METABOLISM OF NUCLEOTIDES AND PHOSPHATE ESTERS IN TEA SHOOTS DURING BLACK TEA MANUFACTURE

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Phosphate esters and nucleotides are a group of phosphate compounds which are intimately involved in almost every process of the living cell. These compounds, which are often complex and unstable, are usually present in small concentrations and as mixtures. Progress in this important field has depended upon the development of methods for identifying and estimating individual esters present in such mixtures. This paper describes the isolation, separation and estimation of acid soluble phosphate compounds in the tea leaf and the changes in these compounds during black tea manufacture. The phosphate compounds detected and estimated in the green leaf were: UMP-5', UMP-3', UDP-sugars (UDPG), UDP, ADP, CTP, UTP, ATP, glucose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate, glucose-1, 6-diphosphate and sucrose-6-phosphate. During withering all the phosphate esters and nucleoside tri-phosphates decreased considerably, whereas the nucleoside diphosphates increased. The changes during fermentation and firing were not predictable because the tissues have been completely damaged by bruising. The fermented leaf and made tea contained appreciably greater quantities of nucleoside triphosphates compared to the withered leaf. Most of the compounds studied play an important role in the biosynthesis of terpenoid compounds of the tea plant. Some of these terpenoid compounds are responsible for tea aroma and flavour.

Of the various solutes in tea leaves, polyphenols, (Roberts 1962; Wickremasinghe & Swain 1965), amino acids (Bhatia & Deb 1965; Roberts & Sanderson 1966), organic acids (Sanderson & Selvendran 1965), chlorophylls (Wickremasinge & Perera 1966) carotenoids (Tirimanne & Wickremasinghe 1965) and volatile constituents (Wickremasinghe & Swain 1965 ; Yamanishi *et al* 1966; Bondarovich *et al* 1967) in fresh leaves and black tea have been studied. These groups of compounds are known to be concerned with the quality and flavour of tea. The quantities of polyphenols in the leaf have been shown to influence the strength, colour and 'briskness' of black tea liquors and thus play an important part in the assessment of quality. The amino acids and volatile constituents have been shown to contribute to the aroma complex of black tea. However, no information is yet available on the phosphate esters and nucleotides in tea leaves, which by analogy with the role they play in other plant tissues might be intimately linked with the metabolism of the groups of compounds mentioned above, and also of carbohydrates and other compounds. This lack of information is because it is difficult to isolate phosphate esters from tissues of plants like tea which contain 25 to 30% of their dry weight as polyphenols. Interest in the phosphate esters and nucleotides is not limited to a study of their occurrence in fresh leaves but extends to the manufacture of black tea. It is known from work on strawberry leaves that phosphate esters are concerned with many of the changes that take place during withering and the same may be true of tea leaves (Selvendran 1967).

The present investigation on tea has been divided into two main stages: the first being the isolation and partial characterization by ion exchange chromatographic and enzymatic methods of some of the phosphate esters and nucleotides in the trichloroacetic acid extract of tea shoots; and the second, the measurement of the concentrations of these compounds during tea manufacture. In addition, the changes in carbondioxide output were also determined.

Experimental

Plant material

The shoots (terminal bud with two leaves attached) plucked from selected bushes at the Tea Research Institute of Ceylon, Talawakele (4500 ft amsl) were used. After the shoots were plucked, samples of green leaf were packed in polythene bags as soon as possible and flown from Ceylon to Cambridge. The samples were kept at 3–5°C during transit and used in the laboratory about two days after being plucked. The term 'fresh leaves' is, therefore, used in a restricted sense because of this delay and it is known that 'Chemical Withering' begins immediately after the leaf is separated from the parent stock. All experiments were carried out on leaves from clone TRI 777.

Manufacture of black tea

The tea was manufactured as is usually done in Ceylon factories, except that the temperature of withering and fermentation was 15°C instead of 21 to 26°C. The flush was spread on a perforated tray and allowed to wither undisturbed for 19 hr until the moisture content was reduced by 25%. The withered leaf was then minced and the macerate spread on a tray, and allowed to 'ferment' at 15°C for two hr. The 'fermented' leaves were then 'fired' (dried at 190°F for 35 min.). As the enzymes in the tissue are not inactivated until the last step, *ie* 'firing' some metabolic processes can take place until that point.

At appropriate time intervals, samples (from 60 g fresh leaves each) were taken and dropped into liquid nitrogen, ground to a fine powder and stored in a room at –15°C under liquid N_2 until required for use.

Carbondioxide emission

Carbondioxide output by the samples was measured by passing CO_2-free air over them and collecting the evolved CO_2 in Pettenkoffer tubes containing barium hydroxide solution.

Extraction and isolation of phosphate esters and nucleotides

All extraction and fractionation procedures were carried out in a room at –3°C. The procedures were similar to those described by Isherwood & Barrett (1967) and Selvendran & Isherwood (1967). On passing the trichloroacetic acid extract (TCA extract from which most of the TCA has been removed by ether extraction) of tea leaves through the cation exchange column, a loss of combined phosphate (about 6%) was observed. Elution of the cation exchange column subsequently with 3N ammonia removed the adsorbed phosphate compounds. Tea leaf like strawberry leaf is rich in polyphenols and the purified extract was passed through a polyclar column to remove residual polyphenols.

Separation of the nucleotides

The purified extract (equivalent to 15 g leaves neutralized with aq. ammonia to pH 7) was added to the top of a column (20 cm × 1 cm diam.) of Dowex–1–resin (2% cross linked ; formate form, 200–400 mesh) and any unadsorbed material was eliminated with water. The nucleotides were separated by gradient elution with aq. ammonium formate using the technique described by Selvendran & Isherwood (1967). The eluate was collected in 5 ml fractions and the elution of the nucleotides was followed by measuring the extraction ($E_{260}^{1\,cm}$) with an Unicam SP 500 Spectrophotometer. One hundred and fifty fractions showing nine different adsorption peaks were collected and analysed.

Enzymatic methods for the estimation of phosphate esters and nucleotides

The enzymatic methods used for the estimation of glucose–6–phosphate (G–6–P), fructose–6–phosphate (F–6–P), glucose–1–phosphate (G–1–P), glucose–1, 6–diphosphate (G–1,6–diP), sucrose–6–phosphate (S–6–P), uridine diphosphate glucose (UDPG), ATP, ADP and AMP were similar to those described by Barker *et al* (1964) and Isherwood & Selvendran (1969).

Estimation of phosphorus

Inorganic and total phosphate were determined by the method of Allen (1940).

Results

The phosphate content in the crude TCA extract and CO_2 output of fresh, withered, fermented and made tea leaves are summarized in Table 1.

TABLE 1 — *Changes in the acid soluble phosphate and CO_2 output of tea leaves during manufacture*

	Constituent analysed (mg/100g fresh leaves)		
	Inorganic phosphate (as P)	Total phosphate (as P)	CO_2 (output)/hr
Fresh leaves	17.7	43.8	47.0
Withered leaves	15.9	51.1	26.4
Fermented leaves	24.1	46.5	4.4
Made tea	30.4	65.1	2.0

Separation and estimation of nucleotides and phosphate esters

The elution sequence of nucleotides from tea leaves is shown in Fig. 1. Some of the nucleotides were identified from absorption characteristics and their concentrations were determined from the extinction ($E_{260}^{1\ cm}$) values. In these calculations no allowance was made for the presence of contaminating nucleotides in individual peaks, as no values were available. The enzymatic estimates of ADP included a small contribution from the other nucleotide diphosphates.

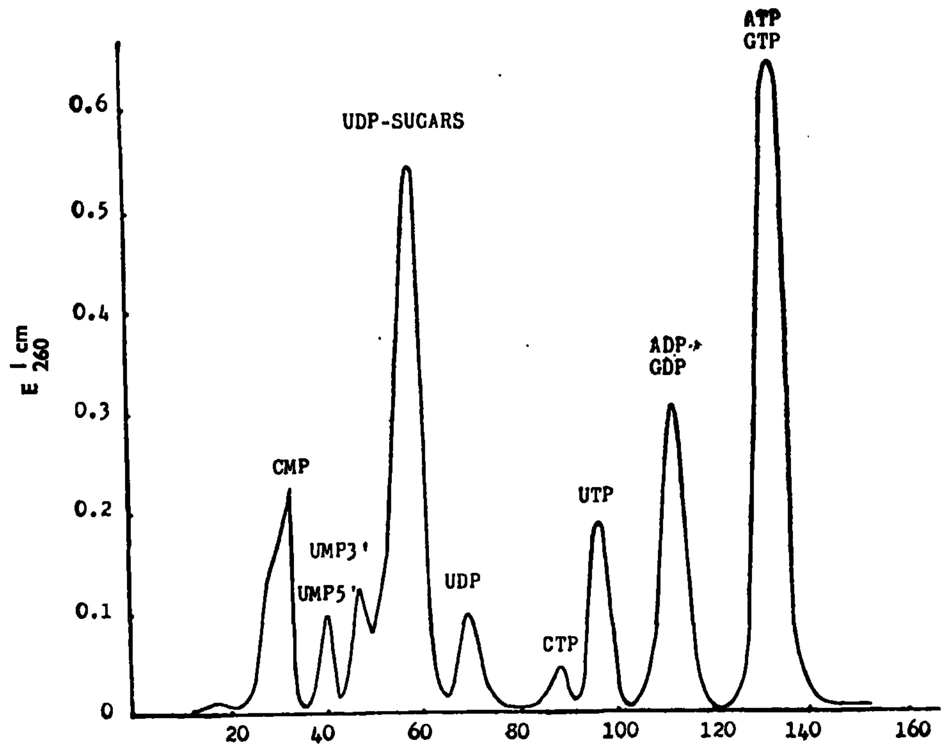

FIGURE 1—*Elution sequence of nucleotides*

Changes in phosphate compounds during the manufacture of black tea

In Table 2 the changes in the concentrations of some phosphate esters in tea shoots during manufacture are given as μ moles/100g fresh weight.

TABLE 2 — *Changes in the concentrations of phosphate esters during tea manufacture as μ moles / 100g fresh weight*

Compound	'Fresh shoots'	'Withered shoots' (19 hr of withering)	Fermented leaves (2 hr of fermentation)	Made tea
Time sampled	0 hr	19 hr	21 hr	21 hr 35 min.
G–6–P	9.7	6.6	5.1	4.7
F–6–P	1.4	0.83	1.22	1.10
G–1–P	0.63	0.52	0.23	0.66
G–1,6–diP	0.06	0.01	0.04	0.13
S–6–P	0.01	0.01	0.01	0.01

It was found that G–6–P, F–6–P, G–1–P and G–1,6–diP decreased on withering. The changes occurring during fermentation and firing were not predictable. This is because the tissues have been completely damaged by bruising. It was noted that G–1,6–diP increased on firing and a similar increase of G–1–P was also observed. S–6–P remained at a low constant level throughout this process.

In Table 3 the changes in the concentrations of the nucleotides in tea shoots during manufacture are given as μ moles/100g fresh weight.

TABLE 3 — *Changes in nucleotides during tea manufacture as μ moles/100g fresh weight*

Compound	'Fresh shoots'	'Withered shoots'	Fermented leaves	Made tea
Time sampled	0 hr	19 hr	21 hr	21 hr 35 min.
UMP-5'	2.1	3.5	2.8	3.3
UMP-3'	1.5	2.74	—	36.3
AMP	(0.02)	(0.02)	(0.02)	(0.02)
UDP–sugars	22	15.7	19.6	11.0
UDPG	(7.1)	(3.4)	(3.6)	(4.5)
UDP	3.8	4.0	4.0	4.7
ADP	7.3 (9.2)	6.5 (9.2)	7.2 (10.2)	12.8 (11.6)
CTP	5.7	0.1	3.6	13.8*
UTP	7.0	0.58	2.8	9.1*
ATP	18.1 (15.0)	0.63 (0.1)	11.6 (9.4)	8.9 (4.7)

The values within brackets were determined by enzymatic techniques.

*In the made-tea sample, the absorption spectra of the peaks corresponding to CTP and UTP were not characteristic of the nucleotides.

The monophosphates (UMP-5' and AMP) changed slightly during manufacture. UDP–sugars and UDPG decreased on withering. The diphosphates (UDP and ADP) changed slightly on withering while the triphosphates CTP, UTP and ATP decreased markedly. Fermentation caused slight changes in the nucleoside diphosphates but resulted in a considerable increase in all the nucleoside triphosphates. The most important change which occurred on firing was the sharp increase in uridine–3' or 2' phosphate. The identity of this compound was established by cochromatography with a mixture of uridine 2' and 3' monophosphates on a Dowex–1 anion exchange column (formate form) using the ammonium formate system. UDP and ADP increased on firing, possibly as a result of the breakdown of triphosphates. The concentration of AMP remained at a very low constant level during the manufacturing process.

Discussion

The concentrations of the phosphate esters and nucleotides in tea shoots were comparable to the values reported for other leafy tissues (Selvendran 1967). The very low concentration of sucrose-6-phosphate in tea shoots compared with the relatively higher values for this compound in mature strawberry leaves could be due to the low capacity of immature leaves to synthesize sucrose. This hypothesis is reflected in the very low concentration of glucose and sucrose in tea shoots compared with mature strawberry leaves. The concentrations of glucose and sucrose in tea shoots (Clone TRI 777) were 32 and 175 μ moles/100g fresh weight respectively, whereas they were 2130 and 6472 μ moles/100g fresh weight in mature strawberry leaves (Selvendran & Isherwood 1969).

The manufacture of black tea is a complex biological process which is affected by the extent to which the tissue is broken down by mechanical means during processing. Withering is accompanied by loss of turgor and an increase in the permeability of cell membranes. The latter could be expected to alter protoplasmic compartmentalization and affect spatial relationships between enzymes and substrates (Sanderson 1968). The disturbance of enzyme-substrate relationships would also alter respiratory processes considerably. These phenomena may be reflected in the changes of phosphate esters and nucleotides. In practice it was found that withering caused a significant decrease in the level of CO_2 output and this change was parallel to the change in the phosphate esters studied, presumably because of the irreversible loss of carbohydrates. The nucleoside triphosphates decreased markedly, while the nucleoside diphosphates changed little. The high energy phosphates are perhaps being used up in some phosphorylation reactions and are not regenerated at the same rate. Mincing causes rupture of the cell membranes and considerably alters the respiratory mechanism of the tissue. This results in extra phosphorylation reactions as seen in the marked increase in the level of all nucleoside triphosphates. It appears, therefore, that kinases are important in tea manufacture and it may be useful to study the biological conditions (pH, temperature, metallic ions like Mg) which affect their activity in order to improve the quality of the finished product. A surprising discovery was that these reactions were proceeding in the presence of the tea polyphenols which are generally assumed to be inhibitory to enzymes and it may be that in tea manufacture the enzymes are protected.

The considerable increase in inorganic and acid soluble organic phosphate on firing indicates that insoluble polyphosphates were being broken down. Preliminary experiments indicated that the residue after TCA extraction contains appreciable quantities of organic phosphate. Partial fractionation of the residue into phospholipids, nucleic acids and phosphoroteins was carried out. It is difficult to comment on the changes in CTP and UDP on firing as the absorption spectra of the corresponding peaks (in the made tea sample) were not identical with that of cytidine and uridine nucleotides.

The marked increase in uridine-3'-phosphate on firing is probably the result of the breakdown of nucleic acids. This is interesting because it is known that 5'-ribonucleotides are important flavouring constituents and the presence of a large quantity of a similar nucleotide may be important in influencing the quality of the finished product (Shimazono 1964). The effect of the 3'-nucleotides on quality has not been investigated, and in view of the marked changes in the level of these constituents during firing, it may be worthwhile investigating their effects on tea manufacture.

Acknowledgement

I gratefully acknowledge the receipt of a Broodbank Post-doctoral Fellowship, awarded by the University of Cambridge, during the tenure of which this work was carried out at the Low Temperature Research Station, Cambridge.

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