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DEVELOPMENT OF A MULTI-COMPONENT ANALYSIS SYSTEM

APPLICATION AND PRELIMINARY RESULTS OF A COMPARATIVE STUDY OF CELLULAR METABOLISM IN HEALTHY AND DAMAGED *PICEA* TREES FROM POLLUTED AREAS

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SUMMARY

A multi-component analysis system is described, based on electrophoresis (for high-molecular-weight analysis), automatic column chromatography (for low-molecular-weight analysis) and flow injection analysis (for total functional analysis). The whole system is interconnected with the LABNET network. In this system chromatography is carried out with the help of automatic analysers (mostly laboratory-made) using high-performance column chromatography on ion exchangers adapted for the specific analysis of essential metabolites grouped by "family" (sugars, amino acids, polyamines, etc.). The most important feature is that all the different "family" analyses are run on a single sample without purification.

This system has been partially applied to a comparative analytical study of cellular metabolism between healthy and damaged *Picea* trees in polluted areas (acid rain). This makes it possible to detect metabolic disfunctioning caused by pollution and to deduce further research goals.

The first results on sugar and amine content showed marked differences between healthy and damaged trees that can be correlated with their physiological state. The results are presented and discussed in the light of the biochemical knowledge of the biological role of the different metabolites with particular emphasis on the precursor–product relationship.

INTRODUCTION

Modern analytical biochemistry is helping considerably in understanding how many, if not all, diseases are linked in some way with deviations from, or alterations in, one or more of the several thousand chemical reactions that normally take place inside the cells and body.

The kinetic analysis of interrelated metabolites, with particular emphasis on the precursor–product relationship including both high- and low-molecular-weight substances, can provide new information on the biochemical reactions taking place in the cells in healthy and diseased states.

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However, studies carried out so far on the reaction sequences in cell metabolism are generally incomplete. It is clear that much additional work is needed to clarify the pathways of the different cell metabolites, to understand the control of their metabolism and to find why in some instances unusual pathways are favoured that differ, often radically, from those which commonly occur.

Multi-component analysis and chromatographic profiling with the help of computers are recent, rapidly developing techniques that are useful for these metabolic studies[1]. For instance, the profiling of urinary organic acids to detect metabolic abnormalities has proved to be a powerful means of identifying new diseases[1–3]. The profile approach to the study of inborn errors of metabolism has been applied via steroid analysis[4]. The use of stable isotopes for *in vivo* studies on metabolic pathways in healthy and diseased states is an alternative approach that is likely to increase rapidly in importance[5].

Most chromatographic profiling studies have been carried out by gas chromatography, gas chromatography coupled with mass spectrometry[6] and classical high-performance liquid chromatography[7] and in these instances it was necessary to apply prior sample purification procedures, which of course are time consuming and may result in losses of minor compounds.

When in 1976 we became interested in computer-assisted multi-component analysis and chromatographic profiling techniques, we decided to develop an analytical system able to use crude biological samples and to analyse in a single sample the largest possible number of metabolites.

We report here the basis of the method and some preliminary results of its application to the study of the biochemical and physiological effects of pollution (acid rain) on *Picea* trees.

EXPERIMENTAL

Samples

Trees from the polluted forest of the Vosges region (Massif du Donon) were selected. They were apparently healthy, damaged or strongly damaged. Needles from these trees were collected and immediately frozen and kept in dry-ice for transport. The needles were then powdered in liquid nitrogen and extracted with a solution (1 ml per ten needles) of 5% trichloroacetic acid (TCA) in 0.05 *M* hydrochloric acid containing appropriate internal quantitation standards.

In addition, experiments were carried out under controlled conditions. Young *Picea* trees were grown in experimental chambers as described previously[8] in the presence of the absence of subnecrotic levels of sulphur dioxide (0.08 ppm). Once a month, ten needles from each young tree were collected and treated as previously described.

Analytical systems

Low-molecular-weight metabolites were analysed by ion-exchange chromatography. All the chromatographic systems used were similar, and some of them have already been described in detail[9–11]. High-molecular-weight substances were analysed by polyacrylamide gel electrophoresis using a Bio-Rad apparatus (Bio-Rad Labs., Richmond, CA, U.S.A.). The total amount of some groups of metabolites (proteins,

sugars, amines, acids, thio derivatives, etc.) where determined by flow injection analysis (FIA). All the components of the multi-component analytical system were interconnected by the Spectra-Physics LABNET network to an IBM-XT microcomputer.

RESULTS AND DISCUSSION

Multi-component analytical system

A schematic diagram of the system and the different steps in sample preparation (in this instance for needles of *Picea* trees) are shown in Fig. 1.

The complete system aims at analysing a single crude sample for the largest possible number of cell metabolites, of both high and low molecular weight. For this, we used for extraction a solution containing suitable concentrations of TCA and hydrochloric acid to precipitate high-molecular-weight products (mostly proteins and

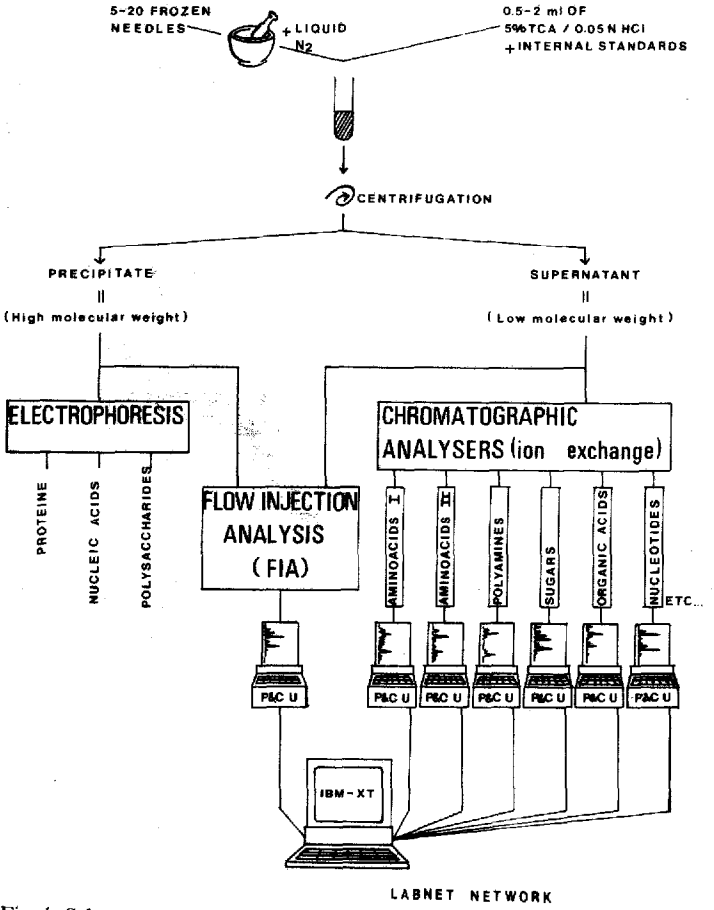

Fig. 1. Schematic diagram of the multi-component analytical system. Automation of the chromatographs is effected using the event external control cable of a 4200 integrator (Spectra-Physics), which serves also for quantification of the chromatographic analysis. The whole system is interconnected with the LABNET network. P&C U = programmer and calculator unit; Amino acids I = amino acids analyser; Amino acids II = modified amino acids analyser; Polyamine = polyamine analyser, etc.

nucleic acids) and to dissolve low-molecular-weight metabolites; the hydrochloric acid helps to fix and to stabilize some volatile compounds, particularly amines. Subsequent centrifugation allows the precipitate (high-molecular-weight substances) to be separated from the supernatant, which contains low-molecular-weight substances. Aliquots of this supernatant are used for high-performance ion exchange chromatographic analysis and the precipitate, after de-lipidation, is dissolved and serves for analysis by electrophoresis.

In this multi-component system a series of chromatographic analysers are used. Each chromatograph is devoted to the analysis of one "family" of metabolites (*e.g.*, one for polyamines, another for sugars and another for amino acids). To make this possible, each analyser uses a specific post-column reaction detection method (*e.g.*, *o*-phthalaldehyde for amino compounds and ethanolamine–boric acid for borate–sugar complexes). Whenever possible we used fluorimetric detection, which allowed us to work at the picomole or nanomole level.

As our ultimate goal is the direct analysis of the crude biological sample, a single solution of appropriate concentrations of hydrochloric acid and TCA (which extracts and fractionates the sample) was used in conjunction with automatic ion-exchange chromatography. Buffer compositions of the appropriate ionic strength and pH allowed in general the retention mostly of the class of compounds to be analysed and the washing out of the other substances before the start of the separation and elution process. After each analysis, the analytical programme included column regeneration and re-equilibration cycles.

This multi-component system, makes it possible to follow and to correlate metabolic chain kinetics and turnovers of the pairs precursor–product (*e.g.*, amino acids–proteins, sugars–polysaccharides, ornithine or arginine-polyamines).

At present four analysers are operational, as follows.

Analyser for usual amino acids. Twenty-five substances are analysed in 90 min at the picomole level: phosphoserine, taurine, phosphoethanolamine, hydroxyproline, methionine sulphone, aspartic acid, threonine, serine, glutamate, glutamine, proline, citrulline, α-aminoadipic acid, glycine, alanine, α-aminobutyric acid, valine, cysteine, methionine, cystathionine, isoleucine, leucine, norleucine (internal standard), tyrosine and phenylalanine.

Analyser for modified amino acids. Twenty-four compounds are analysed in 118 min[11] at the picomole level: tyrosine, phenylalanine, ε-aminocaproic acid, 5-hydroxytryptophan, histidine, 3-methylhistidine, tryptophan, 1-methylhistidine, carnosine, anserine, hydroxylysine, diaminobutyric acid, ornithine, lysine, ammonia, ethanolamine, monomethyllysine, dimethyllysine, trimethyllysine, arginine, carbamylputrescine, unsymmetric dimethylarginine, monomethylarginine and symmetric dimethylarginine.

Analyser for polyamines. Twenty compounds are analysed in just over 1 h at the picomole level[9]: diaminobutyric acid, ornithine, lysine, ammonia, ethanolamine, arginine, acetylputrescine, carbamylputrescine, histamine, aminopropane, diaminopropane, putrescine, cadaverine, norspermidine, spermidine, homospermidine, agmatine, nor-spermine, spermine and 1,7-diaminoheptane (internal standard).

Analyser for sugars. Seven compounds are analysed in 40 min at the nanomole level[10]: raffinose, sucrose, maltose, ribose, fructose, xylose and glucose.

Other analysers will be soon in service to allow the analysis of guanidine derivatives, organic acids, bases, nucleosides and nucleotides.

Comparative study between healthy and damaged Picea trees in polluted areas

This partially developed analytical system has been applied to a comparative study of cellular metabolism between healthy and damaged *Picea* trees from polluted areas, with the intention of detecting metabolic disfunctioning caused by pollution.

Twenty needles of *Picea* trees were extracted with 2 ml of 5% TCA in 0.05 *M* hydrochloric acid[10]. After centrifugation, aliquots of the supernatant were analysed for sugars (5 μl), amino acids (100 μl), modified amino acids (100 μl) and polyamines (50 μl).

Sugar analysis. As can be seen in Table I, damaged trees accumulate free fructose and glucose when compared with healthy trees. This suggests an abnormal situation in the metabolism and biosynthesis of mono- and polysaccharides, caused by the physiological effects of air pollution. A similar situation has been reported in Scots pine trees subjected to sulphur dioxide pollution[12]. The authors suggested that the increased amount of monosaccharides in the needles of damaged trees may contribute to their greater injury by gaseous emissions, as an increase in the level of glucose and fructose increases the turgor of cells, and this in turn causes opening of the stomata through which pollutants can enter and disturb the carbohydrate metabolism. The smaller amounts of glucose and fructose in the seemingly healthy trees may be caused by a more efficient conversion of glucose into polysaccharides; this lowers the osmotic potential of cells in the needles, which could cause closure of the stomata and a reduction in the amount of pollutants entering the leaves.

Amino acid analysis. The most important differences observed concern the contents of histidine and tryptophan. Table II shows the results of the analysis of needles from three healthy and three damaged *Picea* trees. We are not able at present to interpret the differences in the content of histidine. However, the large differences in the tryptophan content could indicate abnormal kinetics of utilization of this compound, which is known to be the precursor of indoleacetic acid, the growth hormone of plants[13].

Arginine and polyamine analysis. The polyamine analyser gave the most interesting results[14]. Table III shows a representative analysis of healthy and damaged needles of polluted *Picea* trees. Noteworthy are the considerable differences observed in the content of arginine, and also of putrescine, spermidine and spermine. Healthy trees contain relatively large amounts of arginine and small amounts of polyamines

TABLE I

FREE SUGAR CONTENTS IN NEEDLES OF POLLUTED *PICEA* TREES

Sugar content is expressed in pmol/μg protein (n = 3).

Tree type	*No.*	*Fructose*	*Glucose*
Healthy	1	9.4 ± 0.12	6.0 ± 0.12
	2	12.5 ± 0.14	11.5 ± 0.21
	3	9.2 ± 0.12	6.0 ± 0.14
Damaged	4	27.8 ± 0.71	23.2 ± 0.10
	5	24.5 ± 0.66	18.8 ± 0.21
	6	31.3 ± 0.57	25.2 ± 0.17

TABLE II

HISTIDINE AND TRYPTOPHAN CONTENTS IN NEEDLES OF POLLUTED *PICEA* TREES

Histidine and tryptophan contents are expressed in pmol/μg protein (n = 3).

Tree type	*No.*	*Histidine*	*Tryptophan*
Healthy	1	21.7 ± 0.13	51.8 ± 0.25
	2	14.5 ± 0.14	38.0 ± 0.25
	3	17.2 ± 0.14	50.6 ± 0.33
Damaged	4	27.2 ± 0.18	83.0 ± 0.33
	5	26.4 ± 0.16	87.3 ± 0.36
	6	29.1 ± 0.21	86.4 ± 0.17

whereas damaged trees contain small amounts of arginine and large amounts of polyamines.

These results indicate clearly that amine synthesis is one of the metabolic responses of plants to acid pollution. Amine synthesis could be induced during a primary process in which sulphur dioxide, once it is absorbed by the plants, is dissolved in the cell water to generate H^+, HSO_3^- and SO_3^{2-} ions, the last being oxidized to SO_4^{2-}. This results in an increase in H^+ concentration in the tissues and in a reduced cation to anion ratio. The metabolic system responsible for the maintenance of cellular pH must modify the kinetics and turnover of organic acids and amines, which are the main components involved in the buffering reactions of the cell.

On the other hand, it is now well established that plants exposed to ionic stress respond by increasing their endogenous level of putrescine[15]. Although this phenomenon was first observed in plants exposed to K^+ deficiency[16], the putrescine content is now known to increase under conditions of Mg^{2+} deficiency, low external pH, high levels of NH_4^+, sulphur dioxide fumigation and osmotic shock[17–19]. It is also well known that putrescine and the polyamines spermidine and spermine derived from it can associate with and affect the behaviour of proteins, nucleic acids and membranes[20–22]. Tracer studies have demonstrated that in plants putrescine is produced from agmatine, the decarboxylation product of arginine. A key rate-limiting enzyme in the conversion of arginine to putrescine is arginine decarboxylase, the

TABLE III

ARGININE AND POLYAMINES CONTENTS OF NEEDLES OF POLLUTED *PICEA* TREES

Contents are expressed in pmol/μg protein (n = 3).

Tree type	*No.*	*Arginine*	*Putrescine*	*Spermidine*	*Spermine*	*Total polyamines*
Healthy	1	308 ± 7.81	30 ± 0.71	132 ± 2.16	4 ± 0.11	166
	2	200 ± 5.31	25 ± 0.51	115 ± 2.42	1 ± 0.03	141
	3	335 ± 7.13	34 ± 0.60	129 ± 1.70	1 ± 0.03	164
Damaged	4	104 ± 2.62	55 ± 1.08	387 ± 3.86	10 ± 0.08	452
	5	189 ± 4.55	46 ± 0.95	361 ± 4.97	6 ± 0.07	413
	6	148 ± 2.45	65 ± 1.16	368 ± 3.40	8 ± 0.12	441

activity of which has been found to be increased by different kinds of ionic stress[23,24], particularly acidic stress[15].

In this work, the differences in amine content observed between samples of healthy and damaged *Picea* trees could be the result of long-term kinetic (healthy) and short-term kinetic (damaged) responses of the plant. Both healthy and damaged trees have initially, as a primary response to acid stress, increased their putrescine and polyamine biosynthesis. The acid environement contributes to raise the arginine decarboxylase activity, the key enzyme in polyamine biosynthesis. In a following step, some trees will be able to modify, as an adaptative response, their metabolic amine paths. The accumulation of arginine could increase the intracellular pH and as a consequence the arginine decarboxylase activity will decrease, resulting in a comparatively high level of arginine and a lower level of putrescine and polyamines as observed in healthy trees (Table III). This is what we call a long-term kinetic response. Those trees which have not been able to modify their amine metabolic pathways will continue to use their arginine to produce putrescine and polyamines as a result of a high arginine decarboxylase activity due to the acidic environment. These trees will remain in what we call a short-term kinetic response and as a consequence they will contain a low level of arginine and high levels of putrescine and polyamines.

As already pointed out, H^+ ions stimulate polyamine synthesis. For the same reason, long exposure of plants to sulphur dioxide induces high polyamine levels. It is therefore possible that the same mechanism by which the cell pH is maintained could cause damaging effects to the plant cell.

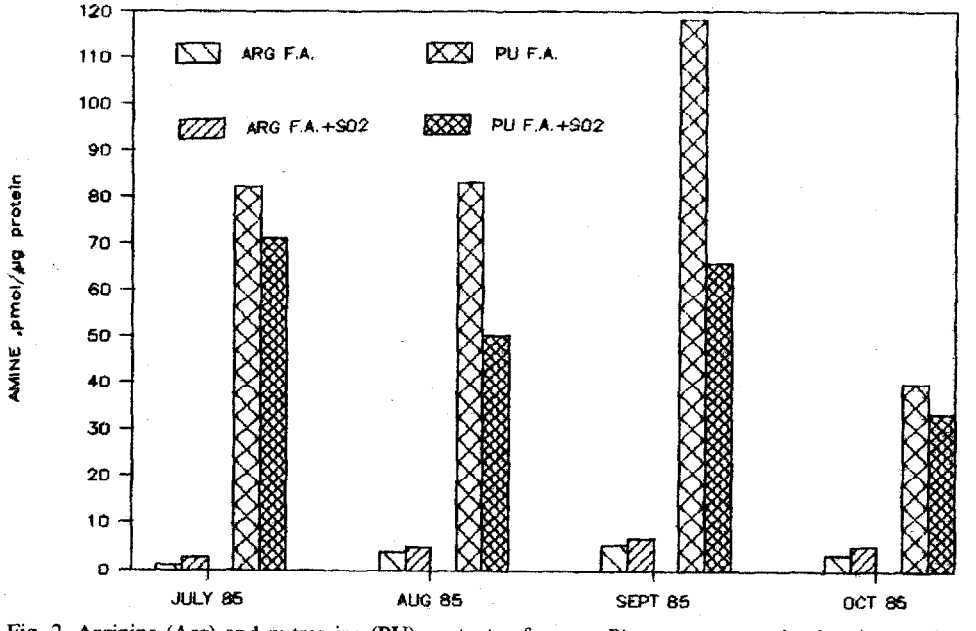

Fig. 2. Arginine (Arg) and putrescine (PU) contents of young *Picea* trees grown in chambers under a filtered air atmosphere (F.A.) and filtered air + 0.08 ppm of sulphur dioxide (F.A. + SO_2). Although the results are not spectacular, a tendency for a high level of Arg and a low level of PU can be observed, as a function of time, in the trees growing in the presence of sulphur dioxide.

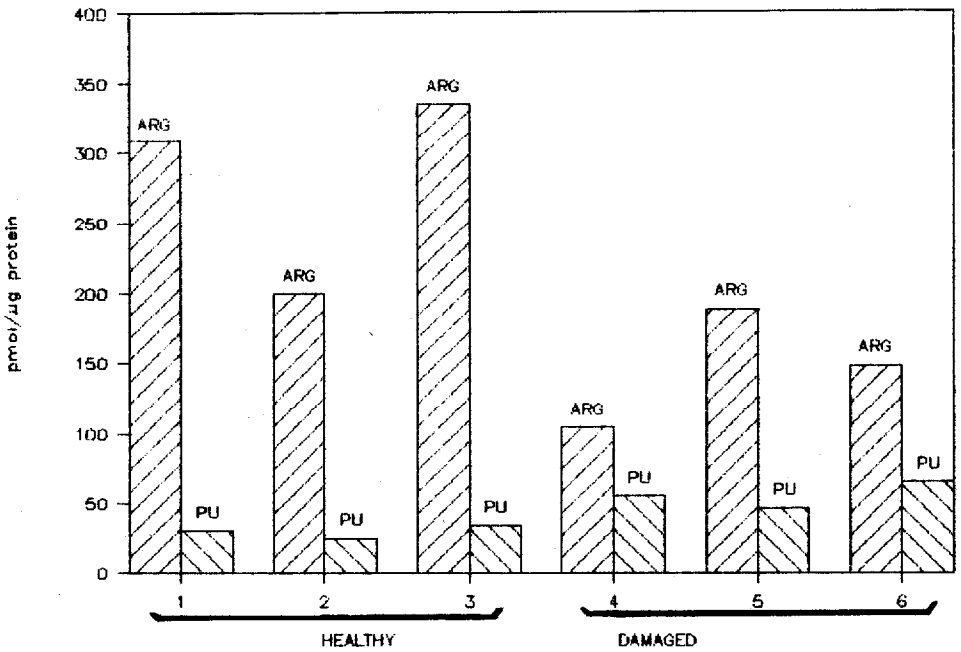

Fig. 3. Histogram showing the arginine (Arg) and putrescine (PU) contents of needles of three samples of healthy (1, 2 and 3) and three samples of damaged (4, 5 and 6) *Picea* trees from a polluted area (Massif du Donon, Vosges, France).

The explanations proposed found some confirmation when experiments under controlled conditions with young *Picea* trees were carried out. They were grown in the absence or presence of subnecrotic levels of sulphur dioxide (0.08 ppm). After 1 month, needles were collected for analysis once a month for a period of 4 months. As can be seen in Fig. 2, the comparative analysis of arginine and putrescine showed a tendency for the trees submitted to sulphur dioxide to increase their arginine content and to lower their putrescine content, as in healthy, resistant trees from a polluted forest (Fig. 3). This strongly supports the suggestion that the differences observed in the contents of arginine and putrescine between healthy and damaged *Picea* trees in the polluted forest may be a consequence of the physiological action of pollution and that the situation in the healthy, resistant trees corresponds well to a long-term kinetic metabolic response.

Of course, more detailed research is necessary in order to understand fully the biochemical and physiological effects of pollution on forest trees. Further investigations of the effects of pollutants cell metabolism may be useful both to obtain and understand functional changes in response to exposure and to provide information concerning the mechanisms of pollutant–metabolism interactions.

This problem provides a good example of the research possible using multicomponent analysis techniques, where the aim is to separate and identify as many constituents as possible. On-line mini- and microcomputer systems now offer the analyst a number of possibilities, such as adaptative program interaction, integrator/calculator functionality, instrument automation, automatic data acquisition and

data reduction, control of the sampling system, automatic peak detection, detection and measurements of overlapping peaks and pattern recognition techniques.

In many instances the need for computer evaluation of the analytical profile may be unnecessary. This may be true when one is dealing with metabolic disorders leading to gross metabolic changes. Other biochemical and physiological conditions, however, may result in more subtle, yet perhaps important changes in the metabolic patterns. It seems reasonable to assume that the best way to detect these minor alterations will be by means of suitable advanced computational methods.

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