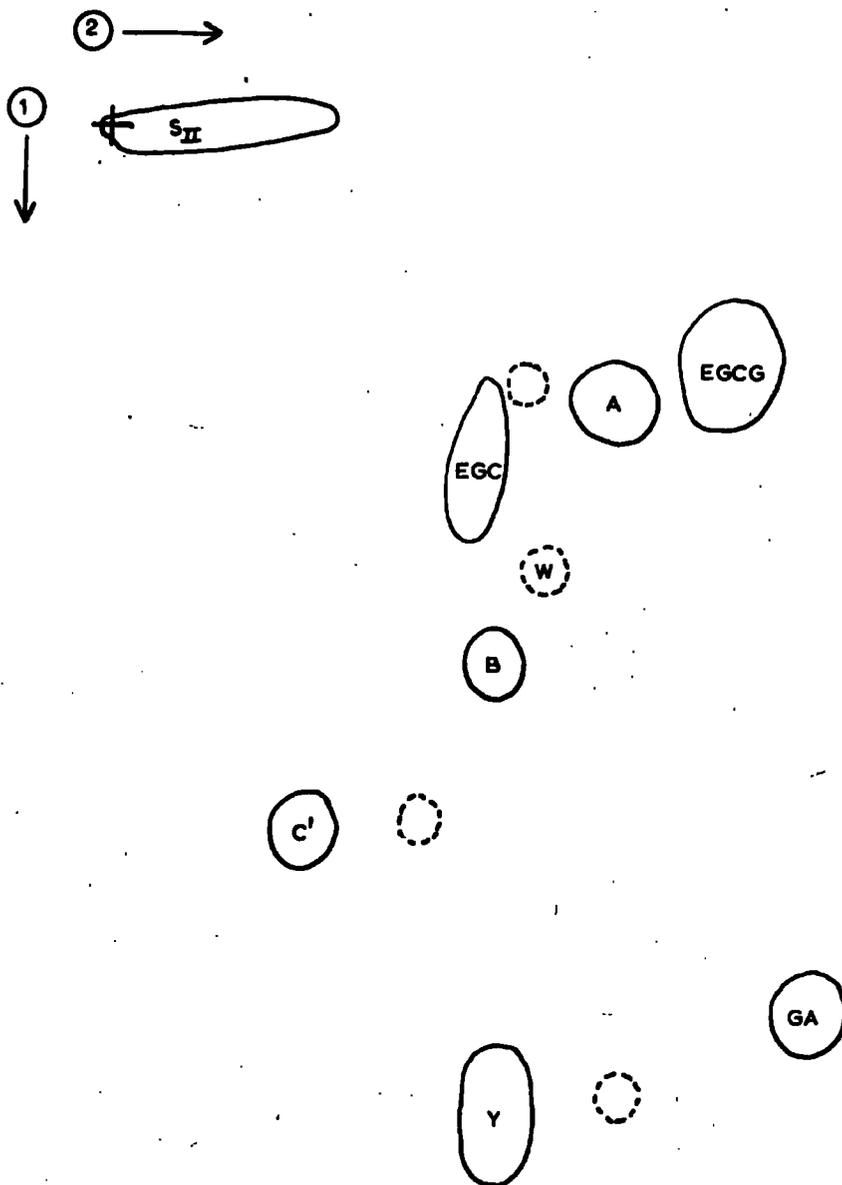
FIGURE 33

Thin Layer Chromatogram taken during
Oxidation of EGCG + EGC

For details of chromatography see Methods chapter.
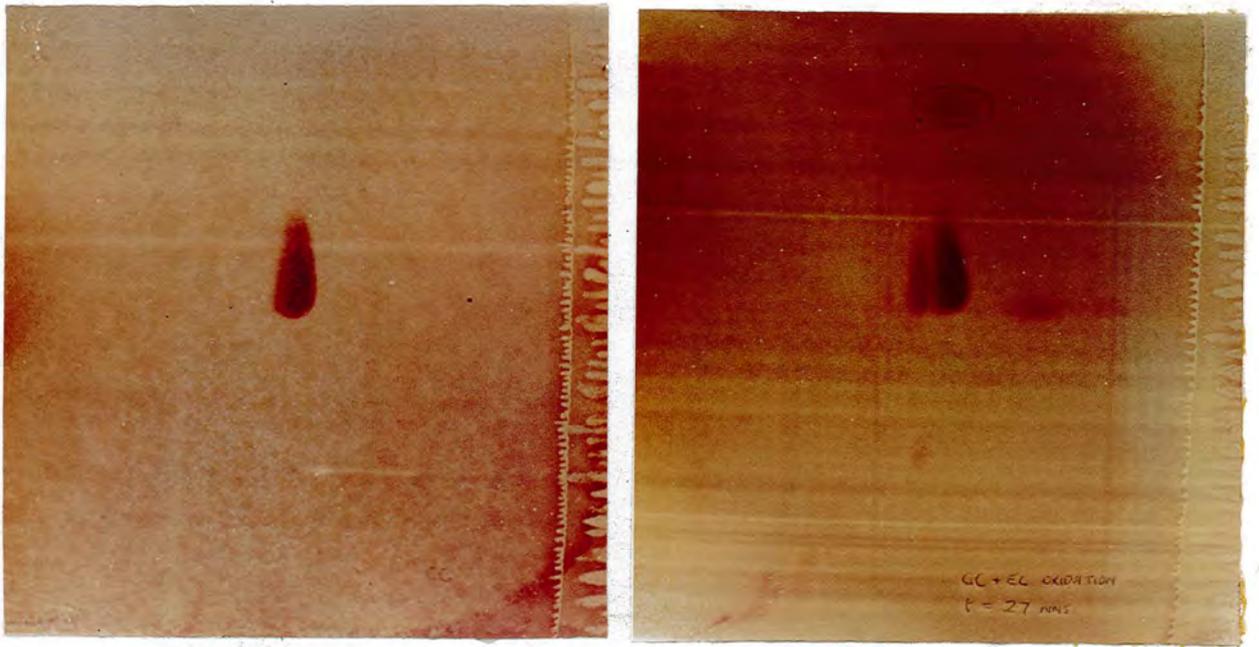
(a)   (b)

FIGURE 34

Enzymic oxidation of GC + EC

Photographs of thin-layer chromatograms taken
before and during incubation. Plates sprayed
with bisdiazotized benzidine. For details of
chromatography see methods chapter.
(a) GC preparation
(b) GC + EC oxidation at 27min.

By inspection of thin layer plates it was observed that GC disappears faster than ECG or EC when oxidised in combination with either of them, but in the control GC + EGC oxidation, EGC was oxidized fastest.

(vii) Enzymic Oxidation of EGC + C

This incubation was performed to see whether C could replace EC in the incubation (1) above. Several minor spots were produced on thin-layer chromatograms, but no traces of TF[1] or TFG were noticed. Neither was any component 8 observed during Sephadex LH-20 chromatography of the final oxidized solution, either from EGC + C, or C alone.

EGC was seen to disappear at a much greater rate than C.

(viii) Enzymic Oxidation of EGC + EGCG

The incubation procedure was as before. Thin layer chromatography showed the developments of the same spots as produced by incubations of these compounds separately (cf. pages 48 and 52), and an additional spot B, which was extracted with ethyl acetate (Figure 33) All spots except spot Y were colourless.

Chromatography on LH-20 Sephadex failed to demonstrate any component 8.

Inspection of thin layer plates indicated that EGC was oxidized marginally faster than EGCG.

6. Enzymic Oxidation of a Mixture of Six Catechins

This series of experiments was designed to investigate

the effect upon TF^1 or TFG production of doubling one particular component of a mixture of six catechins initially existing in proportions similar to that in a tea shoot. (Figures 35,36,37.)

Figure 38 shows a typical recorder trace of the oxidation products of the basic mixture of catechins elating from an LN-20 Sephadex column. Components 1, 3, 5 and 5, T and 8 were very much in evidence, while components 2, 6, and 7 (see Figure 39) were absent. A repeat of the chromatography at lower loadings and higher sensitivity of the optical monitoring system failed to separate the component labeled "4 + 5" into two peaks. Oxidations of the basic mixture with added catechins produced the same components, but quantitatively modified

Where possible the height of peak 4 + 5 was measured as a measure of the thearubigin complex, and in all cases component 8 was integrated to give an estimate of the theaflavin complex from each incubation. Results indicated that doubling the EGC content of the mixture resulted in a 64% increase in the total theaflavin produced. Increasing the EC content made no apparent difference to the theaflavin level, and doubling the EGCG content actually resulted in a 40% reduction in total theaflavins, though a much larger amount of the thearubigin complex was formed. (Table 3).

During the course of these incubations, all the spots
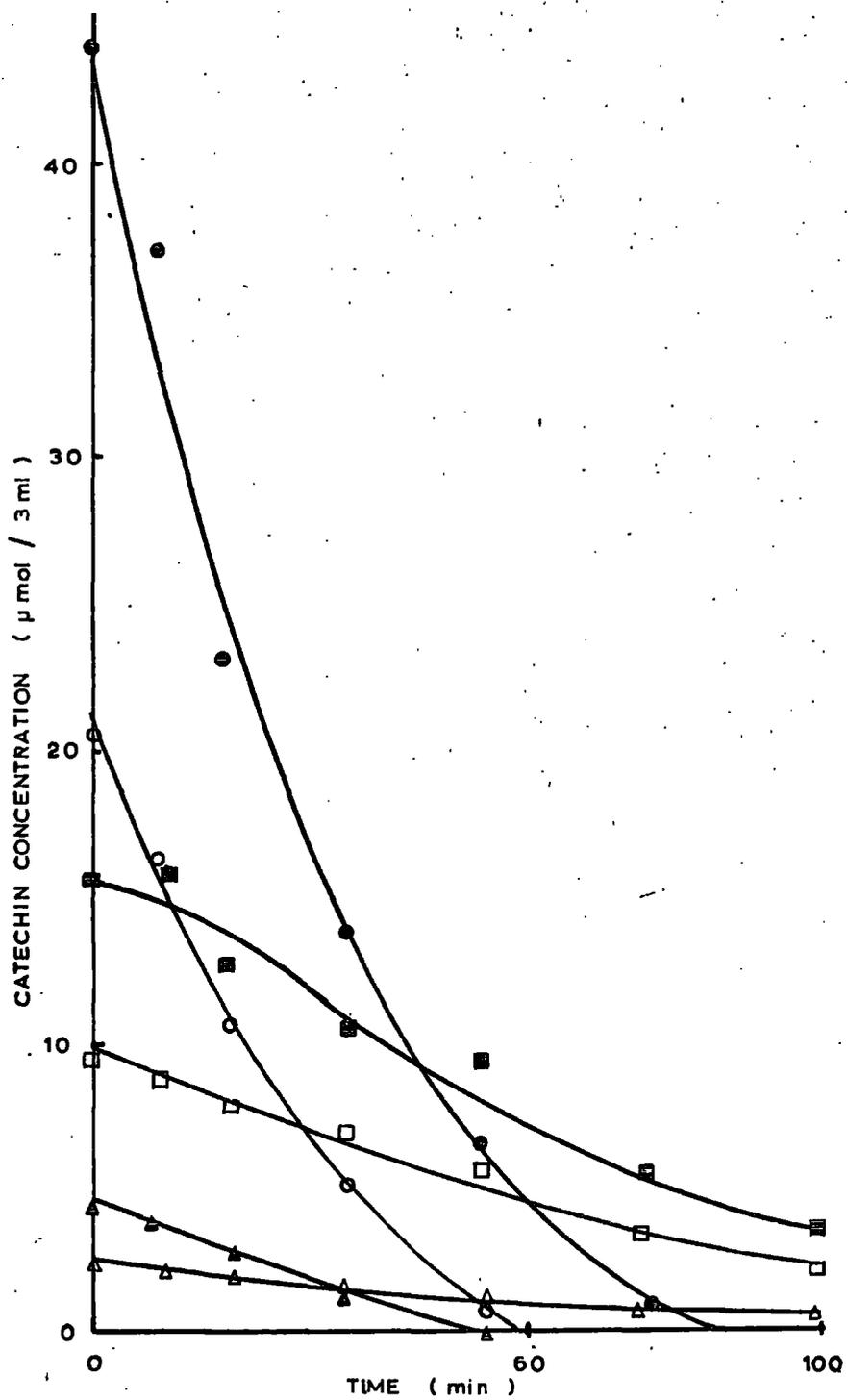
FIGURE 35

Enzymic oxidation of a mixture of six
catechins:

(i) control mixture

O—O EGC   □—□ EC   △—△ C
●—● EGCG   ▨—▨ ECG   ▲—▲ GC
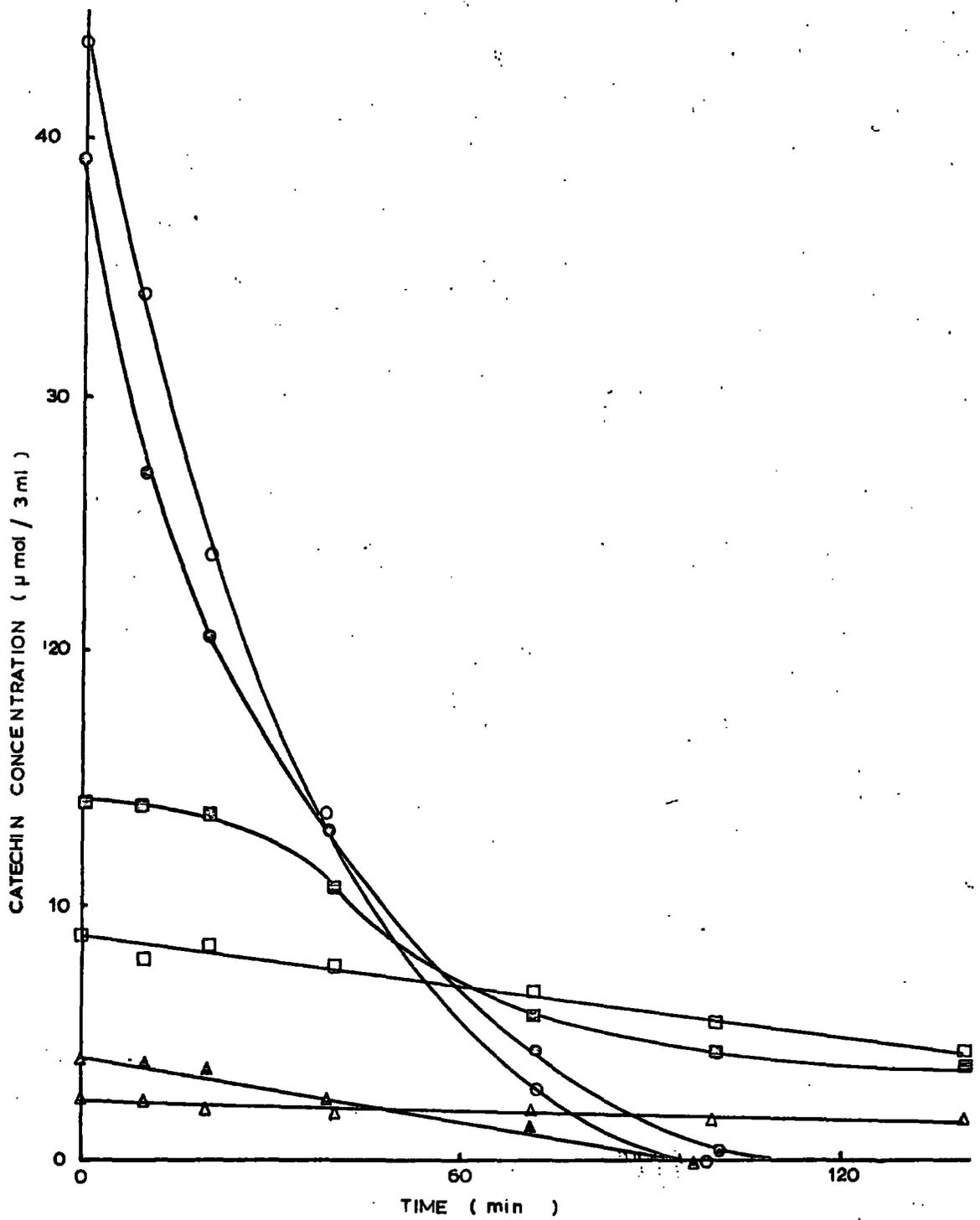
FIGURE 36

Enzymic oxidation of a mixture of six catechins:

(ii)   control mixture + EGC

O—O EGC   □—□ EC   △—△ C
●—● EGCG   ▣—□ ECG   ▲—▲ GC
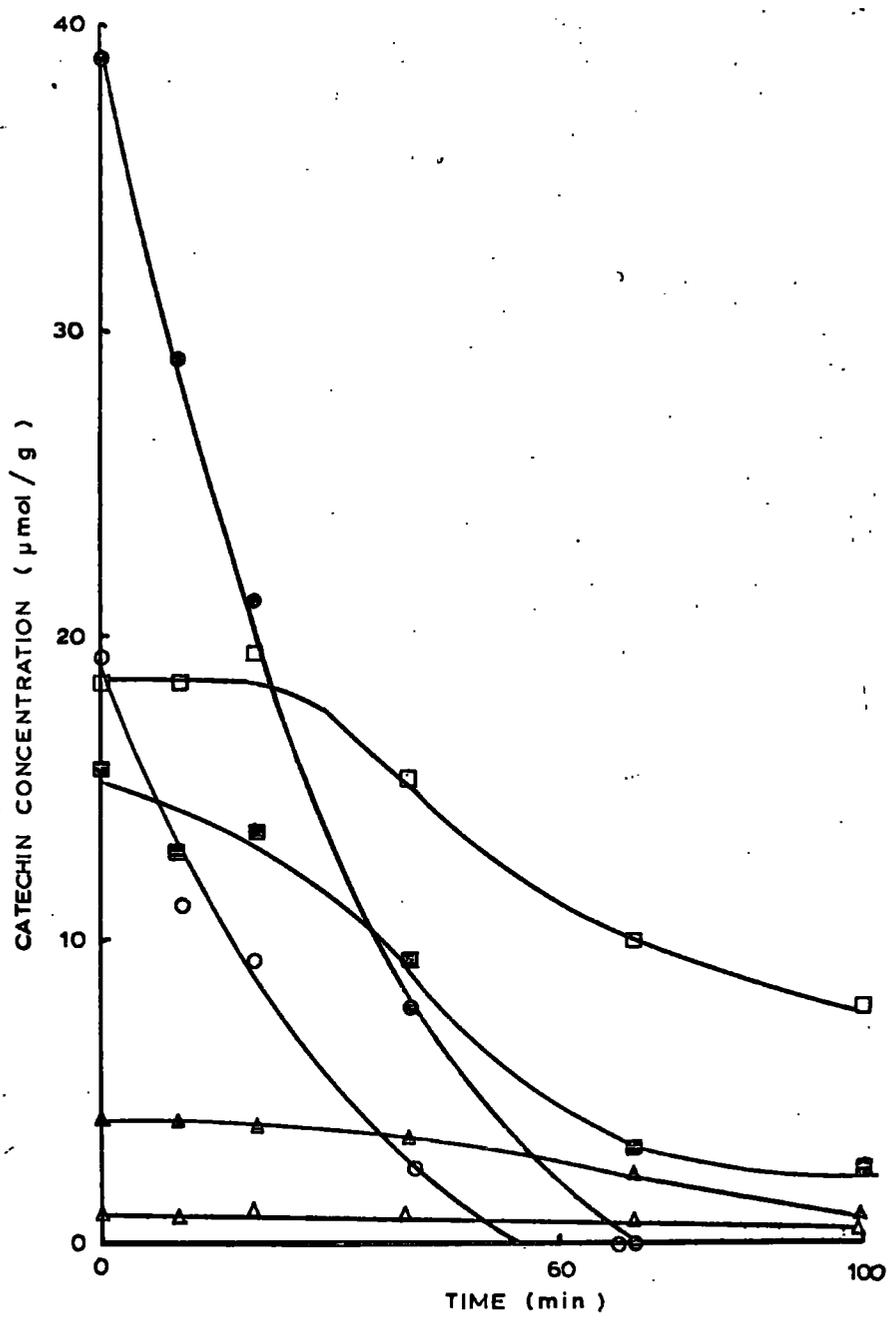
FIGURE 37

Enzymic oxidation of a mixture of six catechins:

(iii) control mixture + EC

O——O EGC   □——□ EC   △——△ C
●——● EGCG   ▣——▣ ECG   ▲——▲ GC

SUBSTRATE	PRODUCTS	
	TF	TR
51.3 mg Control mix	3.50 mg	3.05
50.2 mg Control mix + 11.8 mg EGC	5.74 mg	
50.3 mg Control mix + 4.0 mg EC	3.31 mg	
50.6 mg Control mix + 21.0 mg EGCG	2.05 mg	4.45

TABLE 3

ENZYMIC OXIDATION OF A MIXTURE OF SIX CATECHINS:
EFFECT OF CATECHIN COMPOSITION UPON QUANTITIES OF
TF AND TR PRODUCED.

Theaflavins measured as area under component 8 in
LH-20 Sephadex chromatography, calculated as mg TFG;
thearubigin complex estimated as height of component
4+5 peak in LH-20 Sephadex chromatography, expressed
in arbitrary units.
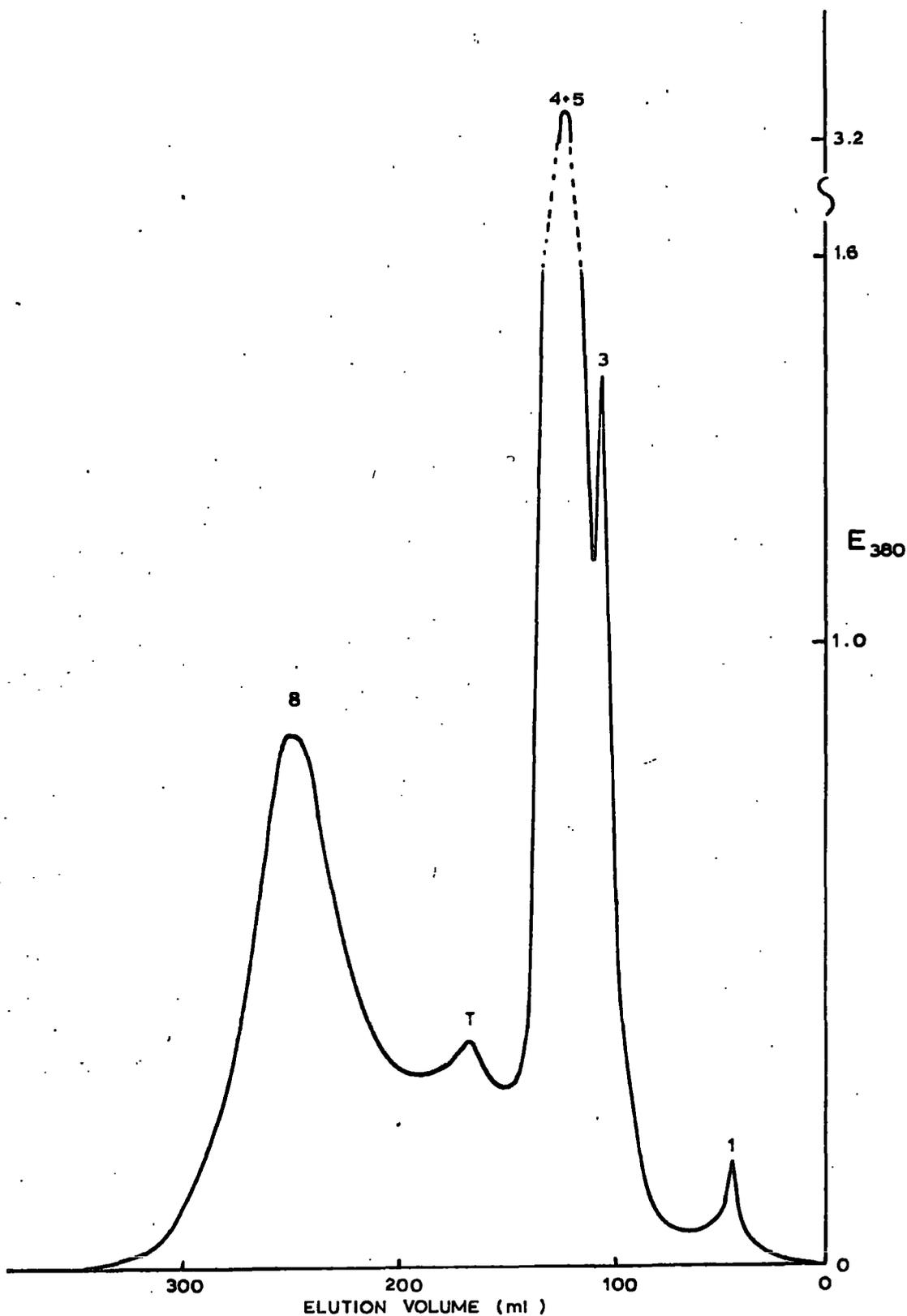
FIGURE 38

Oxidation of a mixture of six catechins: chromatography of products on an LH-20 Sephadex column.

For details of chromatography see Methods chapter.
For numbering of components see Figure 39.
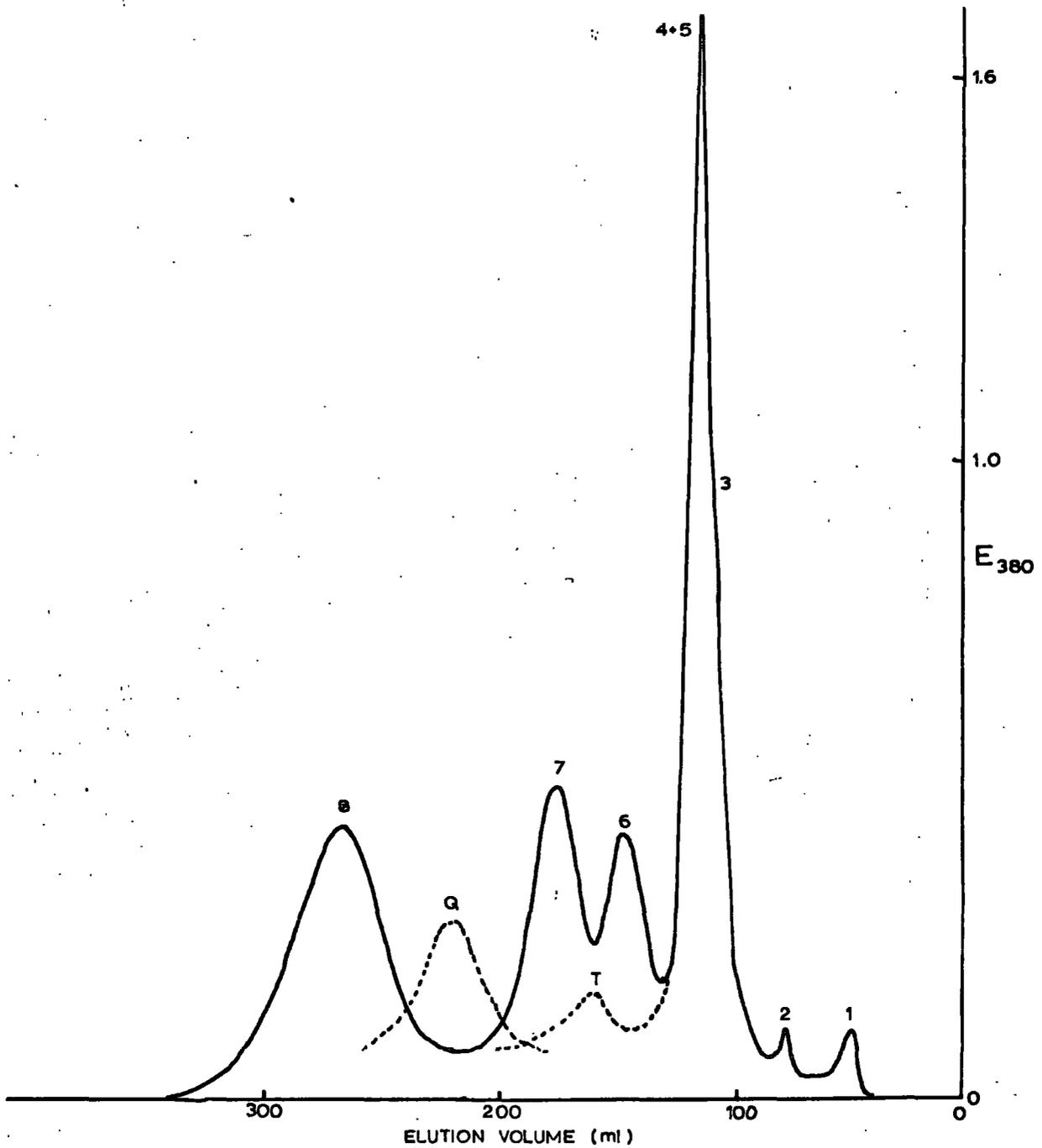
FIGURE 39

Chromatography of a hot aqueous infusion of black tea
on an LH-2O Sephadex column.

For details of chromatography see Methods chapter.
Positions of components Q and T were determined from
elution profiles of products of enzymic oxidation of
mixtures of purified catechins. For identity of
components see text.

in Figure 18 were developed. A definate separation

of TF^1 from TFG was achieved, with partial separation

of the latter into positions TFG_1 and TFG_2.

7. DIFFERENTIATION OF THEAFLAVIN GALLATE COMPONENTS

In view of the considerable variation in R_F

values of the theaflavin gallates produced during

oxidation of catechin pairs, the following experiment

was designed to ascertain whether the product of EGCG +

EC oxidation, TFG_1, was a chromatographically distinct

species from TFG_2, produced as a result of EGC + ECG

oxidation.

During consecutive incubations of EGC + ECG and

EGCG + EC, three dimensional thin-layer plates were

loaded. After elution, the R_F value of the TF^G spot was

plotted against the quantity of TFG loaded (Figure 4?).

It was evident that although loading increased the R_F

value of both TFG components, the degree of variation

differed considerably, and two distinct series were

represented.

8. IDENTITY OF COMPONENTS FROM LH-20 SEPHADEX
CHROMATOGRAPHY

Figure 39 shows the elution profile of a freeze-

dried aqueous infusion of black tea of clone MT 12, after

chromatography on LH-20 Sephadex. The components

discernable in the black tea extracts tested and numbered

1-8, although in this particular tea component 3 is only

just in evidence as a slight shoulder, and components 4
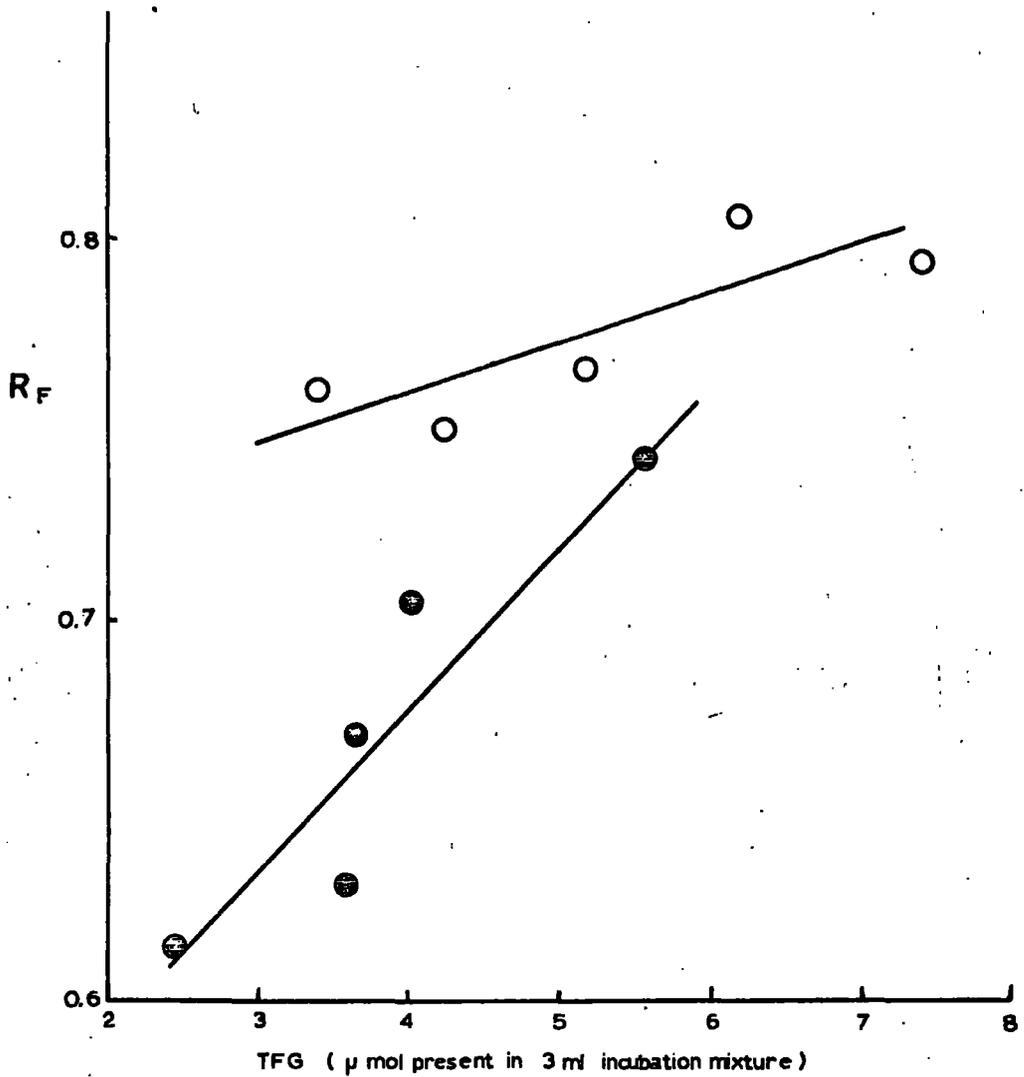
FIGURE 40

Relationship between amount of TFG loaded
and R_F value in t.l.c.

●——● TFG_1 produced from EGCG + EC

○——○ TFG_2 produced from EGC + ECG

and 5 have not separated. Only in very occasional
cases was this peak differentiated into two components.
Components T and Q were characteristic of in vitro
incubations of pure catechins, and were not identified
in black tea infusions, though a connection between
T and component 6 cannot be ruled out.

To elucidate the identity of the various components,
each one was collected in a fraction collector, condensed
under vacuum, and chromatographed on thin-layer cellulose,
under the conditions of Figure 19 . Component 1 produced
a streak at the origin having zero R_F in water, and
appeared to be identical to the streak S_{II}. Components
2-7 were each heterogeneous, spots S_I, R, and others
having zero R_F in water being visible in all of them.
However, spots X and Y seemed exclusively associated with
the 3 + 4 + 5 component. Of the colourless spots identified
on the chromatograms, GA appeared to be associated with
component 3, and the six catechins were eluted with and
after component 7.

Component 8 was soluble in ethyl acetate and produced
spots for TF^1 and TFG, along with a faint streak having
zero R_F in water. The absorption spectrum had maxima
at approximately 380 and 460 nm (cf Figure 26). Mixtures
of TF^1 and TFG produced by in vitro incubation were run
together on the column and were eluted as one symmetrical
component in the position of component 8. At no time was
any heterogeneity of component 8 detected.

Component Q, produced either on its own as a
result of oxidation of ECG, or in combination with
component 8 as a result of oxidation of EGCG + EC,
was soluble in ethyl acetate, and resulted in spot
Q (Figure 18). The absorption spectrum had pronounced
maxima at 275 and 395 nm, and a shoulder at approximately
475 nm.

9. ENVIRONMENTAL INFLUENCES ON POLYPHENOL OXIDASE ACTIVITY

The remaining experiments were conducted in Malawi
to investigate changes in polyphenol oxidase activity
and its catechin substrates in the living bush, and to
attempt to relate these on the one hand to environmental
changes, and on the other to ultimate changes in quality
as assessed by the TF x TC parameter described above.
The first series of experiments deals with changes in
polyphenol oxidase activity.

(i) Seasonal variation in activity

Four clones were selected for investigation :
MT 12, PC1, SFS 204, and SFS 371. Very large week to
week variation was observed, but in general activities
during the cold season months were significantly higher
than those in the hot dry season (Table 4).

The weekly variation in bushes of very different
leaf form (MT 12 and SFS 204) shows some similarity
(Figure 41). It seemed reasonable that the reduction
in activity during the hot dry season may be due to damage
by overheating. Hence Figure 41 includes the total

SUMMER			WINTER		
DATE	PC 1	SFS 371	DATE	PC 1	SFS 371
AUG 20	12.5	14.7	APR 22	19.4	20.8
27	10.9	16.6	29	11.2	20.4
SEP 3	9.8	19.0	MAY 6	13.2	16.5
10	10.5	19.4	13	19.3	20.4
17	11.6	16.8	20	20.6	30.8
24	12.3	18.4	27	14.8	15.6
OCT 1	10.6	15.3	JUN 3	21.5	19.3
8	14.7	15.7	10	15.5	19.0
15	14.3	14.5	17	21.3	19.2
22	13.5	14.7	24	19.5	18.4
29	14.9	14.5	JUL 1	17.9	21.5
MEAN	12.3	16.3	MEAN	17.7	20.2

TABLE 4

SEASONAL VARIATION IN POLYPHENOL OXIDASE ACTIVITY

Comparison of winter and summer activities (U/g fresh wt.)
as estimated colorimetrically, expressed as mean activities
over $2\frac{1}{2}$ months of summer 1968 and $2\frac{1}{2}$ months of winter
1969, for two China hybrid clones: PC 1 and SFS 371. A
Student's 't' test shows the difference between summer
and winter means for each clone to be highly significant
($P < 0.01$).
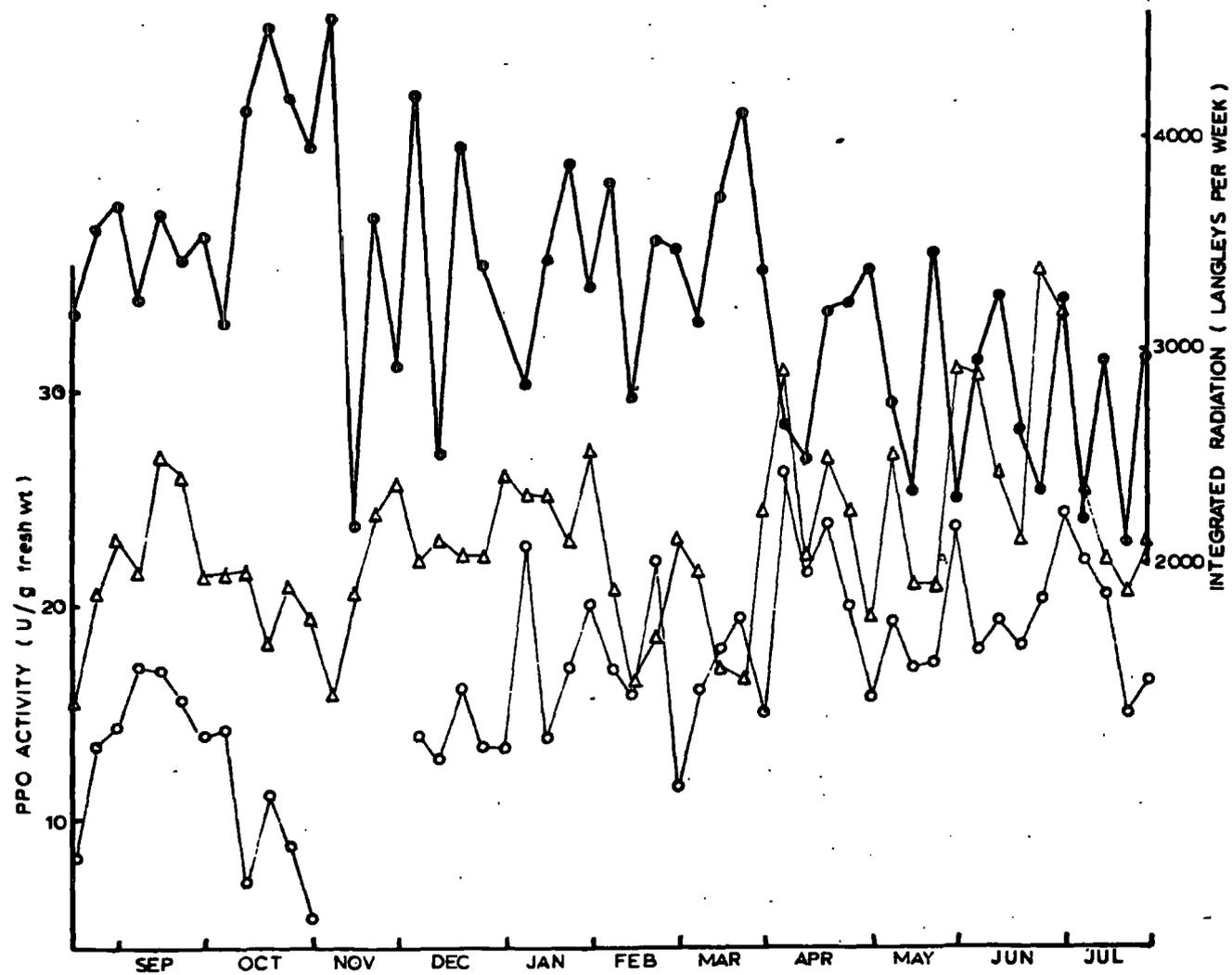
FIGURE 41

Seasonal variation in PPO activity and solar radiation

PPO ACTIVITIES: △—△ SFS 204, O—O MT 12 ; ●—● RADIATION

integrated radiation for each week prior to the day
on which the enzyme assay was made. Some negative
correlation between this figure and the enzyme
activities for the two clones is indicated, but the
regression coefficient of enzyme activity upon
radiation using these figures is not significant at
the P < 0.05 level.

(ii) Effect of shading bushes

To investigate further the radiation effect
suggested in the last experiment, bushes from clones
MT 12 and SFS 204 were shaded (see methods chapter).
Samples from each were assayed for polyphenol oxidase
activity every fortnight, and compared with control
bushes.

During the weeks preceeding the errection of the
shade, no significant difference between sample means
of experimental and control bushes was observed.
Immediately the shade was introduced, there was a marked
increase in the level of polyphenol oxidase activity in
the experimental bushes (Tables 5 and 6). This
difference persisted, although on one occasion the shade
was stolen from the experimental bush of clone MT 12,
and the bush was exposed to direct sunlight for one day
before the shade was replaced. This occurred four days
prior to the next date for assay (March 31) and was
reflected in a drastic reduction in activity of the

| DATE | PPO ACTIVITY | | | AIR TEMP. | | RADI-ATION Cal/cm^2 |
	SHADED	UNSHADED	% DIFFERENCE	HOURS 70–85°F	HOURS >85°F	
JAN 13	18.3	17.7	N.S.			
27	18.7	19.5	N.S.			
FEB 3	22.8	22.1	N.S.			
17	24.2	23.7	N.S.	1124	11	7400
MAR 2	34.4	25.2	36.5	1234	23	7590
16	33.4	26.4	26.5	763	5	5660
31	26.3	24.7	6.5	680	1	6440
APR 13	38.5	30.5	26.2	774	0	5500
27	34.4	27.3	27.4	261	0	5890

TABLE 5

EFFECT OF SHADING BUSHES ON POLYPHENOL OXIDASE ACTIVITY
CLONE MT 12

N.S. = no significant difference at $P < 0.05$.
Experimental bush shaded at 25% transmission on 19 Feb 1970.
Shade stolen from experimental bush, and therefore bush
exposed to full sunlight on 27 Mar for one day. Polyphenol
oxidase activity assayed colorimetrically and expressed in
U/g fresh wt. Difference in activity expressed as percentage
of unshaded control.
(Differences analysed by Student's 't' test.)

| DATE | PPO ACTIVITY | | | AIR TEMP. | | RADI-ATION Cal/cm^2 |
	SHADED	UNSHADED	% DIFFERENCE	HOURS 70-85°F	HOURS 85°F	
JAN 12	25.0	26.0	N.S.			
26	20.5	23.0	N.S.			
FEB 9	40.3	41.9	N.S.			
23	42.2	34.6	21.4	1042	8	7520
MAR 9	42.8	32.1	30.0	1218	26	7160
23	44.8	34.7	29.1	639	1	5490
APR 6	47.3	38.5	23.5	672	0	6270
20	40.0	37.7	6.0*	569	0	5690

TABLE 6

EFFECT OF SHADING BUSHES ON POLYPHENOL OXIDASE ACTIVITY
CLONE SFS 204

Experimental bushes shaded at 40% transmission on
19 Feb 1970. Other details as for Table 5 .

*A power failure during assay of shaded sample
resulted in unreliable readings on this date.

experimental bush when compared to other weeks.

Tables 5 and 6 also include three factors which may be influencing leaf temperature, integrated over the two weeks prior to the date of assay. The greatest difference in activity between shaded and unshaded treatments was observed in both clones following the high radiation and air temperature figures at the beginning of March.

(iii) Effect of nitrogen application

A series of experiments was conducted upon the clonal populations under different nitrogen fertilizer applications in Field 9, Swazi Research Station (see methods chapter), to investigate the effect of different nitrogen levels upon polyphenol oxidase activity. Samples from the different plots were analysed for polyphenol oxidase activity and for leaf nitrogen. A typical example of the results is presented in table 7. Analysis of variance indicates that there is a highly significant (P $<$ 0.01) added variance component due to the nitrogen treatments. Further analysis of these results by the Student-Newman-Keuls procedure (Sokal & Rohlf, 1969) suggests that at P$<$0.05 significance the sample means are distributed as follows:

$$E_o \; < \; E_1 = E_2 < E_3$$

where E_o ...... E_3 represent the mean enzyme activities for the N_o ...... N_3 nitrogen treatments respectively.

Other replications of the experiment, using the same

REPLICATION	NITROGEN APPLICATION				
	N_0	N_1	N_2	N_3	
1	18.0	16.6	22.4	16.3	
2	18.0	16.9	19.1	27.6	
3	17.3	16 9	22.4	26.3	
4	18.0	21.9	27.2	28.8	PPO
5	17.7	24.7	23.4	21.3	activity
6	17.0	28.2	17.5	26.0	
Σ	106.0	132.8	126.9	157.6	
MEAN	17.7	22.1	21.2	26.3	
LEAF N LEVELS	4.72	4.79	4.76	4.92	% dry wt.

ANALYSIS OF VARIANCE - anova table.

Source of Variation	corrected Sum of Squares	degrees of freedom	Variance Estimate	F ratio
Among Treatments	225.4	3	75.1	6.68
Within Treatments	218.3	20	10.9	
Total	443.7	23		

$$F_{0.01 \ 3,20} = 4.94$$

Hence treatment effect is significant at $P < 0.01$

TABLE 7

VARIATION OF POLYPHENOL OXIDASE ACTIVITY WITH
NITROGEN APPLICATION

Enzyme activity expressed in U/g fresh wt.
Four N treatments ($N_0 \ldots N_3$) were respectively
120, 180, 240, and 300 lb N per acre per annum,
applied as ammonium sulphate.

and different clones in the same field all exhibited a

significant added variance component due to the nitrogen

treatments. However, the only consistent factor in

the pattern of distribution of the sample means was that

the enzyme activity for the N_1 treatment was higher

than for the N_0 treatment. At higher levels the effect

was not clear.

10. INFLUENCE OF ENVIRONMENT ON CATECHIN COMPOSITION

(i) Seasonal variation of individual catechins

Attempts to measure the catechin composition of a clone

throughout a complete year failed due to exhaustion of

solvent supplies needed for chromatography. Results

presented here are a result of fortnightly analyses upon

the same bushes of four clones measured between February

and September 1969. This period included the hot rainy

season, the cold season, and the beginning of the hot dry

season.

Figure 42   presents the variation in catechin

composition over this period for clone PC.1., and the

pattern of distribution was typical of the four clones

studied, although in absolute terms PC.1. is abnormally

high in EGC and EC content. A big increase in total catechins

was observed during the cold season months of May, June

and July. During this period there was an increase in

EGC and EC, and a decrease in EGCG and ECG levels.

An attempt was made to correlate the changes in

catechin composition with the rate of growth of the bush,

as measured by the weekly plucking yield of the bushes
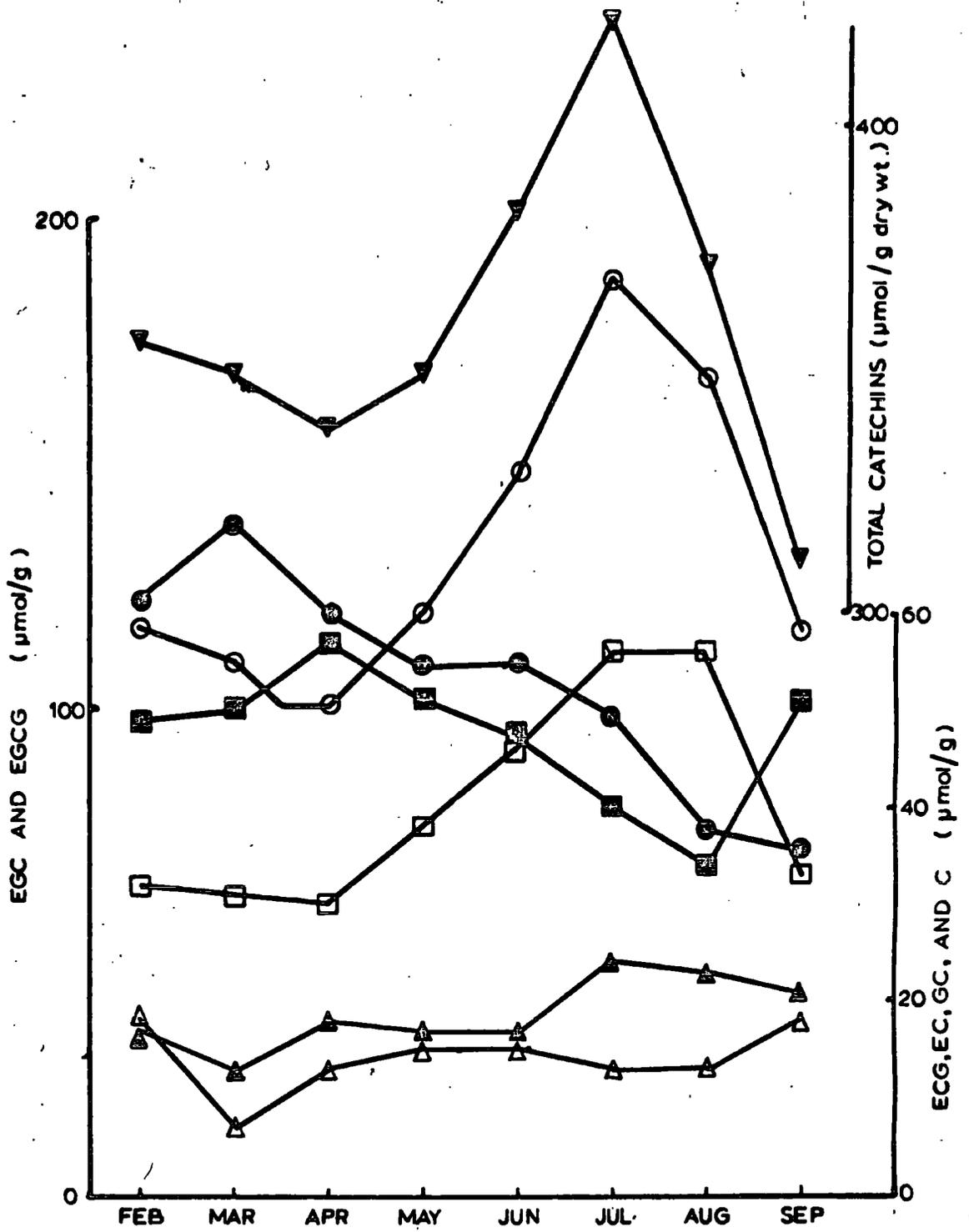
FIGURE 42

Seasonal variation in catechin composition.

O—O EGC   □—□ EC   ▼—▼ TOTAL
●—● EGCG   ▣—▣ ECG
△—△ GC   △—△ C

Monthly means of   fortnightly determinations.

under study. However, plucking yields tend to be so influenced by factors such as time of plucking that the ultimate pattern of yield in no way expresses the week to week variation in growth rate (Figure 43).

In view of the apparent inverse correlation between EGC and EGCG, and EC and ECG suggested in Figure an attempt was made to investigate the significance of such a relationship. Using the figures obtained from each of the four clones under study, however, no regression coefficient was found to be significant at $P < 0.05$. In order to eliminate the total catechin variable, and therefore investigate the EGC-EGCG and EC-ECG relationship further, each catechin was expressed as a percentage of the total (Table 8).

Statistical analysis involved calculation of correlation coefficients for the following quantities:-

Items Compared	Correlation Coefficient	Significance	
EGC with EGCG	- 0.861	P	0.001
EGC with (ECG + GC + EC + C)	-	N.S.	
EGCG with (ECG + GC + EC + C)	-	N.S.	
EC with ECG	- 0.653	P	0.01
EC with (EGC + EGCG + GC + C)	-	N.S.	
ECG with (EGC + EGCG + GC + C)	-	N.S.	

Thus for this clone (PC 1) there was a seasonal variation in total catechin content, and a concurrent change in proportion of the various catechins. The simple
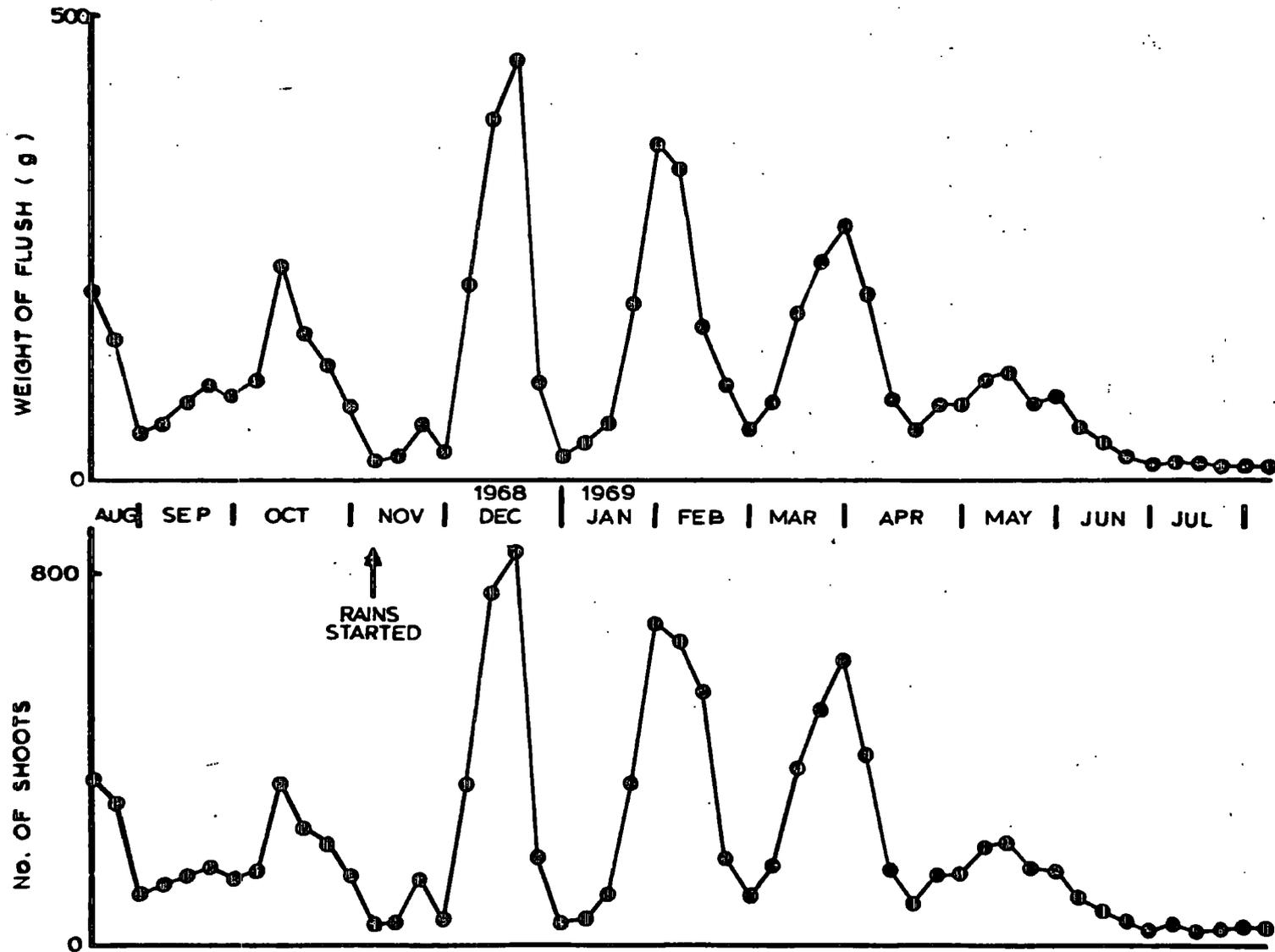
FIGURE 43

Weight and number of shoots in flush plucked each week from six bushes
of clone SFS 371.

	PERCENTAGE OF TOTAL CATECHINS					
DATE	EGC	EGCG	ECG	GC	EC	C
FEB 11	32.4	31.0	15.2	3.9	11.0	6.5
25	33.1	38.0	12.6	5.3	7.2	3.7
MAR 11	32.8	38.4	13.0	4.5	9.0	2.3
25	30.3	40.8	15.4	2.9	8.7	1.7
APR 9	29.1	36.9	16.8	6.0	6.0	3.1
24	30.9	33.7	17.3	4.3	9.6	4.3
MAY 6	37.6	30.5	12.0	4.6	10.9	4.4
20	31.0	31.8	17.6	4.8	10.9	3.9
JUN 3	36.6	30.7	10.8	5.3	13.0	3.6
17	41.2	27.2	13.4	3.5	10.9	4.0
JUL 1	45.3	26.1	8.6	6.5	11.8	3.5
15	48.4	20.5	10.0	4.8	13.1	2.2
29	41.0	23.1	11.1	6.4	15.6	3.9
AUG 12	52.1	18.0	7.7	4.6	14.9	2.6
26	38.0	24.0	10.5	7.6	15.0	4.6
SEP 9	37.7	22.8	16.4	6.7	10.6	5.8

TABLE 8

CATECHIN CONTENT OF SHOOTS OF CLONE PC 1
EXPRESSED AS A PERCENTAGE OF TOTAL CATECHINS.

Data as for figure 42 , and refers to 1969
analyses. All quantities were calculated in
μmol before conversion to percentages.

catechins EGC and EC were at a peak during the cold
months, there being a constant inverse relationship
between simple catechins and the corresponding gallate.

(ii) Effect of shading on catechin composition

The same bushes of clones MT 12 and SFS 204 that
were shaded to investigate the effect of shade upon
polyphenol oxidase activity (Page 59), were also
analysed for catechin content every fortnight. The
results are presented in Table 9 . Shade effectively
lowered the EGC content and raised the EGCG content of
the shoot, and there was a strong indication that the
GC content was also lowered, especially in MT 12.

(iii) Effects of fertilizer application upon catechin
        composition

This series of experiments was designed to investigate
the effects of different levels of N,P, and K fertilizers
on the catechin content of shoots. Established fertilizer
field trials were used (planting and fertilizer histories of
each field are recorded in the materials section).

(a) Effect of Nitrogen

Initial experiments were concerned with nitrogen
differences only, and were performed on clonal material in
Field 9, Swazi Research Station. Results are presented in
table 10 . The trend is for there to be an increase in
EGCG content with increasing nitrogen, and a decrease in
EGC content. For SFS 204 these differences between the
first three nitrogen levels are more than twice the

	DATE	EGC		EGCG		ECG		GC		EC		C		Σ	
		S	C	S	C	S	C	S	C	S	C	S	C	S	C
CLONE SFS 204	FEB 9	61	61	131	128	55	46	16	16	23	21	5	7	291	279
	23	54	62	137	135	47	51	8	11	23	17	7	15	276	291
	MAR 9	50	76	157	124	53	48	8	19	24	23	12	9	304	299
	23	93	105	146	125	48	32	11	14	21	11	6	11	325	298
	APR 6	61	68	135	116	39	46	19	16	20	19	3	8	277	273
	20	72	74	151	142	43	53	8	8	17	15	1	2	292	294
	MAY 4	44	45	124	108	57	49	12	11	17	20	9	8	263	241
CLONE MT 12	MAR 3	91	116	135	111	37	29	6	15	12	20	3	3	284	294
	16	97	97	128	113	28	28	10	13	11	24	2	5	276	280
	* 31	69	65	137	186	36	28	9	17	11	22	4	5	266	323
	APR 13	96	118	150	103	30	24	10	14	14	21	2	5	292	285
	27	77	112	142	104	28	25	3	11	6	21	3	2	259	275
	MAY 4	105	125	127	105	21	44	2	16	5	23	1	2	261	315

Table 9

Effect of Shading on Catechin Composition

S = Shaded Sample   C = unshaded control

* shade stolen during week prior to this reading.

Readings on Feb. 9 were made before shade was errected.

CLONE	TREAT-MENT	N lbs/acre	CATECHINS μmol/g dry wt.						
			EGC	EGCG	ECG	GC	EC	C	Σ
SFS 204	N_0	120	85	118	51	19	28	9	311
	N_1	180	77	128	54	17	28	9	313
	N_2	240	66	132	53	17	25	7	300
	N_3	300	70	131	54	16	27	9	307
MT 12	N_0	120	159	145	39	22	23	5	393
	N_1	180	154	148	40	21	22	9	394
	N_2	240	126	148	45	17	21	6	358
	N_3	300	148	138	37	20	23	8	374

TABLE 10

EFFECT OF NITROGEN APPLICATION ON THE CATECHIN
COMPOSITION. OF TWO CLONAL POPULATIONS.

Nitrogen applied as ammonium sulphate. SFS 204 analysed
on 14 Jan 1970, and MT12 on 4 Feb 1970. Results for
SFS 204 are each a mean of two determinations, and
those for MT 12 means of four determinations.

standard error of the method of analysis (see methods
section), and can be considered significant; the fourth
nitrogen level is not significantly different from the
third, and there are no significant differences in the
other catechin levels, including the sum total.
Differences may thus be summarized:

EGC levels : $No > N_1 > N_2 = N_3$

EGCG levels : $No < N_1 < N_2 = N_3$

all other levels : $No = N_1 = N_2 = N_3$

Although these same trends were discernable in the
MT 12 samples, statistical treatment of the four
replications produced no significant differences at the
$P < .05$ level.

Foliar analysis showed nitrogen levels in the
shoots of the SFS 204 samples to be

No 4.45%

N_1 4.75

N_2 4.73

N_3 4.55

Yields for both SFS 204 and MT 12 showed the N_1 plots
to be yielding significantly more than the No plots, but
yields were irregular at higher N levels.

The experiment was repeated using a genetically
mixed population of tea in Field 3, Mimosa Research Station,
which had been under the same four different nitrogen
applications for 14 years, and yields were responding well
to nitrogen. The experiment was conducted at two levels

| NITROGEN TREATMENT | REPLIC^N | CATECHINS μM/g dry wt. | | | | | | |
		EGC	EGCG	ECG	GC	EC	C	Σ
No	A	90	139	56	25	24	10	344
40 lb/acre	B	102	142	47	14	26	14	350
per year	C	92	141	37	21	21	8	320
	D	103	141	42	19	20	9	334
	MEAN	96.7	141	46	21	23	10	337
N_1	A	99	146	47	18	23	15	348
80 lb/acre	B	87	142	52	19	22	10	329
per year	C	89	164	46	18	23	11	349
	D	84	169	50	17	26	8	359
	MEAN	89.7	158	49	18	24	11	350
N_2	A	74	178	41	13	20	6	332
160 lb/acre	B	67	173	42	8	15	7	312
per year	C	71	158	41	10	14	4	298
	D	76	167	40	11	16	3	313
	MEAN	72.0	164	41	11	16	5	309
N_3	A	71	160	45	13	15	2	306
240 lb/acre	B	91	176	51	15	28	11	372
per year	C	75	157 .	42	9	13	3	299
	D	60	157	33	6	11	7	274
	MEAN	74.3	163	43	11	17	6	314

TABLE 11

Effect of Nitrogen Application on Catechin Composition

For details see text. (Field Sampled 28 Apr 1970)
Four samplings from one replication block of four treatments

NITROGEN TREATMENT	REPLIC[N] BLOCKS	CATECHINS $\mu m/g$ dry wt.							YIELD	FOLIAR NITROGEN %
		EGC	EGCG	ECG	GC	EC	C	Σ		
No	I	72	112	46	4	14	1	249	494	4.00
	II	54	106	33	15	15	3	226	537	4.16
	III	77	114	39	8	21	3	262	593	4.13
	IV	68	122	43	12	18	2	265	555	4.17
	MEAN	67.8	114	40	10	17	2	251	545	4.12
N_1	I	54	118	38	4	12	4	231	726	4.31
	II	55	132	37	6	12	2	247	747	4.34
	III	53	130	46	17	18	5	269	700	4.44
	IV	54	132	41	11	14	7	259	789	4.87
	MEAN	54.0	128	40	10	14	4	250	740	4.49
N_2	I	44	124	41	6	10	0	225	814	4.86
	II	46	112	40	11	15	4	228	748	4.97
	III	49	118	41	7	11	8	234	849	4.83
	IV	56	141	46	10	15	6	274	856	4.90
	MEAN	28.8	124	41	8	13	4	239	817	4.89
N_3	I	33	126	40	13	12	2	212	593	4.92
	II	49	119	39	9	15	3	234	841	4.96
	III	56	132	45	8	13	3	257	771	4.93
	IV	53	130	36	8	15	3	245	814	4.96
	MEAN	47.8	127	40	10	14	3	242	755	4.94

<u>TABLE 12</u>

Effect of Nitrogen Application on Catechin Composition

Nitrogen application as in Table 11. Yield expressed in total pounds of green leaf for each plot to date this season. For further details see text. (Field sampled 28 April 1970).
One sampling from each of four replication blocks of four treatments.

of replication: first,four samples were selected at
random from each of the four treatment plots in one
block, and catechin analysis performed on the fresh
leaf; then samples were taken from each of the four
treatment plots in each of four replication blocks,
the leaf dried, each plot homogenized together, and
one analysis performed per plot. Thus in each case
sixteen analyses were performed. The results are
presented in tables 11and12. Also included in table 12
are the yields of the respective plots and the foliar
nitrogen values as measured by the Kjeldahl technique.
An analysis of variance was performed on each factor
which appeared to be affected by the different
nitrogen treatment, and the means compared by an a
posteriori test - the Student-Newman-Keuls test
(Sokal & Rohlf,1969). An example of this analysis,
using the foliar nitrogen levels in table 12 follows:

<u>Effect of Nitrogen application on foliar nitrogen
levels</u> (Data from Table 12)

Nitrogen treatment:	N_0	N_1	N_2	N_3	
Replication I	4.00	4.31	4.86	4.92	% N dry wt.
II	4.16	4.34	4.97	4.96	
III	4.13	4.44	4.83	4.93	
IV	4.17	4.87	4.90	4.96	
Treatment total (ΣnY)	16.46	17.96	19.56	19.77	
Mean $\overline{Y}$	4.12	4.49	4.89	4.94	

If there are a treatments, each comprised of n
readings of Y % N

Grand total $\sum_a \sum_n Y = 73.75$

Correction term $CT = \frac{1}{an} (\sum_a \sum_n Y)^2 = 339.9$

Corrected Sum of Squares (total) $= SS_T = \sum_a \sum_n Y^2 = 2.026$

Corrected Sum of Squares between sample means

$$= SS_b = \frac{1}{n} \sum_a (\sum_n Y)^2 - CT = 1.794$$

Analysis of variance - Anova table

Source of Variation	df	SS	MS	F
Between samples	3	1.794	0.598	30.8
Within samples	12	0.232	0.0194	
Total	15	2.026		

(where df is degrees of freedom, MS is the mean squares

variance estimate, and F the variance ratio $\frac{MS\ between}{MS\ within}$)

$F_{0.001(3,12)} = 10.8$ from tables, < 30.8 .

Hence there is a highly significant ($P \lll 0.001$)

added component due to treatment effects in the mean

square among treatments, i.e. the different nitrogen

treatments have a significant effect upon the foliar

nitrogen levels of the young shoot.

Comparison among means : Student-Newman-Keuls test

Standard error of sample mean

$$S\bar{Y} = \sqrt{\frac{MS\ within}{n}} = \sqrt{\frac{.0194}{4}} = .0696 \text{ for df} = 12$$

Least significant range (LSR) for K means of standard

error SY and v degrees of freedom is given by

$$LSR \text{ (for k samples)} = Qx(k, v) \times S\bar{Y}$$

Where $Q_x(k,v)$ is the tabular value of the
studentized range for k means, v degrees of freedom, and
at $P < 0.05$ level of significance.

Hence:

k =	2	3	4
Q =	3.08	3.77	4.20
LSR =	0.21	0.26	0.29

Comparing means Y above:

N_3-N_0 = 4.94 - 4.12 = 0.82 $>$ LSR (k=4)   Significant at P 0.05

N_2-N_0 = 4.89 - 4.12 = 0.77 $>$ LSR (k=3)   "   "   "

N_3-N_1 = 4.94 - 4.49 = 0.55 $>$ LSR (k=3)   "   "   "

N_1-N_0 = 4.49 - 4.12 = 0.37 $>$ LSR (k=2)   "   "   "

N_2-N_1 = 4.89 - 4.49 = 0.40 $>$ LSR (k=2)   "   "   "

N_3-N_2 = 4.94 - 4.89 = 0.15 $<$ LSR (k=2) Not Significant at P 0.05

This result may be summarised:

With respect to foliar nitrogen levels $N_0 < N_1 < N_2 = N_3$

or   Nitrogen application N_0 N_1 N_2 N_3

Foliar nitrogen levels (%N)   4.12   4.49   <u>4.89   4.94</u>

Where those means underlined are not significantly different

(Sokal & Rinkel, 1963).

Treatment of the contents of tables 11 and 12 in this
way indicated that the different nitrogen treatments had a
significant effect upon foliar nitrogen levels in the shoot,
the yield, and content of EGC and EGCG. GC levels are
also significantly affected in table 11 , though the
differences in table 12 , while showing the same trend,

are too small to be significant. The Student-Newman-Keuls
test showed the relation between means to be as follows,
using the underlining convention to indicate that means
are not significantly different at the P 0.05 level.

EFFECT	N_0	N_1	N_2	N_3
Foliar N %	4.12	4.49	4.89	4.94
Yield (lbs leaf)	545	740	817	755
EGC (Table 11)	96.7	89.7	72.0	74.3
EGC (Table 12)	67.8	54.0	48.8	47.8
EGCG (Table 11)	141	155	169	163
EGCG (Table 12)	114	128	124	127
GC (Table 11)	21	18	10.5	10.8

Reducing the level of significance to $P < 0.1$, all
differences between N_0, N_1, and N_2 become significant
except in the case of EGCG (Table 12).

(b)   Effect of Nitrogen, Phosphate, and Potassium in
       combination

An experiment was conducted upon a clonal population
of tea planted in a factorial 3^3 fertilizer trial at
Two Rivers Research Station. The population had only
been planted two years previous to sampling, and yields
were not responding to fertilizer treatments. A
significant effect of nitrogen upon EGC and EGCG levels was found,
but the effects of phosphate and potassium were not
significant.

The experiment was repeated upon a genetically mixed

TREATMENT		CATECHINS μM/g dry weight							FOLIAR ANALYSIS		
PLOT	NPK	EGC	EGCG	ECG	GC	EC	C	Σ	N%	P%	K%
1	201	68	135	41	19	20	9	292	4.62	0.35	1.48
2	220	69	132	47	27	30	15	320	4.57	0.31	1.18
3	102	75	117	45	21	27	10	295	4.27	0.42	1.24
4	022	81	128	45	23	33	11	321	3.92	0.30	1.34
5	110	77	115	39	19	26	13	289	4.36	0.47	1.24
6	000	63	106	46	21	25	9	270	3.98	0.31	1.28
7	212	67	115	41	16	23	6	268	4.02	0.41	1.34
8	011	62	116	36	9	18	7	248	4.24	0.35	1.48
9	121	70	122	34	8	19	6	259	4.14	0.28	1.28
10	122	63	100	28	8	13	3	215	4.07	0.32	1.42
11	202	45	108	26	5	12	2	198	4.61	0.35	1.24
12	012	54	99	27	2	9	1	192	4.06	0.31	1.24
13	100	61	100	25	6	11	1	204	4.28	0.40	1.42
14	111	63	100	26	5	12	1	206	4.28	0.41	1.24
15	221	69	87	35	14	21	7	233	4.68	0.37	1.38
16	020	75	141	58	16	26	8	324	4.03	0.34	1.38
17	210	62	117	40	13	18	5	255	4.83	0.40	1.26
18	001	70	137	38	10	19	5	279	4.06	0.39	1.62
19	222	71	131	36	11	20	5	274	4.67	0.39	1.42
20	211	49	121	33	9	14	5	231	4.93	0.32	1.50
21	021	66	112	40	10	17	2	247	4.20	0.39	1.56
22	101	69	137	35	11	15	1	268	4.26	0.37	1.38
23	200	57	110	28	11	15	5	226	4.51	0.35	1.22
24	112	61	130	43	13	17	6	270	4.27	0.38	1.50
25	002	64	134	33	9	18	4	262	4.42	0.37	1.62
26	010	64	120	40	14	25	1	264	4.17	0.36	1.50
27	120	83	102	37	3	20	2	257	4.21	0.44	1.42

TABLE 13

Effect of Nitrogen, Phosphate and Potassium upon Catechin Composition
Field sampled on 6.5.70 For analysis of results see text.

population of mature tea in Field 13 Swazi Research
Station, which had been under the same factorial
fertilizer treatments for ten years. Each of the 27
plots was sampled (one shoot from each bush, dried,
and shoots from each plot homogenized) and analysed
for catechins, foliar nitrogen, phosphate, and
potassium. Results are presented in Table 13 . The
fertilizer levels for each treatment are given in the
materials section.

It was thought advisable to check the way in which
the plant was responding to the various fertilizer
applications in terms of foliar levels of nitrogen,
phosphate, and potassium. The statistical analysis for
each foliar mineral analysis was the same as for each
catechin analysis and the following treatment of
potassium levels is given as an example. The figures
in this table were obtained a month prior to those
presented in table 13 .

Plotting data from 8th April 1970 (K% dry weight):

	N_0			N_1			N_2		
	P_0	P_1	P_2	P_0	P_1	P_2	P_0	P_1	P_2
K_0	1.96	1.74	1.68	1.70	1.58	1.56	1.44	1.38	1.58
K_1	1.68	1.82	1.88	1.68	1.76	1.90	1.64	1.62	1.82
K_2	1.82	2.06	2.14	1.90	1.82	1.96	1.96	1.82	1.74

Summing over K, P, and N respectively:-

	N_0	N_1	N_2		N_0	N_1	N_2		P_0	P_1	P_2
P_0	5.46	5.28	5.04	K_0	5.38	4.84	4.40	K_0	5.10	4.70	4.82
P_1	5.62	5.16	4.82	K_1	5.38	5.34	5.08	K_1	5.00	5.20	5.60
PP_2	5.70	5.42	5.14	K_2	6.02	5.68	5.52	K_2	5.68	5.70	5.84

where each entry is the sum of 3 readings.

Summing over P, N, and K respectively:-

	N_0	N_1	N_2	K_0	K_1	K_2	P_0	P_1	P_2
Σ	16.78	15.86	15.00	14.62	15.80	17.22	15.78	15.60	16.26
MEAN	1.86	1.75	1.67	1.62	1.76	1.92	1.75	1.74	1.81

where each entry of Σ is the sum of 9 readings.

Grand total = 47.64

Correction term $CT = \dfrac{total^2}{N} = \dfrac{47.64^2}{27} = 84.0581$

$SS_N = \frac{1}{9}(16.78^2 \ldots . 15.00^2) - CT = 0.176$

$SS_P = \frac{1}{9}(15.78^2 \ldots . 16.26^2) - CT = 0.0259$

$SS_K = \frac{1}{9}(14.62^2 \ldots . 17.22^2) - CT = 0.3766$

$SS_{NP} = \frac{1}{3}(5.46^2 \ldots \ldots 5.14^2) - SS_N - SS_P - CT = 0.0133$

$SS_{NK} = \frac{1}{3}(5.38^2 \ldots \ldots 5.52^2) - SS_N - SS_K - CT = 0.0458$

$SS_{PK} = \frac{1}{3}(5.10^2 \ldots \ldots 5.84^2) - SS_P - SS_K - CT = 0.0695$

SS Total $= (1.96^2 \ldots . . 1.74^2) - CT = 0.8347$

Anova table:

NATURE OF VARIANCE	SOURCE	df	SS	MS	F
MAIN	N	2	0.176	0.088	6.88
FACTORS	P	2	0.0259	0.013	N.S.
	K	2	0.376	0.188	14.7
FIRST	NP	4	0.0133	0.00333	N.S.
ORDER	NK	4	0.0458	0.01145	N.S.
INTERACTIONS	PK	4	0.0695	0.01738	N.S.
TOTAL		26	0.8347		
RESIDUAL		8	0.1276	0.0150	
REVISED RESIDUAL		20	0.2562	0.0128	

(residual values revised to include variation due to first order interactions when these have been shown to be not significant).

$$F_{0.01} (2,20) = 5.86 < F_N$$

$$F_{0.001} (2,20) = 9.95 < F_K$$

Hence the influence of potassium fertilizer application upon the level of potassium in the leaf is highly significant ($P \lll 0.001$) and the negative influence of nitrogen fertilizer application upon foliar potassium levels is significant ($P < 0.01$)

The Student-Newman-Keuls test can then be used to determine the relation between each of the three means.

Both potassium and nitrogen fertilizers significantly
(P <0.01) raised the foliar levels of these elements, and,
as shown above, nitrogen also had an antagonistic effect
upon potassium uptake. The effect of phosphate fertilizer
upon leaf phosphate levels was not significant. (In
previous months this effect was never significant above
P < 0.1). Nitrogen had a negative influence upon EGC
levels, but only at a low level of significance (P < 0.2),
while phosphate had a significant (P < 0.05) positive
influence upon EGC levels. Relationships between means
of all these quantities were determined by the Student -
Newman - Keuls test, and are expressed in Table 14 .
Levels of other catechins, including the total, were not
significantly affected by fertilizer application, nor
could any catechin effect be correlated to potassium
application.

The relationship between EGC content and nitrogen and
phosphate application was further investigated by
performing a regression of EGC upon foliar N and P levels,
using the information in Figure 20 . When EGC is
measured in μmol/g, and the mineral levels in percentages the
following partial regression equation is obtained:

$$EGC = 64.5 \ P - 14.1 \ N + 103$$

in which the factor for N is significant at the P < 0.02
level, and the factor for P is significant at the P < 0.1
level.

FERTILIZER	ELEMENT OR CATECHIN	FOLIAR ANALYSIS			UNITS	LEVEL OF SIGNI- FICANCE P<
		FERTILIZER LEVELS				
		0	1	2		
N	N	4.12	4.24	4.60	% dry wt	0.001
N	K	1.45	1.35	1.34	"	0.05
P	P	0.37	0.38	0.35	"	N.S.
K	K	1.32	1.44	1.37	"	0.05
N	EGC	66.6	69.1	61.9	μmol/g	0.10
P	EGC	63.6	62.1	71.9	"	0.05

TABLE 14

INFLUENCE OF FERTILIZER APPLICATION UPON MINERAL
AND CATECHIN LEVELS IN THE SHOOT: COMPARISON OF
MEANS.

Means of each of three levels of fertilizer
application calculated from Table 13. Probability
level in 'level of significance' column refers to
the significance of the fertilizer treatment being
a component of variance. In the comparison of means,
those terms which are not significantly different at
the P< 0.05 level are underlined (Student − Newman −
Keuls range test.)

11. RELATION BETWEEN CATECHIN COMPOSITION AND TF PRODUCTION

The following series of experiments was designed to investigate the significance of each particular catechin component of the shoot in determining the ultimate quality of the made tea liquor as assessed by TF content or TF x TC product (cf Page 41).

(i) Seasonal Variation of TF

At the same time as the experiment to investigate seasonal variation in catechin composition (9 (i) of this section) the same clones were ground with sand and fermented under standard conditions (see methods section), and the maximum quantity of theaflavin, as determined by the miniature Roberts[1] method (see methods section). The results for clone PC 1 showed a good correlation between EGC and TF content, both these factors being low in March, rising to a peak in July, and then dropping in August and September (Figure 44). These results were typical of the four clones studied. Also plotted on Figure 44 are the monthly averages of the TF content of a local commercial tea for comparison.

(ii) Direct Correlation between Catechins and TF production

Over the period February and July 1969 a number of clones from Swazi Research Station were analysed with respect to catechin content, enzyme activity (one assay per sample) and production of TF during fermentation after grinding with sand. The results are presented in table 15 .
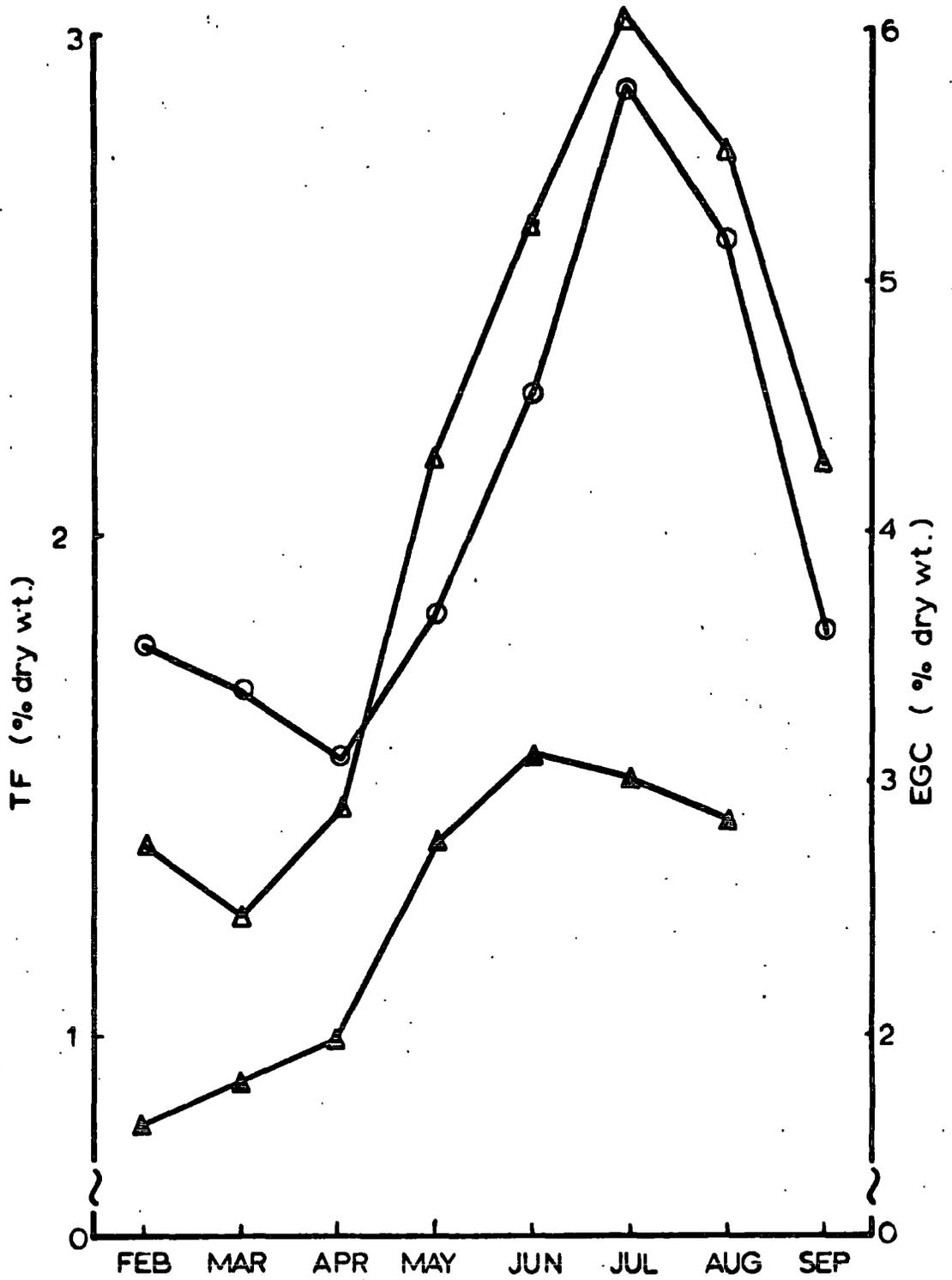
FIGURE 44

Seasonal variation of TF and EGC for clone PC 1

O——O EGC
△——△ TF produced during fermentation after sand-grinding.
△——△ TF in black tea of local commercial manufacture.

CLONE	DATE		CATECHINS (μm/g dry weight)							FERMENTATION PRODUCTS		ENZYME ACTIVITY
			EGC	EGCG	ECG	GC	EC	C	Σ	TF%	TFxTC	
MFS 143	FEB	11	64	164	69	21	20	10	348	1.78	20.1	16.8
FR 1		11	24	112	70	26	52	22	306	0.82	7.1	11.7
MFS 120		18	81	148	60	25	32	17	363	1.37	12.8	10.5
SFS 420	MAR	18	48	158	62	14	21	17	320	1.32	11.4	13.2
SFS 421		18	51	152	65	20	22	14	324	1.76	14.3	7.9
SFS 475		18	64	141	70	18	20	22	335	1.93	18.5	24.2
SFS 423	APR	1	38	144	56	12	10	20	280	1.62	12.0	14.9
SFS 430		1	61	160	67	15	16	22	341	2.02	17.5	14.8
SRS 441		8	43	155	34	14	15	12	273	1.57	13.8	22.1
SFS 442		8	51	148	52	22	15	8	296	1.05	6.3	14.7
SFS 446		15	69	163	84	16	12	24	368	1.96	19.3	16.4
SFS 447		15	53	160	46	11	9	18	297	1.66	14.4	17.2
MT 7	JUN	10	79	129	62	17	35	9	331	2.35	25.9	26.1
MT 11		10	36	85	38	17	11	9	196	1.37	10.2	16.4
MT 14		10	101	142	55	20	29	12	359	2.88	27.6	26.7
K6/97	JULY	1	137	143	63	19	37	12	411	3.21	36.4	13.6
CL 17		8	84	179	47	19	32	11	372	2.58	25.3	17.3

TABLE 15

Catechin Composition and TF Production of Various Clones

Units of TF x TC : TF% x TC E_{460} Polyphenol Oxidase activity in International Units per g fresh weight
Readings of TF and TF x TB for each clone were made on a model fermentation initiated by sand grinding
during the same week as the catechin analysis and enzyme assay were performed.

Regressions of TF and TF x TC upon EGC, $\frac{EGC}{EGCG}$, and

catechins all gave positive regression coefficients at least at the P < 0.05 level, while regressions of TF and TF x TC upon EGCG were definately not significant. A regression of TC upon Σ catechins was also significant. The regression of TF x TC upon EGC was highest in significance (Figure 45). A partial regression equation of TF x TC upon EGC and Σ catechins was derived, but the partial regression coefficient relating to Σ was not significant.

In Figure 45 it can be seen that the greatest deviations below the regression line are of low polyphenol oxidase activity, while the greatest deviations above the line are of high activity. Hence a partial regression of TF x TC upon EGC and polyphenol oxidase activity was derived:

TF x TC = 0.249 EGC + 0.325 E - 4.00

where E is the polyphenol oxidase activity in International Units, and the partial regression coefficients for EGC and E are significant at the P < 0.001 and P < 0.2 levels respectively.

The experiment was repeated, using 21 different clones from Swazi Tea Research Station, but analysing the liquor after miniature factory manufacture instead of the sand-grinding fermentation system. The results were exactly the same, except that slightly more error was introduced

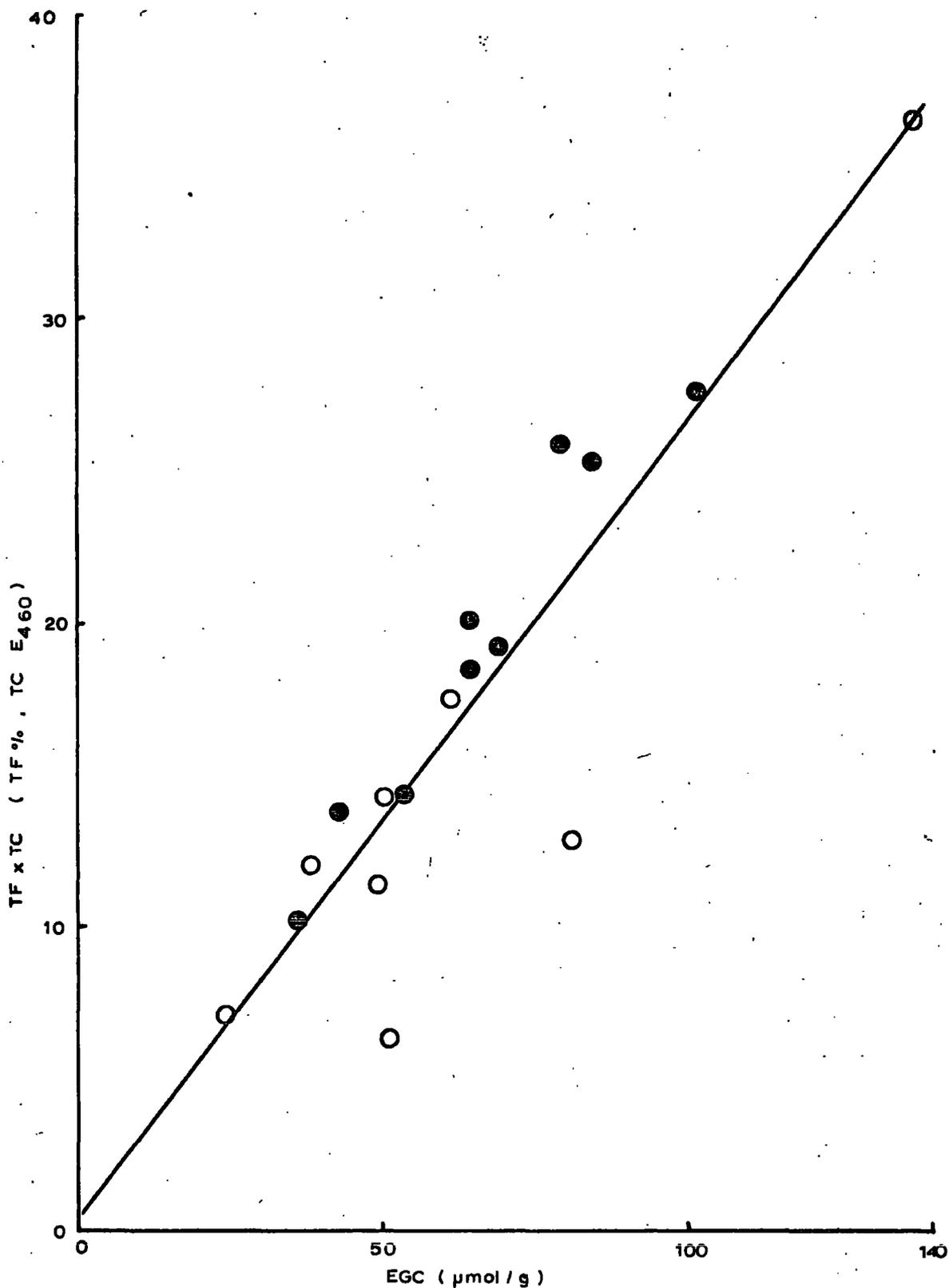FIGURE 45
Regression of TF x TC upon EGC

●—● Clones having PPO activity above average.
○—○ Clones having PPO activity below average.

Data taken from 17 different clones analysed between
February and July 1969 (Table   ). Line drawn
represents the regression equation

$$TF \times TC = 0.263 \times EGC + 0.400$$

(units as above)
in which the regression coefficient is highly
significant (P≪0.001).

into the analysis procedure, and significances were correspondingly lower.

12. EFFECT OF SHADING UPON MADE TEA QUALITY

Apart from regular enzyme and catechin analysis from the shaded bushes of MT 12 and SFS 204 described in sections 9(ii) and 10 (ii) of this chapter, samples of leaf were also manufactured in the Research Station miniature factory and analysed by the modified Roberts' method (Table 1 6). In every case analysed, the quality of the resultant tea liquor, as assessed by the product TF x TC, was higher in the unshaded control bushes than in the shaded experimental samples.

13. DISTRIBUTION OF CATECHINS IN THE SHOOT

Leaves from young vegetative shoots of clone SFS 204 were divided into six categories according to size, the smallest being the apical bud and the largest the third leaf. Mean weights were determined for each category, the samples dried, homogenised, and analysed for catechin content (Figure 4 6). Results indicated that after an initial increase in the bud stage, the sum total of catechins fell as the leaf matured, as did the gallates EGCG and ECG. Between bud and second leaf, there was an increase in the simple catechins EGC and EC, while GC and C were at too low a level in this clone to be accurately assessed.

DATE	CLONE	SHADED OR CONTROL	ROBERTS' ANALYSIS				
			TF	TC	$E\frac{380}{460}$	B%	TFxTC
4 MAR 70	MT 12	shaded	1.54	7.32	2.79	21.2	11.3
"	"	control	1.77	7.11	3.38	23.3	12.6
14 APR 70	"	shaded	1.50	7.80	2.69	20.1	11.7
"	"	control	1.96	7.53	3.22	23.2	14.8
21 APR 70	SFS 204	shaded	2.37	7.44	3.25	29.8	17.6
"		control	2.59	7.92	3.55	29.8	20.5

TABLE 16

ANALYSIS OF MADE TEA LIQUORS FROM SHADED AND
UNSHADED BUSHES OF CLONES MT 12 AND SFS 204.

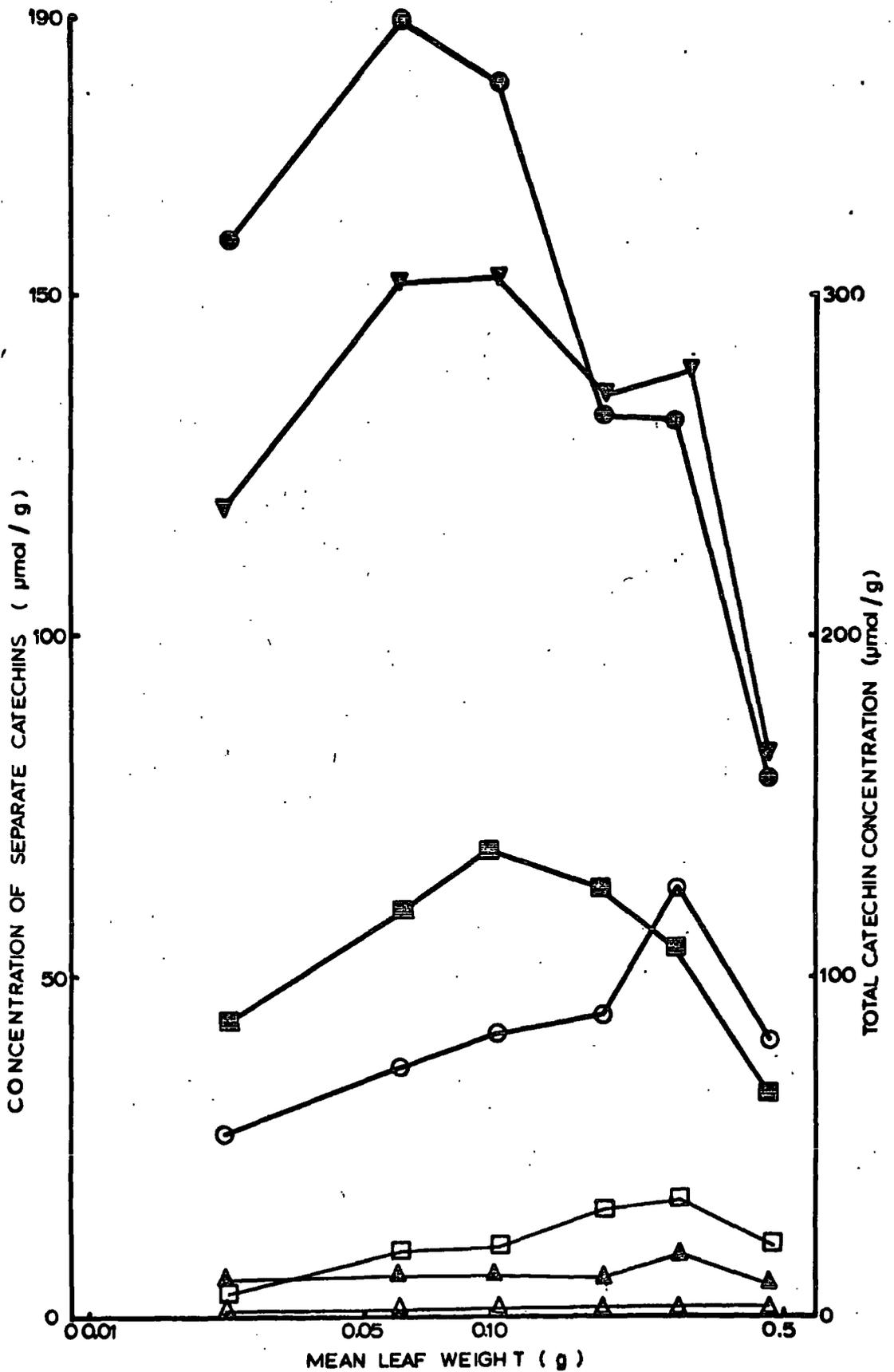For units of analysis see Methods chapter.

FIGURE 46

Distribution of catechins in leaves of
different age.

O—O EGC   □—□ EC   △—△ C   ▽—▽ TOTAL
●—● EGCG ▦—▦ ECG ▲—▲ GC

Clone SFS 204: heaviest leaf analysed corresponds
to third leaf on shoot.

DISCUSSION

Assay and solubility of polyphenol oxidase

Early investigations into the mechanism of tea fermentation demonstrated a high peroxidase activity in the tea leaf (Lamb & Sreerangacher, 1940 a,b), though it was also shown that fermentation would proceed without the involvement of hydrogen peroxide (Sreerangacher, 1943a), and was dependent upon the presence of atmospheric oxygen (Sreerangacher, 1943b). Roberts (1939) failed to correlate peroxidase activity with the rate of the fermentation reaction, and subsequently accepted Sreerangacher's views that "tea oxidase" was a polyphenol oxidase of the copper-protein type (Roberts, 1962a). While PPO is undoubtably the main oxidising enzyme concerned in fermentation, interest in peroxidase was revived when Bendall (1965) pointed out that hydrogen peroxide may be produced in benzotropolone formation, and Bokuchava and Skobeleva (1969), in a recent review of the relevant Russian literature, express their view of the importance of peroxidase. Multiplicity of PPO has been demonstrated (Takeo and Uritani, 1966; Gregory and Bendall, 1966) and Takeo and Uritani showed that various fractions had different substrate specificities.

It is thus unlikely that any single assay method will express the activity of the oxidising system as it occurs in vivo. Methods used have involved the measurement of the rates of product formation, either directly (Baruah and

Swain, 1953; Bendall, 1959; Sheen et.al. 1969) or by
coupled oxidation of an added reducing agent (Ashraf
and Frieden, 1957; Sisler and Evans, 1958; Sanderson,
1964 e); of oxygen consumption (Sreerangacher, 1943a)
Bendall and Gregory, 1963; Harel et.al., 1964), or of
consumption of a phenolic substrate (Mayer et.al.,
1966; Warren and Routley, 1970). However, the initial
products of catechol oxidase activity are extremely
reactive compounds reacting not only with each other
but with unchanged substrate, and hence different
methods of assay give very different results (Mayer et.al.,
1966)

The polarographic method used by Gregory (1964) is
most convenient, and a large number of replicates can
be handled rapidly. It permits the determination of
initial rates, and is apparently the most accurate
method available (Mayer et.al. 1966). Hence the
polarographic method was used in this study, though for
routine work in Malawi the necessary apparatus was not
available, and a colourimetric method originally developed
by Bendall (1959) was employed. In this method the
oxidation of pyrogallol to purpurogallin is multistage,
but the Beer - Lambert Law is obeyed (Figure 1   ).
However, both these assay systems employ pyrogallol as
phenolic substrate; if, as Takeo and Uritani (1965)
suggest, different PPO fractions have different
specificities towards o-diphenols and vic-triphenols, and

the proportions of these fractions vary in different material, then these assay systems may be misleading when comparing the enzyme activities of different clones, or of the same clone under different cultural conditions.

PPO from tea is almost completely particle-bound (Gregory, 1964). However, Sanderson (1964 c) reports that the enzyme may be rendered completely soluble by removal of the phenolic material with an insoluble polyamide absorbent, polycaprolactam or polyvinylpyrrolidone (Sanderson, 1965 a). Firenzuoli et.al. (1969) report that enzymes inactivated by phenolics may be reactivated by the use of soluble polyvinylpyrrolidone and the detergent Tween 80, while on the other hand Havel et.al. (1964) report inhibition of catechol oxidases by soluble polyvinylpyrrolidone. It seemed reasonable therefore, though by no means certain, that polyvinylpyrrolidone, in either soluble or insoluble form, would assist in extracting PPO from an acetone powder. Results (see Methods Chapter, Section 11) indicated that while it was possible to remove phenolics from solution with insoluble polyvinylpyrrolidone, PPO was also removed from solution; and while it was possible to solubilize a large portion of the PPO activity using soluble polyvinylpyrrolidone, the enzyme remained in association with phenolic material, and attempts to precipitate it from solution by the usual methods failed. Some batches of acetone powder were made from leaf which had been damaged, and had fermented

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slightly. Other batches had been made from undamaged
leaf, but had been allowed to approach room temperature
for about 24 hr. Thus it would appear that whatever
the properties of polyvinylpyrrolidone might be in
removing phenolics and assisting the extraction of PPO
from green leaf, it was unable to break the association
between PPO and its oxidation products, and hence was no
use in the present situation.

Analytical Assessment of Quality

Methods used by Roberts to assess the theaflavin and
thearubigin content of made tea liquors (Roberts and
Smith, 1961) make it clear that Roberts used the term
"thearubigin" to describe all the coloured components
except theaflavin, which thus represents a very heterogeneous
group of compounds, which, as discussed later, cannot be
assumed to be related.

Roberts obtained his TR% factor from solutions left
after TF extraction from a tea liquor, and after
acidification with oxalic acid. This test was necessary
to keep all the TR components in acidic form rather than
the more deeply coloured anions. However, a tea-taster's
assessment of a liquor will be affected by the presence
of the anions, and in the present investigation, where
an attempt was being made to orientate the analysis to
conventional quality assessment, the acidification step
was not included. Furthermore, expression of TR as a
weight for weight percentage supposes a knowledge of the

light extinction value of a standard solution of TR.
In view of the heterogeneous nature of TR, such a
value is bound to be highly arbitrary. In the present
investigation the TR% factor is not quoted, the total
colour value of the liquor (TC) being more useful.

Table 2 gives an indication of how closely the
characters of the Roberts' analysis (Roberts and Smith
1961, 1963) corresponds to the market value of a
particular sample of tea. The analysis was modified
as described in the methods chapter, and the TC factor
included in lieu of TR%.

Roberts always placed great emphasis on the TF
value in judging the market value of a tea liquor
(Roberts, 1962; Wood and Roberts, 1964). He reported
that a tea taster's opinion of the colour of a tea
liquor is largely determined by TF content, and that
the generally superior colours of CTC teas when compared
with the colours of those of conventional manufacture
are due to a high TF content, and high ratio of TF to TR,
as will be expressed in the B% factor in Table 2 .
Since the development of this analysis, many other workers
have confirmed that a high TF content is a necessary
quality in a superior tea (Subrahmanyan et.al., 1963;
Ramaswarmy, 1963; Sanderson, 1965b; Bokuchava et.al.,
1966; Nakagawa, 1970).

While it may not be possible to correlate TR content
to the overall commercial value of a tea (Nakagawa, 1970),
TR has been positively correlated to organoleptic quality

scores for the "strength" and "body" of an infusion
(Roberts, 1962a; Subrahmanyan et.al., 1963; Kharebava
and Nikolaishvili, 1964; Nikolaishvili, 1966). Thus
TC to which the TR complex is the principal contributor,
will be a measure of the strength of the liquor as well
as its depth of colour.

Direct tasting of purified and semi-fractionated
components of tea liquors (Millin et.al., 1969) confirms
Roberts view of the importance of TF, which is the main
contributor to the astringency of the tea taste. At the
same time Millin and co-workers found that lower molecular
weight fractions of the TR complex also contributed to
astringency, and certainly to a good colour, but that
a preponderance of higher molecular weighs fractions were
responsible for soft, flat, thin liquors. Also, the
effects of all these components are likely to be modified
by interaction with caffeine.

It thus becomes clear that a good tea liquor generally
requires a high TC, and a high relative proportion of TF.
It would be difficult to combine all of the factors of the
Roberts' analysis into a formula for determining the
ultimate price-value of a tea, and Roberts never attempted
to do so. Even if it were possible to stabilize market demand,
the Roberts method of analysis makes no assessment of
volatile components of tea quality, the "flavour" components,
such as would be produced by low-boiling compounds initially
present in the green leaf, or produced by reaction of

oxidized flavanols with amino-acids, carbohydrates, or organic acids, (Sanderson, 1965b). The purpose of the present investigation was to develop a single factor from the Roberts' analysis which could be significantly related to the market value, and used as an assessment of the quality of teas, in experiments concerning the phenolic properties of tea, where a tea-taster's assessment would not be sensitive enough to differentiate between samples, or would be too much affected by market fluctuations to be useful in long-term compensions. The product TF x TC (Table 2 and Figure 10) was derived for these ends. In a sample selected for standard grade of leaf, and absence of "flavour", there was a high degree of correlation ($r = 0.788$, $P < 0.001$) between the product TF x TC and the price these teas realised on the same day on the London Auction floor.

The regression equation derived from this information (Figure 10) would predict the selling price within, on average, 3.7d/lb; but this prediction is merely academic, for a change in market demand would affect both slope and intercept of this line. Nor is all the variation in the market price accounted for by this or any other single factor (Eden, 1954). The quantity $1 - r^2$, sometimes known as the coefficient of non-determination (Sokal & Rohlf, 1969) expresses the proportion of variance of a variable (i.e. Selling Price) that has not been explained by another variable (i.e. the product TF x TC). In this example the coefficient of non-determination is 0.38; i.e. only 62% of the

variation in selling price of the teas in table 2

is explained by its association with the product

TF x TC. Other sources of variation are the inaccuracy

of the analytical method, the limits of discrimination

of the tea-taster, and the peculiarities of an auction

system. Over and above these sources is the variation

due to the "mark" under which the teas are sold : it is

clear from figure 10 that teas from Kenya were selling

above the mean predicted price, while a large proportion

of teas from Assam are selling below that price. This

would be explained by Kenya having a reputable "mark"

at the time, and Assam an unpopular one. In addition,

there may be other facets of quality which have not been

assessed by the analytical method.

Even at a time when market demand places little

emphasis on the contribution to quality measured by this

particular analytical method, there is unlikely to be

a reversal in its assessment : i.e. the slope of the line

in figure 10 will vary, but its sign will remain the

same. Thus any changes induced in the tea plant, or

changes in manufacturing technique, which result in an

increase in the product TF x TC are almost certain to

result in an increase in market value, though the extent

of this increase will vary.

It must be added that the TF value measured by the

Roberts analysis does not differentiate between simple

theaflavin and theaflavin gallates. No mention is made in the literature of any difference in taste between these fractions; their absorption spectra, and hence their contribution to colour, are very similar (Roberts and Williams, 1958). Millin and co-workers have only tested the "month-feel" of theaflavin, and not of its gallates (Millin et.al., 1969). It is suggested that better quality teas have a lower $E \frac{380}{460}$ ratio for the TF complex (Kharebava and Nokolaishvíli, 1964), which by Roberts' reasoning indicates that the ratio of TF^1 to TFG is greater (Roberts and Smith, 1963). It is possible then that TF^1 may be more beneficial than TFG in determining the character of a tea liquor.

Development of oxidation products during fermentation

An uncharacterized in vitro system was used to study the development of characters assessed by the Roberts method during a simulated fermentation. The results presented in Figure 11 indicate that the TF level reaches a maximum and then declines. This can also be seen in normal factory fermentation (Figure 12), and the phenomenon is well documented (e.g. Roberts, 1958; 1962a). It has been reported that production of TF is preceeded by an initial delay period (Bendall, 1965), although this is not particularly pronounced in the in vitro system used here. The TC of the liquor reaches a maximum some time later than optimum TF production, and then remains static. Consequently, the product TF x TC reaches a rather ill-defined

peak shortly after peak TF production. This is the time prefered by professional tea makers for stopping the fermentation process (Bendall, 1965); a tea fired earlier than this would be termed "brassy" and "lacking in body" by the tea taster, while a tea fired appreciably later would be termed "good body and strength, but lacking in briskness".

The above considerations contributed to the concept of assessing the market value of a tea by the product TF x TC. The use of such a term puts equal weight on TF and TC in assessing the value of a tea : hence teas fired at 40 and 70 min in the example illustrated (Figure 1 1   ) would be valued the same, though their cup characteristics would be very different. Theoretically, it cannot be shown that an equal weighting of TF and TC is justified, and hence a multiple regression equation of the form calculated in the Results Chapter should be used. However, such an equation has a standard error of estimate as high as that for the simple regression equation of selling price upon TF x TC (3.83 d/lb and 3.73d/lb for multiple and simple regressions respectively), and since the multiple regression is considerably more cumbersome to use, the product TF x TC was retained as the factor for routine assessment of values related to market requirements.

Disappearance of catechins during fermentation

Roberts (1962^{a}) claimed that only EGC, EGCG, and ECG are consumed during the fermentation process, but the

results presented in Figure 12   illustrate that EC
and GC are also consumed. This discrepancy is no doubt
due to the increased powers of resolution of thin-layers
as opposed to paper in chromatography, Roberts having
used the latter medium. It is possible that still
better accuracy of the method would indicate the
participation of C also.

Forrest (1967) in similar investigations concluded
that the level of ECG remained constant until the bulk
of the gallocatechins EGC and EGCG had been consumed. This
view cannot be substantiated by the evidence presented
here : the level of ECG follows a normal decay curve which
is apparently unrelated to the decay curves of either
EGC or ECG.

The TF content of the fermenting leaf reached a
maximum at 110 min, and thereafter declined. At this point
most of the EGC was consumed, though there were still
appreciable amounts of EGCG and ECG, and the level of EC
was still about 70% of its original value. Forrest (1967)
claimed that of the catechins involved in TF synthesis
EGC and EC were most likely to be limiting, especially
the latter. This view was partly based on the fact that
dried leaf powders rich in these catechins caused a
significant increase in the rate of TF synthesis when
added to an incubated sample of shoots in which fermentation
had been initiated by grinding with sand. The results
presented in Figure 12   are certainly in agreement with

EGC being a limiting factor in TF synthesis, but EC
is far from exhausted upon cessation of TF synthesis.
However, a much higher initial level of EC, resulting
in a higher proportion of the corresponding quinone
after oxidation (q.v.), could lead to an increase in
TF synthesis : thus EC may be limiting at the onset of
fermentation, and EGC later on.

Between 110 and 130 min there is still some TC
production, which, in the absence of any TF production,
must be due entirely to production of TR. Between these
times there is still considerable consumption of EGCG
and ECG, but virtually none of EGC, which does not
therefore appear to be essential to TR production.

In Vitro Incubations

The relation of PPO activity to quality is a matter
of some dispute. Large variations in PPO activity have
been reported between genetically different populations
(Table 15 ; Ota et.al., 1969; Sanderson, 1964 d); in
different parts of the shoot (Forrest, 1967; Ota et.al. 1968);
as a response to different nutrient conditions (Table 7 ;
Akhundov, 1967), with a change in season (Figure 41 ;
Table 4 ; Takeo, 1966a; Ota et.al., 1968); as a
diurnal variation (Bellizo & Soule, 1967); and during the
withering stage of manufacture (Sanderson, 1964e; Takeo
1966c, 1969). Consequently, attempts have been made to
relate these differences to aspects of quality. Ota et.al.
(1968) claimed that the activity of PPO is more important in
the production of quality than the polyphenol content,
though it is not clear by what criteria quality was assessed.

Using samples of leaf in which fermentation was initiated by grinding with sand, Bendall (1960) showed that the rate of total colour production was dependent upon the PPO activity, but could not demonstrate a correlation between PPO activity, and the amount of TF produced. Sanderson (1964) also showed that the rate of fermentation is dependent upon PPO activity. However, while these observations confirm that PPO is rate determining in fermentation, as illustrated here for both TC and TF production (Figure 16), the problem is not whether components of quality can be produced faster under conditions of higher PPO activity, but whether they attain a higher ultimate value.

Forrest (1967) examined clones of different PPO activity but similar catechin composition, and also mixed material of high PPO activity and poor catechin composition with that of low PPO activity and good catechin composition. His results led him to the conclusion that relatively low activities were quite sufficient to ensure that the best use was made of the available catechin components in terms of TF and TR synthesis, though there was a threshold below which PPO activity could be limiting. Results presented here confirm this viewpoint (Figure 13 and text), though it appears there may be an upper limit of PPO activity above which TF levels are likely to be reduced. However, the ultimate level of TC produced is also directly related to the PPO activity (Figure 14). Thus, using the product

TF x TC as a criterion of equality, it appears that PPO
activity will be related to quality (Figure 15), but
the relationship is not linear : low activities will
result in a disproportionate loss in quality.

It is difficult to assess how far these results
are applicable to fermentation under factory conditions,
when the activity of the enzyme in contact with the
substrate will be more important than the overall PPO
activity of the material. Furthermore, multiplicity of
tea PPO has been reported (Takeo & Uritani, 1966; Gregory
and Bendall, 1966), and the substrate specificities of
the different fractions may vary: Takeo and Uritani found
that their fractions differed with respect to their
reactivity towards o-diphenols and vic-triphenols, which
could have a profound effect on their ability to catalyse
TF production, since this must involve the oxidative
coupling of equimolar quantities of a di- and a tri-hydric
phenol (q.v.).

In modern factory methods of fermentation, utilizing
troughs, skips, or perforated belts, it has been recognised
that to ensure optimum quality a good flow of air is
necessary, and in some instances gaseous oxygen has been
introduced into the system (Harler, 1963). Figure 17
shows that the increase in quality which may be experienced
under these conditions is not merely due to acceleration of
the oxidative process, but because, while ultimate TC levels
are similar, the level of TF attainiable is greater.

In an in vitro system, incubating preparations of

individual catechins, extracted from tea leaf and
purified by counter-current distribution (Roberts &
Myers, 1960a), with a washed PPO preparation (Roberts &
Wood, 1950), Roberts obtained chromatographic spots
corresponding to TF^1, TFG, A, B, C^1, GA, Q, P and R
(Roberts and Myers, 1959). All these compounds had
previously been identified as being produced as a result
of the fermentation process (Roberts et.al., 1957;
Roberts, 1958 a). Incubation of EGCG alone gave TFG, A,
and GA, together with traces of Q and Z. A similar
incubation of EGC gave C^1, and traces of R. Oxidation of
a mixture of these two gave TF, TFG, A, B, C^1, GA, and
traces of R, Q, & Z. Roberts therefore maintained that
all the end products of fermentation that could be
recognised on a paper chromatogram, except TR, could be
derived from EGC and EGCG (Roberts 1962a).

All Roberts' compounds were identified in the series
of incubations reported in the results chapter, section 5,
but their occurrence does not confirm Roberts' views as to
their origins: incubations of EGC and EGCG separately, or
in combination, failed to produce either TF^1 or TFG. This
finding is also reported by Takino and Imagawa (1963a),
although Nakagawa and Torii (1965) appear to have produced
TFG from an incubation of EGCG alone.

The production of spots A, B, and C^1 in incubations of
EGC and EGCG (Figure 3.3 and text) is in agreement with the
results of each of the above authors. Roberts concluded
that substances A, B, and C^1 were dimeric condensation

products of EGCG, EGCG + EGC, and EGC, respectively, and
suggested a possible linkage between the "B" rings of the
flavanoid residues (Roberts and Myers, 1959)ᵤ, the
feasability of which was investigated by Takino (Takino
et.al., 1963); Roberts' original structural formulae were
recently confirmed (Ferretti et.al., 1968).

Working on the assumption that TFG could be derived
from EGCG, and TF^1 only from a mixture of EGC and EGCG,
Roberts suggested structural formulae for TF^1 and TFG
(Roberts, 1962 a) which have never been confirmed.
Subsequently, two independent teams reported the following
structural formulae for TF (Brown et.al., 1966; Takino
et.al., 1965, 1966):
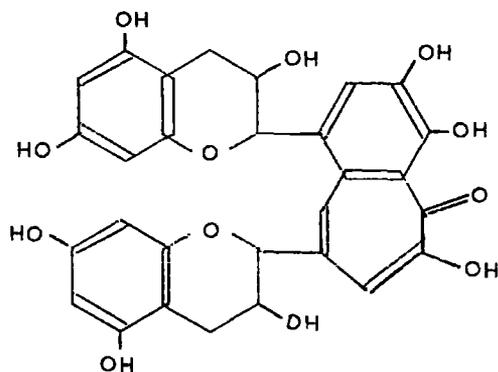
Theaflavin

At the same time Takino and co-workers developed
a model system using catechins purified by fractional
precipitation with lead acetate (Takino and Imagawa 1963 a).
An incubation of EGC and EC together produced TF^1
(Takino and Imagawa, 1963 b;). This result was also
obtained by Nakagawa and Torii (1965), and confirmed
in the present investigation (Figure 20).

Takino proposed the following scheme of formation
of TF in the EGC + EC incubation (Takino et.al., 1964):
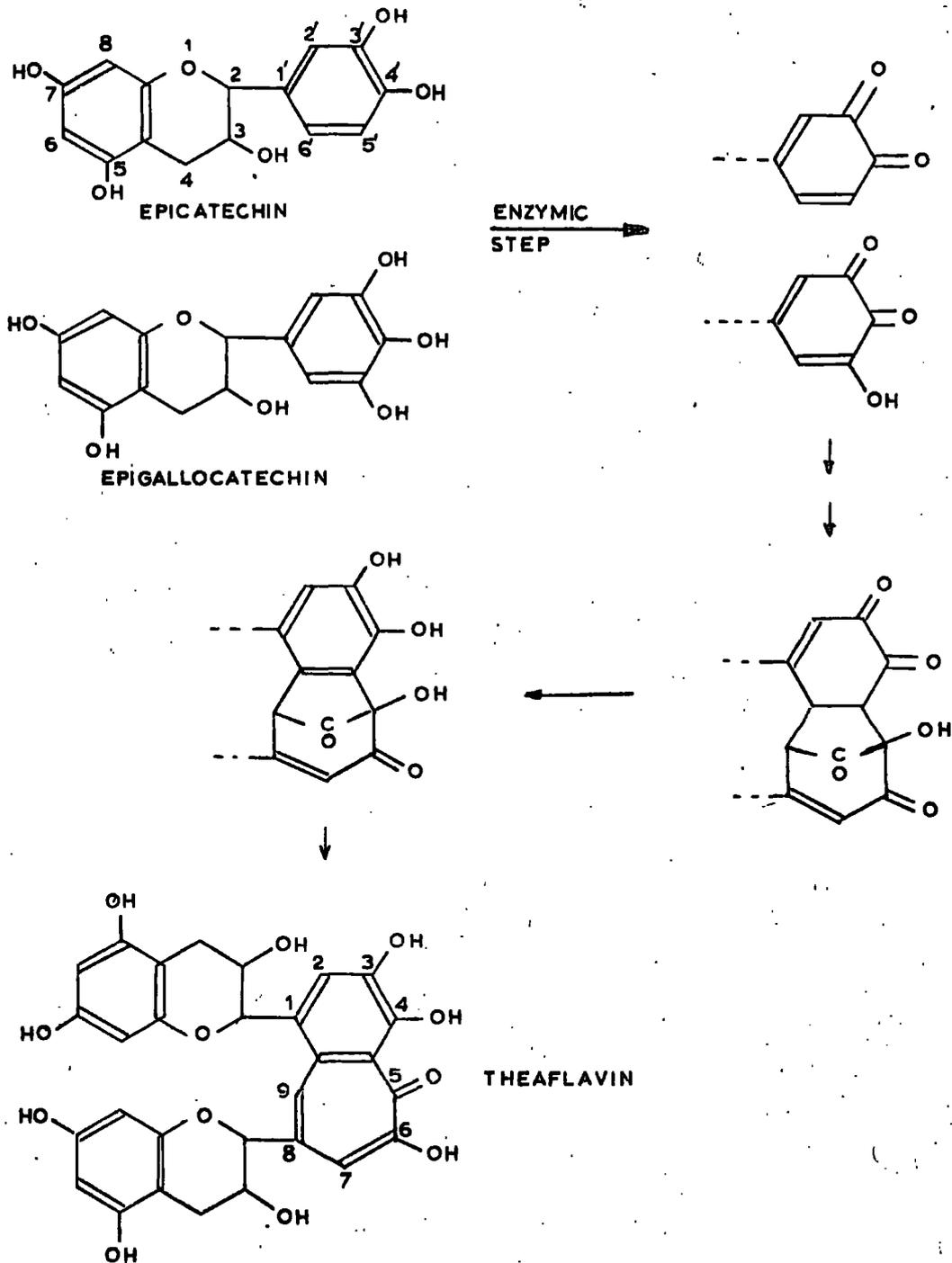
The formation of orthoquinones as an early step in
the enzymic or non-enzymic oxidation of polyphenols
has been well established (Hathway and Seakins, 1955, 1957).
The initial oxidative step appears to involve semi-quinone-
type free radical formation (Hathway and Seakins, 1955;

Soboleva et.al., 1966) and the kinetics of the enzymic reaction have been discussed by Gregory (1964).

If the condensation of the quinones occurs as suggested by Takino, then the benzene moiety of the benzotropolene nucleus in the TF^1 molecule has been derived from the o-diphenol (i.e. EC), and the 7 membered ring from the vic-triphenol (i.e. EGC). Incubation of EGC with catechol and pyrogallol (Takino and Imagawa, 1964) resulted in the formation of categallin and pyrogallin respectively, compounds shown to have the following formulae (Takino et.al., 1967):
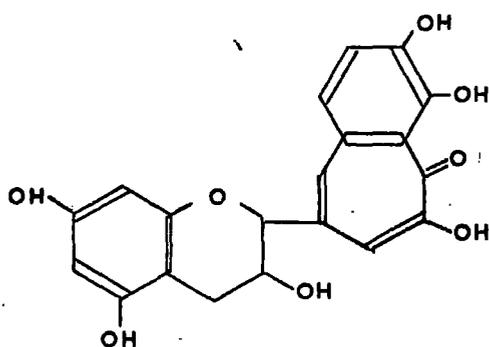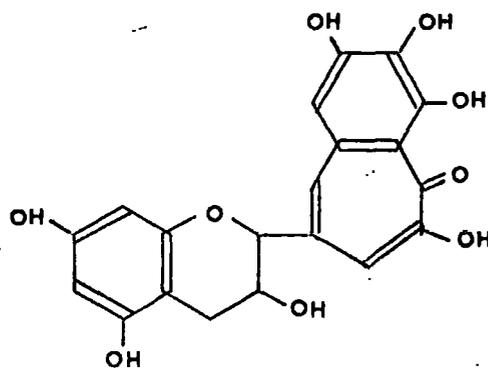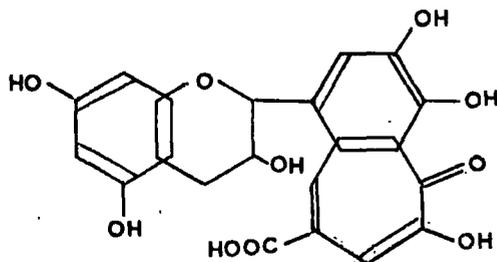

categallin   pyrogallin

Furthermore, an incubation of (+) - catechin and gallic acid, using an inorganic oxidising system, produced a compound shown to have the following formula (Horikawa, 1969):

Thus in this series of experiments only vic-triphenols, either simple phenols or polyphenols, will contribute to the 7-membered moiety of the benzotropolene nucleus; and for a benzotropolone nucleus without hydroxylation at position 2, only o-diphenols, either simple phenols or polyphenols, will contribute to the benzene moiety.

Thus if Roberts had formed a TF compound from an oxidation of EGC + EGCG, it would have been hydroxylated at position 2 of the benzotropolone nucleus. Takino reported that categallin and pyrogallin have different physical and chemical properties including spectral characteristics; thus the hypothetical TF suggested above would have different properties from the TF[1] of proven formula, and could not have been the compound observed by Roberts. We must therefore regard Roberts results as being a result of other contaminating catechins in his incubation mixes. For the same reason we must regard the production of TFG from EGCG alone as being due to similar contamination.

From the formula of TF[1] and its origins, it follows that esterification at the 3-hydroxy position of either or both flavanoid residues could give rise to three different theaflavin gallates, according to whether EGCG, ECG, or both were used in the incubation mixture. Although Takino verbally claimed to have produced TFG in his incubations (Takino et.al., 1957) the results have not been published. Results presented here show that theaflavin gallates are

indeed formed from incubations of EGC + ECG (Figure 23)
and EGCG + EC (Figure 27). The production of traces
of TF^1 in the former incubation is probably the result
of some esterase action of the enzyme preparation upon
ECG, as GA is also evident on the chromatogram. These
results are in agreement with those of Nakawgawa and
Torii (1965).

Chromatographic heterogeneity of TFG from made
tea infusions has been reported (Vuataz and Brandenberger,
1961), and Nakagawa and Torii reported chromatographic
heterogeneity of the TFG's produced in their incubations,
the product of EGC + ECG oxidation having a higher Rf
value in organic solvents on paper, chromatograms than
the product of EGCG + EC oxidation. While this appeared
to be the case in the present investigation, it was
possible that differences in Rf were due to different
compounds but different loadings of the same compound,
as the phenomenon of Rf value varying with loading is well
known on thin layers (Randerath, 1963). Running the two
preparations together produced a diffuse spot which was not
conclusive evidence of heterogeneity, but using a series
of different loadings and studying the variation in Rf
it was evident that the two TFG preparations were
chromatographically distinct (Figure 40).

Nakagawa and Torii also reported that the TFG produced
from oxidation of EGCG + EC had a different absorption
spectrum from the oxidation product of EGC + ECG, or TF^1

itself, having no defined maximum at 460 nm., but their
observations were made on a butanol extract of the total
incubation mixture, and not on the pure TFG_2. A similar
absorption spectrum was found in this investigation, but
after purifying the TFG_2 by chromatography on LH-20
Sephadex. The absorption spectrum was indistinguishable
from either TFG_1 produced from EGC + ECG, or TF^1 produced
from EGC + EC. (Figure 2 6   )

The above authors report production of a further
species of TFG from an incubation of EGCG + EGC. Although
TFG production is observed in a similar incubation in the
present work (Figure 3 0   ), the TFG spot is large and
diffuse, and there are traces of TF^1 and a strong GA spot
which indicates considerable splitting of the gallate ester
bonds of both EGCG and ECG. Thus TFG, and TFG_2 are to be
expected on the chromatogram, and the resolution is not
good enough to show conclusively the existence of a third
TFG component. However, there is no difficulty in
constructing a spacial model of a theaflavin digallate, so
it is unlikely that there is a steric hinderance effect
which prevents its formation.

The traces of TF^1 produced in the incubation of GC
(Page 5 3   ) cannot have originated from this molecule
alone for the reasons discussed above, and must be a result
of contamination of the GC preparation in spite of its
apparent chromatographic purity (Figure 3 4   ). The product
of GC + EC incubation, which is produced in much greater

yield, has a slightly higher Rf value in the organic
solvent (Figure 34   ), and is presumably an epimer
of TF^1. Similarly the incubation of GC with ECG
produces an epimer of TFG_2. Thus it appears that GC
can replace EGC in incubations producing various
theaflavins, though the yields of theaflavin are smaller
than when EGC is used.

Contrary to the results presented here, and to those
of Roberts and Myers (1959), Nakagawa and Torii produced
a TF spot from an incubation of EGC + C. This spot was
found to run very close to TF^1 produced from EGC + EC,
and may well have been due to contamination of the
reaction components. On the other hand, since the
oxidation of C produces a very strong brown streak having
zero Rf in water, the production of traces of a further
epi-theaflavin in the present work may have been overlooked.

The significance of these last two sets of reactions in
the normal fermentation process must be slight: an
epi-theaflavin has not been reported, and the TF^1 spot on
a chromatogram of a made tea liquor is relatively precise
compared to that corresponding to TFG, and does not appear
to be a collection of epimers.

Thus it would appear that of the six catechins considered
appreciable quantities of TF can be produced from the following
combinations:

             EGC   +   EC
             EGC   +   ECG
             EGCG +   EC

In these combinations EGC and EC occur both in TF[1] and TFG synthesis. ECG only occurs once, and since in the leaf the level of ECG is not much less than that of EGC, it is unlikely to be limiting.

Forrest (1967) found that addition of powders derived from whole shoots to a fermenting system caused significant increases in TF synthesis which could be correlated to the EGC content of the powders added. At the same time he found a significant positive correlation between the content of EGC and EC in a large sample of different clones, and concluded that the increases in TF production he observed may therefore have been due to addition of EC rather than EGC. However, doubling the content of EC in a mixture of the six catechins in an <u>in vitro</u> incubation does not lead to an increase in TF production, while doubling the EGC content leads to a very significant increase (Table 3). This suggests very strongly that EGC is limiting in TF production.

The reduction in the peak TF level and large increase in the TR level experienced when the EGCG level in the same system is doubled indicates that this catechin is most important in TR production, as would be expected from its relative abundance, and that quality, in terms of TF production, may actually be impaired when EGCG is present in too great a concentration.

Quantitative aspects of TF production in the <u>in vitro</u> experiments using purified catechins must be regarded with

some reserve. Since the TF production curve reaches
a maximum and then declines, the peak TF level can
only be accurately determined by continual analytical
monitoring in terms of TF. When incubations using crude
leaf extracts were performed, the Roberts' analysis was
adequate for this purpose, but insufficient quantities
of purified catechins were available for this analysis
to be used in the series of experiments at present under
discussion. When only one species of TF or TFG was being
produced in an incubation, it was possible to remove the
spot produced on a thin layer chromatogram to obtain a
quantitative estimate using the vanillin reaction. But
when more than one species was being produced, as in
incubations of six catechins together, differentiation and
separation from TR streaks was difficult, and resulted in
considerable experimental error. A series of preliminary
incubations was therefore performed on the same weight of
the basic mixture of six catechins, using the same quantity
of enzyme preparation, and monitoring the reaction rate in
the oxygen electrode. Each reaction was stopped at a
different time, the reaction rate in terms of oxygen uptake
noted, and the TF content of the whole incubation mixture
determined accurately after chromatography on LH-20
Sephadex (see methods chapter). This procedure resulted
in an accurate assessment of the maximum TF level which
could be produced from a specified weight of the basic
mixture of six catechins, and the reaction rate in terms of
oxygen uptake corresponding to the time at which maximum TF

was produced. Subsequent incubations in which the proportions of the six catechins were varied were thus stopped when the reaction rate had fallen to the predetermined level, and the TF content determined after LH-20 chromatography. This was assumed to be the peak TF level. Small errors in timing were acceptable, as the TF peak was not particularly sharp in these incubations. Furthermore, while a lower TF value than the control may be suspect, a higher level cannot be the result of not stopping the reaction at the peak of TF production.

Several workers have reported that the disappearance of EC and ECG in fermentation is not appreciable until the gallocatechins EGC and EGCG are consumed (Roberts, 1962 a; Mileshko et.al., 1966; Forrest, 1967; Buzun et.al. 1968). This is not so obvious in the normal factory process (Figure 12), as it is in the in vitro system (Figures 35, 36, and 37). Roberts observed the phenomenon to be more noticeable when oxidizing pairs of catechins in vitro (Roberts and Myers, 1960 b). He proposed that in a situation where two catechins are incubated together, the substance with higher redox potential will act as a carrier for the oxidation of the substance of lower redox potential; this is compatible with the knowledge of oxidation reactions of phenols (Brown, 1967). Thus as long as substances of lower redox potential are present, the carrier will remain unoxidized. This can readily be seen in the oxidation of EGC + EC (Figure 21), though

in the production of TFG_1 and TFG_2 while the oxidation rate of the dihydroxy catechin is much less than that of the galloyl catechin, it does not increase appreciably upon exhaustion of the galloyl catechin. (Figures 24 and 28). However, Roberts also reports that while TF^1 and TFG are not substrates for PPO, they may undergo coupled oxidation (Roberts and Myers, 1960 b), so an increase in the rate of the simple catechins in the above reactions would not be noticed until TFG was exhausted.

For a substance to act as an efficient carrier in the system at present under discussion, it must be a substrate for PPO, have a relatively high turn-over number with the enzyme, and have a high redox potential. By comparison of their reactivities in the paired catechin incubations, the six catechins may be arranged in descending order of redox potential as follows: EC or C, ECG, GC, EGCG, EGC. (An incubation of EC + C was not performed, so it is not clear which of these substrates has the highest redox potential. The incubation of ECG + EC was not performed here, but Nakagawa and Torii (1965) report ECG to be consumed first). Gregory (1964) reports the maximum reaction velocity, which will be related to turn-over number, for the oxidation of EC with a purified PPO preparation to be almost twice that for C. Thus EC is likely to be the most efficient carrier.

None of these compounds can be perfect carriers, as

some consumption of EC and ECG can be observed in all
incubations studied, regardless of whether substances of
lower redox potential are present. Indeed, since
the benzene ring of the theaflavin benzotropolone
nucleus is derived from the dihydroxy catechins,
production of TF is dependent upon concurrent
consumption of dihydroxy and trihydroxy catechins.

It has been reported from one laboratory that in
the fermenting leaf high EC or ECG levels result in
increased rates of EGG and EGCG consumption (Buzum et.al.,
1968; Mileshko et.al., 1966). If this is true, it was
not observed when the EC level is raised in an *in vitro*
incubation (compare Figures 35 and 37). In a system
in which either C, EC or ECG are acting as carriers, the
system will become saturated with these molecules at
catalytic rather than substrate concentrations, as lohg
as substances of lower redox potential are present in
sufficient concentration to ensure reduction of the
carrier as soon as it is oxidized. Thus it seems highly
unlikely that additions of either EC or ECG will produce
increases in the overall rate of EGC or EGCG consumption;
nor, as shown here, (Table 3), will an increase in EC
bring about an increase in TF production.

Given that there is an initial and continuous slow
consumption of carrier molecules even in the presence of
unoxidized substances of lower redox potential, the
oxidized carrier can undergo further independent reaction,
reaction with another molecule of its own species, such as

the co-polymerisation process described by Hathway &
Seakins (1957), or reaction with another molecular
species, which, if it be a galloyl catechin, will
result in theaflavin formation. The likelihood of
this last happening will depend upon the concentration
of galloyl catechin - derived quinones in the medium.
This in turn will depend upon the concentration of
the galloyl catechin, and the life of the corresponding
quinone before it undergoes further reaction. The
quinone derived from EGC would be expected to have the
longest life, since EGC has the lowest redox potential
of the six catechins studied. But the increase in TF
production which occurs when high concentrations of EGC
are used in a fermenting system (Table 3 ; Forrest, 1967),
would indicate that the EGC - derived quinones are still
not present in sufficiently high concentration to ensure
that all the ECG - EC - derived quinones available are
converted to theaflavins.

Roberts' scheme of fermentation (Roberts 1962a)
suggested that the TR complex was derived indirectly from
EGC and EGCG, by suitably coupled oxidations of the
bisflavanols, TF[1], and TFG. He explained that his failure
to produce TR in _in vitro_ incubations was due to the
absence of suitable coupling agents in his incubation
mixes (Roberts, 1958b). Contrary to Roberts findings
some TR is formed in all the incubations performed in
this work, and possibly by the coupled oxidation of
bisflavanols as Roberts suggested (Figure 3·1   ).

Forrest reported that the leucoanthocyanins (proanthocyanidins) present in monomeric state in the shoots of the tea plant are consumed during fermentation (Forrest, 1967; Forrest and Bendall 1969b), and suggested that they may be involved in TR synthesis. The association of TR with chebulinic acid, corilagin, and glucogallin has been reported (Wickremasinghe, 1967). Two reports claim to have isolated protein amino-acids from the TR complex (Vuataz and Brandenberger, 1961; Millin et.al., 1969). Brown et.al. (1969a, 1969b) gave evidence that the thearubigins are polymeric proanthocyanidins. Their analysis failed to reveal any nitrogen or benzotropolone derivatives as an integral part of the TR structure, and no evidence was found to support the involvement of any of the compounds listed by Wickremasinghe. In fact, the structure for TR proposed by these authors (Brown et.al. 1969b) is compatible with its formation from the six catechins alone, although the inclusion of leucoanthocyanins is not precluded. They proposed an interflavanoid c - c linkage between the 4 and 8 positions, though other linkages including c - o bond formation are possible (Seshadri, 1967).

Millin and co-workers have used chromatography on alkylated Sephadex to separate TF from the remainder of the coloured components of a tea liquor (Crispen et.al. 1968), and have fractionated the TR complex into at least seven components by this technique (Millin et.al., 1969).

Most of these components have been reproduced in the

present investigation from <u>in vitro</u> incubations of the

six catechins (compare Figures 3 8 and 3 9), the

major components 3, 4, and 5 (as numbered in Figure 3 9)

being produced from an enzymic oxidation of EGCG

alone (Figure 2 9). But the conspicuous absence of

components 6 and 7 from TR produced in any <u>in vitro</u>

incubation suggests that either the complete TR complex

cannot be produced from the six catechins alone, or that

oxidative conditions are not suitable in the <u>in vitro</u>

system for production of these particular components.

With regard to the identity of components, 6 and 7,

it would be tempting to say that these components have

the lowest molecular weight of the TR complex, and indeed

fractionation of flavonoid polymers with respect to

molecular weight has been achieved (Forrest and Bendall,

1969b; Somers, 1966). But while the non-dialysable

material of components 1 - 5 is probably eluted from the

Sephadex in order of descending molecular weight (Millin

et.al., 1969), as is the normal behaviour of macromoleules

on Sephadex gels (Andrews, 1964), considerable adsorption

between Sephadex and phenols takes place (Brook and

Housley, 1969): the simple catechins are in fact eluted

before the larger catechin gallates (Buzun et.al., 1965,

1966; Bokuchava and Oragvelidze, 1965), and TF later still

(Millin et.al., 1969), thus casting some doubt as to the

precise molecular size of components 6 and 7.

Sanderson (1965b, 1966)b proposed a scheme of fermentation in which many compounds, such as amino-acids, carbohydrates, and organic acids, may interact with the oxidized flavanols to produce low-boiling volatile components of "flavour", and also modify the TR structure. While it is well established that amino-acids and organic acids will affect the rate of the flavanol oxidations (Nakagawa, 1967; Soboleva, 1966), and the production of low-boiling aldehydes has been demonstrated in the reaction between tea quinones and amino-acids (Bokuchava and Popov, 1954; Popov, 1956), the results presented here show that a large proportion of the TR complex can be formed without the involvement of free amino-acids at all. Roberts (1961) demonstrated that the oxidation products of catechins were likely to form insoluble protein complexes, and soluble complexes of caffeine, amino-acids, and polyphenolic condensation products have also been reported (Vachnadze, 1966). Smith (1968) also found non-caffeine nitrogen in the complexes of TF, TR, and caffeine which constitute the "cream" which forms when high grade tea liquors cool (Roberts, 1963). Thus, while it is unlikely that nitrogen is contained as an integral part of the TR polymers, the superficial association of amino-acids or proteins with TR is highly likely, which will undoubtably alter the character of the infused tea liquor (Roberts, 1962b).

Spots X and Y (Figure 18) appear on thin layer chromatograms from all incubations performed, and from aqueous extracts of black tea. They are faint yellow in

colour, and stain red-brown with benzidine. They have
a higher mobility in water than any͜ spot reported for
tea extracts chromatographed on paper usihg 2% (v/v)
acetic acid as solvent. They are not extracted by
ethyl acetate, and when thin-layer chromatograms are
made of the fractions of a tea liquor eluting from a
column of LH-20 Sephadex, they appear to be part of
components 4 and 5 (Page 57). They would thus appear
to form part of the TR complex. Their identity as anions
of components of the TR complex would explain why they
have not been reported on chromatograms using acidic
aqueous solvents, and their variable Rf values in water
(Page 47).

Roberts showed that while substance Q and purpurogallin
carboxylic acid were chromatographically inseparable
(Cartwright and Roberts, 1954), they showed different
colour reactions with added sodium hydroxide (Roberts
et.al., 1957), and different absorption spectra (Roberts
and Williams, 1958). He produced substance Q in an
incubation of ECG alone (Roberts and Myers, 1959), which
is confirmed here (Page 50), and in an incubation of
EGCG alone. This last finding cannot be confirmed here,
though an incubation of EGCG + EC produced appreciable
quantities of Q. The absorption spectrum for the sample
of Q formed as a result of ECG oxidation and purified
by chromatography on LH-20 Sephadex (Figure 26) agrees
with that given by Roberts, and is the same as that for
substance Q produced by an incubation of EGCG + EC.

Roberts (1962a) suggested that Q was a mixture
of purpurogalim carboxylic acid, a flavanotropolone
derived from pyrogallol and EGCG, and the main oxidation
product of ECG. It seems likely that two compounds as
different in size and structure as purpurogallin
carboxylic acid and a benzotropolone compound would be
separated by chromatography on LH-20 Sephadex. The
symmetry of the peak observed on elution (Figure 25)
thus indicates that Q is a single compound, though a direct
attempt to separate a mixture of purpurogallin carboxylic
acid and substance Q by this method was not attempted.

Pyrogallol is not present in any of the incubations
performed here, but Roberts claimed that the pyrogallol
moiety may be provided by a galloyl catechin. The absence
of substance Q from incubations of EGCG, EGC, EGCG + EGC,
or EGC + EC, however, obviates this possibility.

The substance Q produced by EGCG + EC oxidation may
be a flavanotropolone derived from EC and GA, having the
formula
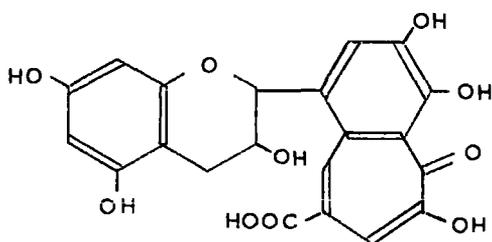
This compound has been produced by an incubation of
C + GA, using an inorganic oxidizing agent (Horikawa,
1969). The same compound, or its gallate, could be derived
from an oxidation of ECG.

Extension of the results of <u>in vitro</u> experiments to
the <u>in vivo</u> process must always be subject to reservation.

The universal critisism of _in vitro_ work is that it
cannot take into account sub-cellular organisation
in the living tissue; this critisism is less valid in
the present study, as tea fermentation is a morbid
process which only takes place after the disruption of
tissue and cellular organisation. However, many more
compounds are involved in the reactions under investigation
than have been studied here, and this has special
significance where coupled oxidations are concerned. Thus,
while it is shown that various forms of TF may be
produced from the six catechins studied, this is only
indicative, and not proof, that such reactions occur in
the normal fermentation process.

In Vivo Variations in Polyphenol Oxidase Activity

Since there is considerable variation in activity of
PPO activity in different parts of the tea plant (Popov,
1965; Bokuchava and Soboleva, 1966; Forrest, 1967), studies
on the variation in PPO activity due to seasonal and
agronomic factors were conducted on shoots of a standard
size only (see methods section). Seasonal variations
in PPO activity reported for Georgian tea (Tedeshvilli -
Razmadze, 1966) are small compared with the order of
fluctuations recorded here (Figure 41). Forrest (1969a)
reported that the PPO activity of tissue cultures of tea
was inversely correlated to growth rate. An attempt was
therefore made to correlate the weekly fluctuations
observed with growth rate, using the weekly plucked yield

of bushes as a measure of growth rate. However, the
pattern of distribution of yield throughout the season
(Figure 43   ) is very much influenced by the frequency
of plucking (Fordham, 1970): after the dry season, growth
starts suddenly at the onset of the rains, and the
majority of shoots will be ready for plucking between
5 - 7 weeks later. Plucking shoots at this time will
result in a wave of shoots after a further 5 - 7 weeks.
Thus the large yield experienced 6 and 12 weeks after
the beginning of the rains in Figure 43   are not indicative
of high growth rates at these times. Other measurements in
the rate of growth are currently in progress.

The fluctuations in PPO activity may be partly due
to leaf damage due to overheating or sunscorch, since
activities were lowest during the cold season (Table 4   ).
Protection of the leaf from direct sunlight resulted in
an increase in PPO activity which was greatest at times
when most solar radiation was received (Tables 5 and 6   ).
Shading bushes also results in a decrease in growth rate
(McCulloch et.al. 1966), and the drop in PPO activity may
be a reflection of this, as would follow from Forrest's
observations. But the drastic drop in activity recorded
several days after the shade had been removed from the
experimental bushes for just twenty four hours (Table 5   )
would suggest that a reduction in activity due to heat
damage is more likely.

Work on Georgian soils indicates that increases in
fertilizer levels result in an increase in the activities of

oxidase and peroxidase in the tea plant (Akhumdov,
1968)b, though while this may be the case in Malawi at
moderate nitrogen levels, the effect is inconsistent
at higher levels (Page 61). Sheen et.al., (1969),
working with tobacco seedlings grown in pot culture
found that while some nitrogen application resulted in
increases in the oxidase activity of the leaves, higher
levels produced a reduction in activity, which is
compatible with the above observation.

In Vivo Variations in Catechin Composition

Studies on the seasonal changes in catechin composition
were made on several different clones growing under
irrigation and balanced fertilizer applications. Since
very considerable diurnal variations in catechin composition
have been reported (Dzhemukhadze, et.al. 1960; Zaprometov and
Kolonkova, 1965) all samples were plucked at 7 am. throughout
the season.

No consistent seasonal variations in polyphenol content
have been reported from Ceylon: Sanderson and Kanapathipillai
(1964) concluded that season had no significant influence
upon levels of flavanols, and while a correlation between
rainfall and total vanillin reacting polyphenols has been
reported (Wickremasinghe et.al., 1967) the level of
significance was inacceptably low (P $\not<$ 0.05 for any clone
studied. In N.E. India, however, large seasonal variations
in polyphenol content do occur (Wood et.al., 1964). The
main flavanols EGC, EGCG, and ECG were found to be maximal

during August, which in India is a hot, wet month, when growth is rapid. (Bhatia and Ullah, 1968).

In Malawi, Forrest (1967) concluded that the catechin composition of any particular clone under regular plucking was a very stable character, not much influenced by climatic changes. However, the results for clone PC 1 presented here (Figure 42), which are typical of four clones studied, show a very pronounced seasonal variation. Total catechins, and EGC, GC, and EC are all maximal during July, while the catechin gallates EGCG and ECG are lowest at this time. The period May-July constitutes the cold season in Malawi, and growth is very slow.

A comparison between results from Malawi and India or Russia is complicated by their lying in opposite hemispheres and at different degrees of latitude. Growing seasons are very different. In Russia, tea stops growing during the winter months, and the polyphenolic content falls during this period (Dogonadze, 1969). In Malawi, the reverse occurs. The factors which are limiting for growth of the tea plant in different parts of the world, whether temperature, daylength, or intensity of radiation (all of which have been suggested), are very imperfectly understood. It may well be that the factors which limit growth in the winter in Russia or Assam are different from those in Malawi. For instance, winter dormancy in Assam, at latitude 27°N is thought to be a day-length effect (Barua, 1969), which is unlikely to be

the case in Malawi, where at latitude 16°S there is
only a very small change in day length over the year,
and experiments with day-length have proved negative
(Fordham, 1970).

The pattern of variation in catechin composition for
clone PC 1 (Figure 42   ) suggests the following
correlations between components:

$$EGC \quad \propto \quad \frac{1}{EGCG}$$

$$EC \quad \propto \quad \frac{1}{ECG}$$

$$EC \quad \propto \quad EGC$$

$$ECG \quad \propto \quad EGCG$$

Demonstration of these relationships in the present
instance is complicated by the variation in total catechin
content. Forrest (1967) reports the correlation between
EGC and EC at a high level of significance ($P < 0.01$)
amongst a large number of clones, and the correlation
between ECG and EGCG has also been reported, though the
level of significance is not given (Bhatia & Ullah, 1968).
But in a situation in which all the six catechins are
derived from a single precursor during biosynthesis, a rise
in the level of the precursor, whether as a result of genetic
or environmental differences, can result of general increase
in the level of all catechins. Thus positive correlations
are not indicative of any further relationship between the
entities concerned. Negative correlations, however, would
be indicative of a change in balance of catechins,
regardless of their overall level, though this effect will
be disguised if greater changes in overall level are taking

place at the same time. For example, consider the situation in which a substrate A gives rise to a product B, which is in equilibrium with another compound C:

Reaction 2

B $\longleftrightarrow$ C

Reaction 1

A
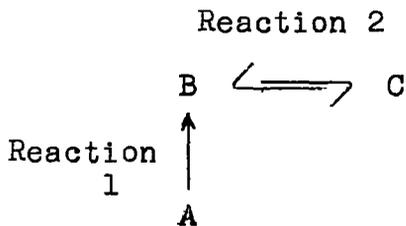
A change in rate of reaction 1 while the equilibrium of reaction 2 remains undisturbed would result in positively correlated fluctuations in the levels of B and C. A change in the equilibrium of reaction 2 while reaction 1 was constant would result in negatively correlated fluctuations of B and C. But when fluctuations in the rate of reaction 1 are concurrent with changes in equilibrium of reaction 2, there will not necessarily be any correlation at all between B and C.

In an attempt to clarify the interrelationships between catechins further, quantities of separate catechins (originally calculated in μ moles) were expressed as a percentage of the total catechin content of each sample. Table 8 is thus a representation of the data in Figure 42 . From these results a significant positive correlation is found between EGC and EC (P < 0.01) and EGCG and ECG (P < 0.02), confirming the findings of Forrest (1967) and Bhatia and Ullah (1968) respectively. However, this could not be repeated with every clone examined, though this may have been due to experimental

error masking the effect.

Since in Table 8 all quantities are converted to percentages, a variation in any one quantity is bound to have a negative correlation with the remaining quantities. Hence, when looking for negative correlations between two quantities, it is necessary to demonstrate a higher degree of correlation than would be explained by the percentage conversion. When comparing EGC with EGCG, therefore, it was first determined that no negative correlation also existed between either of these catechins and the remaining four together. (Page 62). With clone PC 1 this treatment showed highly significant negative correlations between EGC and EGCG ($P < 0.001$) and between EC and ECG ($P < 0.01$).

This method of analysis is not perfect, however, and it did not invariably solve numerical models developed to illustrate the suspected situation. However, evidence for the negative correlation between EGC and EGCG was found in other experiments where the variation in total catechin content was not great. Thus several determinations on each of two clones indicated the effect of shade is to decrease EGC levels and increase EGCG levels (Table 9); and increasing nitrogen application leads to decreased EGC levels and increased EGCG levels (Table 11), when a significant ($P < 0.02$) negative correlation between these components can be demonstrated.

Early experiments on the biosynthesis of flavanoids in the tea plant indicated that synthesis for all catechins

proceeded via shikimic acid (Zaprometov & Silina, 1960;
Zaprometov, 1962; Zaprometov and Bukhlaeva, 1963), and
the enzyme dehydroshikimic reductase was shown to be
operative (Sanderson, 1966a). Results of $\left[^{14}C\right]$-tracer
experiments led Zaprometov (1964) to propose the
following scheme of biosynthesis:

quinic acid
↓
shikimic acid ⟶ GA
↓
prephrenic acid
↓   GA
EC ⇌ ECG

hydroxylation ↓
    GA
EGC ⇌ EGCG
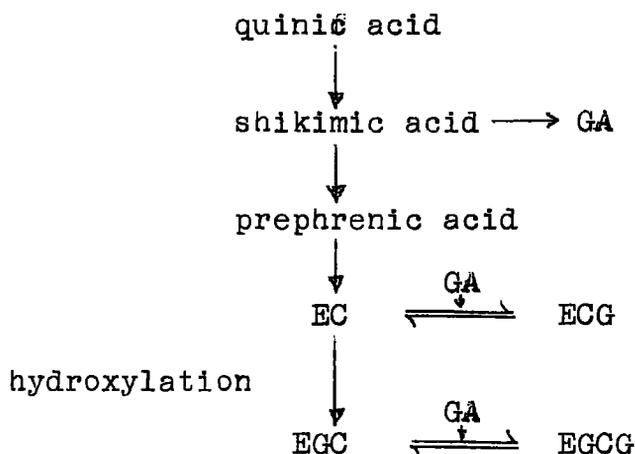
This scheme would explain the observed negative
correlation between EGCG and EGC, and EC and ECG, but
not the positive correlation between EC and EGC. However,
the authors later modified there scheme when it was shown
that $^{14}(C)$ was incorporated equally fast into EC and EGC
(Zaprometov and Bukhlaeva 1967a, 1968a). Subsequently
the radioactivity of EC and EGC was observed to fall, and
that of EGCG and ECG increased (Zaprometov and Bukhlaeva,
1968b). Independent formation of flavanols of different
degrees of hydroxylation is thus indicated, and the above
scheme may be modified thus:

shikimic acid
↓
common precursor

GA
EGCG ⇌ EGC ⟵ ⟶ EC ⇌ ECG
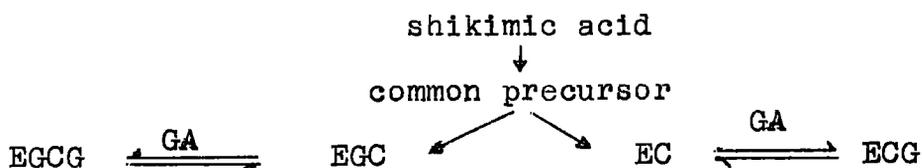
Such a scheme would explain both the negative correlations between EGCG and EGC, and EC and ECG, and also indicates that under certain conditions EGC and EC could be positively correlated.

Labelled catechins introduced into shoots of the tea plant are rapidly metabolised, and $^{14}(CO_2)$ is excreted in the dark (Zaprometov and Bukhlaeva, 1967b), which suggests a "catechin cycle". Such a cycle is proposed by Yuan and Chen (1965). These authors confirm the hypothesis of independent formation of dihydroxy and trihydroxy catechins, and their rapid metabolism whereby catechins may be reutilized in the plants growth processes. They also suggest that the dynamic balance of catechins is related to climatic conditions and different growth phases, ample evidence for which is presented here. (q.v.)

Thus the relationships between catechins suggested on page 13 are supported by some degree of experimental evidence, and are compatible with present knowledge of the biosynthesis of catechins. But in the absence of more suitable statistical analysis of the results, they must remain as likely, but unproven, trends.

In experiments where young shoots and seedlings of the tea plant were growing in complete darkness or light, light was found to increase the rate of overall flavanoid synthesis and alter the state of hydroxylation of the catechin B ring, while the degree of esterification with gallic acid was much less affected (Forrest 1969 b). These findings are in direct contrast with the results of

analyses performed on young shoots growing under shade
or in full sunlight in the field (Table 9), when
there is little difference in total catechin content,
no differences in the degree of hydroxylation of the
catechin B ring, but considerable increases in the
proportion of gallic esters as a result of the shading.
Iwasa (1968) used much heavier shade (1.6 - 6.4% of open
field as opposed to the 25 - 40% used here) and found
a decrease in total catechin content which was entirely
due to reduction in EC and EGC levels, while levels of
ECG and EGCG remained the same. No differences were
reported in the ratio of dihydroxy to trihydroxy catechins.

There is a considerable reduction in growth rate of
tea under shade, well known in Malawi, and also reported
by Iwasa (1968) in China, and McCulloch et.al. (1966) in
Kenya. But the reduction in total catechin content and
increase in the ratio of catechin gallates to simple
catechins is unlikely to be a result of a reduction in
growth rate; for the opposite effect is observed during
the season when growth is slowest (Figure 42). If the
observed changes are a direct effect of light, then shoots
growing in partial sunlight respond to increased light
levels in a very different manner from those growing in
complete darkness.

Experiments on tea seedlings grown in sand culture
suggest that addition of potassium and phosphorus assists
the accumulation of all catechins in the shoot, while a
complete nutrient application including nitrogen results

in the accumulation mainly of EGCG. (Mgaloblishvili, 1966a, 1966b). Field experiments show that nitrogen application leads either to a general lowering in catechin content (Bhatia and Ullah, 1962; Guseinov et.al. 1966; Akhindov, 1968) or an increase in the proportion of EGCG (Dzhemukhadze et.al., 1968). These findings agree well with the results obtained in this work, where moderate increase in nitrogen lead to small reductions in total catechins, an increase in EGCG levels, and a corresponding decrease in EGC levels (Tables 10, 11, and 12). The results at higher nitrogen levels were variable, but in such instances the plant did not appear to be responding significantly to nitrogen in terms of yield or foliar nitrogen levels. (Table 12). It would thus appear that raising of EGCG levels and lowering of EGC levels is correlated to a growth effect rather than the direct influence of nitrogen application, and that rapid growth resulted in high levels of EGCG and lower levels of EGC. However, the causal relationship may be reversed : Dzhemukhadze et.al. (1968) suggest that gallic esters of catechins have a stimulating effect and simple catechins an inhibiting effect on the growth processes of the plant.

The increase in EGC levels which was found to occur with phosphorus application (Table 14) is not reported in the literature, although high phosphate levels have been observed to produce high levels of total polyphenols

(Shavishvili, 1966). Contrary to the effect noticed
here, Forrest (1967) reported a slight depression in
EGC levels with phosphorus application, though he
doubted the significance of these findings. Foliar
phosphorus levels show little increase with increasing
phosphorus application (Table 14), which suggests
that the phosphorus applied in the fertilizer programme
is hot being fully utilized by the plant. Under these
conditions it is bound to be difficult to demonstrate
the effects of phosphorus application on any metabolic
process.

All the differences in catechin composition due to
fertilizer application are small in comparison with
inter-clonal differences, and are therefore difficult to
detect in mixed populations. A repeat of these experiments
on clonal material established for several years on
factorial fertilizer treatments may produce more decisive
results. While results presented here are sufficient to
show that nitrogen and phosphorus applications do
significantly effect catechin composition, the degree of
significance would not justify quantitative extension of
the results; for example, to determine a formula for
nitrogen and phosphorus application which, while
increasing yield, would not result in an alteration in the
balance of EGC and EGCG.

Relation of Catechin Composition to Quality

Climatic factors, while limiting growth appear to

result in increased TF levels. Thus TF levels in Assam
are high during the cool months of the early part of the
growing season, and lowest when the tea is growing fast
(Roberts and Smith, 1963). A similar effect is observed
in the present work, and the high TF values which are
general for the cold season months of June - July in
Malawi, appear to be correlated with EGC levels (Figure
44   ).

Forrest's finding of a significant regression of TF
upon EGC (Forrest, 1967) was confirmed (Page 74), though
contrary to Forrest's observations no correlation between
EC and TF could be found in any series of clones studied.
While the EGC level was obviously the greatest contributing
factor to the quality as assessed by the product TF x TC.
(Figure 45   ), the PPO activity may occasionally be
limiting, though this may be only when PPO activities do
not exceed a threshold level, as a partial regression
coefficient of TF x TC upon PPO activity is not significant.
This would agree with results from in vitro incubations
(Figure 15).

From these results it is evident that all effects
which result in a change in catechin balance in the shoot
will have a correlated effect upon the quality of the tea
liquor made from this shoot : any treatment which results
in a disturbance of the EGC - EGCG equilibrium in favour
of EGC will result in increased quality.

It follows that tea grown under shade, when the EGC
to EGCG ratio is lowered (Table 9), will have a lower

TF x TC value than that grown in full sunlight, as is confirmed by manufacturing samples from these situations (Table 16). Nitrogen application will also lead to a reduction in quality, which is reported by tea-tasting panels in other parts of the world (Mirzoyan, 1963; Sanderson, 1964; Willson and Choudhury, 1968), while phosphate application will lead to an increase in quality, and will therefore offset the deterimental effect of nitrogen (Sanderson, 1964a; Willson and Choudhury 1968).

Changes in quality throughout the season appear to be negatively correlated with growth rate. If one were therefore to remove the limiting effect of climatic influence, for example, by irrigating during the dry season, the resulting manufactured tea may not have the same good quality expected of teas harvested in that season. Just such a case is reported : Dzhemukhadze et.al. (1968) observed an accumulation of EGCG and reduction in EGC after irrigation. Similarly, tea growing slowly on land of marginal fertility often shows excellent quality. Any increase in the yield which might be obtained by nitrogen application in these instances will only be achieved at the expense of loss in quality, though this effect may be balanced by concurrent phosphate application.

There is a general decrease in catechin levels as the components of the shoot age (Bhatia, 1963; Torii & Ota, 1960), and a concommittent increase in the ratio of simple catechins to catechin gallates (Figure 46 ; Forrest and Bendall, 1969a). The quality of the tea from different

parts of the shoot, however, falls with the maturity of the leaf material, as is evident from an analysis of the TF content (Forrest, 1967) or a direct price valuation by tea tasters (Barua, 1968; Choudury, 1968). Thus tea made from buds and first leaves is high in TF, while tea made from the third leaf is lacking in TF, though the TR levels are similar (Forrest, 1967). This finding is anomolous with the above evidence that high EGC levels are correlated with high TF production.

Forrest (1967) also reported that addition of dried leaf powders made from whole shoots of clones high in EGC to a fermenting system caused large increases in the rate of production and final level of TF. But addition of powders made from mature leaves only, and therefore rich in EGC, caused an increase in the rate of production and final level of TR, but the TF level was only increased slightly; while addition of powders made from buds, low in EGC, caused large increases in TF production.

Forrest suggested that mature leaves contain substances not present in younger parts of the shoot, which were capable of complexing with EGC, and thus diverting EGC from TF to TR synthesis. However, this would not explain why TR levels produced in fermentation by the buds should be as high as those produced by mature leaves, unless this is a result of the higher total catechin content of buds.

An alternative suggestion is that younger tissues contain substances, present in higher concentrations than in mature leaves, which favour the conversion of EGC to TF

rather than to TR. As was discussed earlier, TF production is dependent upon consumption of EC and ECG, which are normally acting as carriers in the oxidation process. Substrates for PPO which have higher redox potentials than either EC or ECG may predominate in young tissues, and accelerate the oxidation of EC and ECG. This would result in an increase of their respective quinones available for TF synthesis.

Whether there is any truth in either of these explanations or not, it seems likely that there are substances other than catechins involved in TF synthesis, and that they are not evenly distributed throughout the shoot. But for there to be such a high level of significance in the regression of TF upon EGC (Page 74) when whole shoots of standard size are sampled, such substances must be present in similar concentrations in similarly sized shoots from different clones.

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APPENDIX

Abbreviations

Abbreviations used throughout this thesis are as recommended in 'Instructions to Authors', Biochem. J. 116, (1), with the following additions:

EGC epigallocatechin

EGCG epigallocatechin gallate

ECG epicatechin gallate

GC gallocatechin

EC epicatechin

C catechin

GA gallic acid

CA chlorogenic acid

QA p-coumarylquinic acid

TG theogallin (galloyl quinic acid)

A digalloylbisepigallocatechin

B galloylbisepigallocatechin

C^1 bisepigallocatechin

TF theaflavin (collective)

TF^1 theaflavin (specific for that form of theaflavin whose structural formula was described by Brown et.al. (1966).

TFG theaflavin gallate (TFG_1 and TFG_2 refer to specific spots on thin layer chromatograms).

TR thearubigin

S_I, S_{II} fractions of TR as described by Roberts et.al. (1957).

P,Q,R,Z oxidation products as described by Roberts et.al. (1957), and not identified.

X,Y,W fermentation products not yet identified.

PPO polyphenol oxidase from tea leaf

CTC cut-tear-crush machine: a commercial device for initiating fermentation.

TF%, TC, $E\frac{380}{460}$, B% parameters of Roberts' Made Tea analysis, described in Methods Chapter.

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