Biochemical Analysis in Crop Science

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Foreword

This book will interest many people in plant biochemistry as an extremely relevant science for securing progress in crop production for the future. The importance of such progress is obvious at a time when parts of the Third World are already seriously short of food, when many more parts have increasingly grave difficulties in the face of population explosions, and when in both Western and centrally controlled economies the importance of reliable sources of food and feeding stuffs has, once again, been made dramatically clear.

Many young scientists, striving to master recent explosions of knowledge, find it hard to turn their appreciation of basic concepts and related theory to practical purposes, particularly in such broad and complex subjects as crop production and utilization. They have great difficulty in finding brief, readable accounts of the intricate results already obtained, and of the practical methods of obtaining them. They need a reliable guide to purposes and techniques related to the taxonomy of molecular types of proven importance. They also need careful selections of references so that particular matters can be pursued in greater depth as purposes and interests may later require. And many with years of experience of applied biochemistry will also find value in such a wide-ranging guide to give them a fresh view of developments outside their own particular specialities, and perhaps even to change their area of specialization.

Dr. Draper has planned his useful book so as to attract and help both those who specialized early in biochemistry and those whose first interests were in other sciences and their integrations in agriculture and horticulture. He has produced a comprehensive, but brief and readable, guide which also has a full bibliography, making it a useful reference book.

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1. Introduction

THE efficient production of food by the cultivation of crop species is the essential basis of intensive agriculture. The crop scientist has already played a central role in many of the major developments which have allowed our present systems of farming practice to evolve, and it is unlikely that further improvements will be achieved without a continued scientific analysis of the factors limiting crop production. To the crop scientist involved in these future developments, a knowledge of biochemistry will become increasingly important. There are two main arguments to support this statement. First, the improvement of crops by agronomic or genetic means involves the manipulation of the physiological and biochemical characteristics of the plant, and it is only by an appreciation of the cellular mechanisms involved that the likelihood of success in a particular project can be guaged, the causes of failure understood, or the future possibilities identified. Secondly, the evaluation of crop varieties or changed methods of husbandry often involves the chemical analysis of material in order to assess its nutritive value, storage characteristics, or dietary acceptability.

In the title of this book the term 'biochemical analysis' is intended to describe both of these aspects of plant biochemistry and this is reflected in the arrangement of the subject matter. Chapters 2–4 deal with the technical procedures associated with the sampling and analysis of temperate and tropical crops and their products. Many of these methods are applicable to a wide range of materials including grass and other forages, cereal grains, leguminous seeds, roots, tubers, and fruits. The major constituents found in plant tissues are the subject of Chapter 5, which comments on the physiological and metabolic significance of individual compounds and also outlines the appropriate analytical techniques required for their examination. It is hoped that this dichotomous approach will enable plant biologists with only an elementary knowledge of biochemistry to initiate experiments, and at the same time develop a greater appreciation of the function and importance of the particular compounds under examination.

The historical development of biochemistry in agriculture has centred on a study of the *nutritive value* of crops and their products. There are many excellent books and reviews covering this approach and these are referred to where appropriate. Although no detailed discussion of the nutritional significance of compounds is attempted here, *selected* observations have been included, especially when they appear to be particularly topical and the subject of current investigation. Mention is also made of toxic compounds which occur in plant material. These include natural constituents and exogenous substances such as pesticides.

Many scientists have forecast changes in our current approach to food production, with a shift towards the direct exploitation of plants for human consumption, rather than for animal feeding. Undoubtedly, developments of this nature will demand a greater understanding of the mechanisms controlling the yield, composition, and quality of crop species and this is one of fields in which the biochemist will play an increasingly important part.

Although this book describes, as a continuous theme, the application of biochemical methods and principles to the scientific study of *crop* plants, it is intended that students in the pure sciences, as well as in agriculture, will find the subject matter of direct relevance to their studies, project work, or research. Each section includes a list of references which should enable the research worker to pursue a particular topic in far greater depth than is possible within this book. It is appropriate to make special mention of two standard works which are referred to extensively in the following pages. The first of these, *Modern methods of plant analysis*, edited by Paech and Tracey, was published as a series of volumes during the period 1956–64. The second, more recent work, *Phytochemical methods* (Harborne, 1973) also gives practical details of many useful techniques.

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2. Sampling, storage, and extraction of plant material

BEFORE the initiation of a programme of biochemical analysis, great attention must be given to the proposed methods of sampling, storage, and extraction. Although it is impossible to formulate exact procedures for all possible circumstances, an awareness of the problems likely to be encountered and how to solve them is sufficient to promote a critical attitude towards these preliminary aspects of analysis.

2.1 Sampling procedures

The biochemist working with crop plants may obtain tissue for analysis in a number of different ways. The sample may be derived from a growing crop in the field, from a bulk store of grain or other harvested material, or from laboratory experiments in which the conditions of growth or storage have been carefully controlled. The approach required in each of these situations depends on the purpose of the experiment and the likely variability in composition of the material in question.

As an example, consider two separate experiments in which the amino acid lysine is to be determined. In the first experiment the analyst may wish to compare the amounts of lysine in two different varieties of a cereal, perhaps barley. To carry out this task the varieties would have to be grown at a number of different centres, in replicated plots, over a period of several years. Sub-sampling procedures would have to be carefully standardized and replicated chemical analyses would be required in order to assess error inherent in the laboratory method. Only in this way would it be possible to confirm that any small difference encountered was due to a genetic rather than an environmental determinant.

In the second experiment, the aim might be to determine the lysine content of a protein fraction isolated from plant tissue. Say the fraction represented a single molecular species, perhaps an important enzyme such as ribulose diphosphate carboxylase, present in large enough amounts to influence the nutritive value of leaf protein. In this case it can be assumed that the fraction would have

a fixed composition *even when the environmental conditions of growth are varied.* Thus as long as the protein could be obtained in a purified form, the error associated with the lysine estimation would be entirely due to the *chemical* procedures employed, and replication would be necessary only in order to assess the extent of this particular source of error. This illustration shows that by applying the accepted dogma of modern biology, in this case by assuming that each specific enzyme protein has a unique primary sequence, it is possible to modify sampling procedures in appropriate circumstances and so reduce the number of analyses required. In the following discussion of field and laboratory experiments it will be assumed that similar arguments have been examined prior to the finalization of an experimental design.

2.1.1. *Field experiments*

One of the main difficulties associated with the collection of material in the field is the problem of obtaining a representative sample. For biochemical work this is especially true, due to the fact that usually only very small amounts of tissue will be used in the final assay. If samples are taken from a full-scale crop before harvest, a large number will be required from many positions in the field. Small plots are easier to examine and it is possible to carry out sequential studies by harvesting several plots at each of a number of occasions during the growing season.

The total number of samples analysed should be increased if it is found necessary to work with fresh, rather than with dried and milled material. This is because in the latter case, sub-sampling can be carried out much more easily with less chance of bias.

A further major problem arises when the compounds to be analysed are metabolically labile. For example, the analysis of free amino acids or simple sugars in leaves should ideally be carried out using extracts obtained immediately after removal of the leaves from the plant. An alternative is to freeze samples with solid carbon dioxide and transfer them to the laboratory in this condition. They should then be extracted *without thawing*. These precautions are necessary because at normal temperatures many enzymes associated with the metabolism of these compounds remain active in the leaves even after the tissue has been removed from the parent plant. Less difficulty is encountered with stable components such as lignin

or other cell wall materials, where samples can be stored in polythene bags and transferred to a drying oven later in the day.

2.1.2. *Greenhouse and laboratory experiments*

With experiments carried out in controlled, or semi-controlled conditions, there is generally less difficulty in effecting rapid transfer of material to the laboratory. Usually plants in growth cabinets are raised in pots or shallow trays and each of these units would normally constitute a single sample in any subsequent statistical treatment. For this reason the sampling procedures adopted in growth cabinet studies are usually worked out before the start of the experiment, the design of which may be limited by a restriction of space.

Detailed biochemical studies often involve complex analyses on many different components and it is important to remember that fluctuations in the levels of some of these components may occur in diurnal rhythm. Thus it is sometimes important to standardize the time of day at which samples are taken.

Usually it is the aerial part of the plant which is required, and samples are easily removed by excising the shoot system just above soil level. However, root samples of growing plants may also be under examination, in which case the removal of surface contamination is often extremely difficult. Plants grown in sand, using liquid nutrients, are well suited for root studies as the sand particles can be freed from the roots by careful washing in cold water.

2.2. Storage of samples

Samples must be stored in such a way that changes in chemical composition are avoided. The method of storage should always be chosen with due regard to the nature of the eventual analyses to be carried out. Changes in composition can be due to the action of enzymes, either occurring naturally in the cells of the sampled tissue, or produced by a microbial population present in the sample. Oxidative changes may also occur due to the reaction of molecular oxygen with compounds such as the unsaturated fatty acids. A less common problem is the loss of low boiling point constituents due to volatilization; this occurs if the conditions of drying are inappropriate. Individually, each of these processes can be minimized, but occasionally a storage procedure which prevents one unwanted event may favour a second, deleterious process. In such cases

TABLE 2.1
Occurrence and prevention of chemical changes in stored samples

Process	Example	Means of reducing rate of process
Oxidative deterioration	Autoxidation of unsaturated fatty acids	Storage under nitrogen
Metabolic transformations	Breakdown of proteins by proteolytic enzymes	Storage at low temperature or rapid drying
Microbial activity	Utilization of stored sample as a substrate for the growth of bacteria or fungi	Storage at low temperature or rapid drying
Volatilization	Loss of volatile fatty acids	Avoidance of heat treatment and storage in sealed containers

separate samples may be required for particular analyses. The application of different storage procedures in the prevention of the deterioration of samples is outlined in Table 2.1.

2.2.1. *Low temperature storage*

Fresh plant material may be preserved by freezing at −12°C to −18°C using plastic self-seal bags or plastic boxes as containers. Freezing should be carried out as quickly as possible and for this reason small samples are preferred. The rate of freezing can be increased by placing tissue into liquid nitrogen or by freezing with solid carbon dioxide. Deep-freeze cabinets of a commercial type, particularly those with access from above, are suitable repositories for samples, but trial experiments should be carried out to check on the long term stability of the biochemical components which are under examination. Deep-freeze rooms are useful for large scale storage but it should be noted that often a 'de-frost' period is programmed each day for this type of installation and the temperature may rise to an unacceptable level.

Storage at low temperatures above freezing point is unsatisfactory, as many biochemical transformations continue even at 2–3°C. Some physiological processes are even accelerated under these conditions. Samples which are allowed to thaw and are then re-frozen

should also be treated with caution. Freezing usually results in a loss of the permeability properties of the membranes of the cell and, upon thawing, enzymes and substrates previously in separate subcellular compartments may be brought into contact.

2.2.2. *Drying of samples*

For the routine examination of leafy material with respect to major constituents such as fibre, fat, nitrogen, and ash, the usual procedure is to dry the sample in a forced draught oven, at around 105°C, over a period of several hours or perhaps overnight. The sample can then be milled to a powder, the fineness of which can be checked by sieving. However, when certain plant metabolites are being studied, rapid drying may produce unwanted changes and, of course, in the case of enzyme assays such treatment would be completely inappropriate. However, if drying can be accomplished efficiently then this approach to sample preservation has wide applications and material may be stored for long periods of time. Indeed, Harborne (1973) cites reports of the analysis of essential oils of dried herbarium material dating back to the eighteenth century.

In addition to the usual form of laboratory oven equipped with a fan and an air venting arrangement, microwave ovens, which offer an alternative and extremely rapid means of drying samples are also available. However, their use is not yet widespread and they offer few advantages except in special circumstances—for example, when a large number of samples must be handled in a short period of time.

One of the most useful techniques for drying biological material involves the initial freezing of the tissue followed by sublimation of the ice at reduced pressure and low temperature. This procedure is known as *freeze drying*. It is extremely useful for drying protein precipitates and similar materials, which are obtained as light fluffy powders. Commercial equipment is available for processing fairly large quantities of wet tissues, slurries, or precipitates.

2.3. Extraction of samples

Samples for analysis may consist either of post-harvest material, often in a fairly stable physiological state, or of fresh material of high water content, such as leaf tissue. During extraction, care must be taken to minimize changes in chemical composition and to ensure

that the percentage recovery is satisfactory. When extracting fresh tissue with aqueous solvents, it may be difficult to break all, or nearly all, of the cells. Other reasons for low efficiency of extraction include the use of too small a volume of solvent, failure to make a sufficient number of sequential washings, the choice of an inappropriate solvent, and the loss of components by denaturation or chemical modification.

Fresh material may be dried and milled prior to extraction or it can be extracted immediately by disrupting the tissue in the solvent using some type of manual or mechanical technique. The simplest procedure is to use a conventional *pestle and mortar*, preferably of glass, with a little acid-washed quartz sand as an abrasive. *Cone-shaped pestles* which are rotated within glass mortars of the appropriate shape are also available and these can be operated by hand or by the drive from an electric motor, usually coupled to the pestle by a flexible mounting. They are suitable only for small volumes of liquid. Another alternative is the *Waring blender*, which consists of a glass container within which a rapidly rotating knife-edged propellor breaks up the tissue in the presence of an appropriate volume of solvent (Fig. 2.1). Additional techniques which are especially suitable for dried and milled material include *mechanical shaking* with solvent for a defined period of time, and continuous extraction, involving the percolation of condensed solvent in a refluxing system, through the sample contained in a porous thimble. This latter

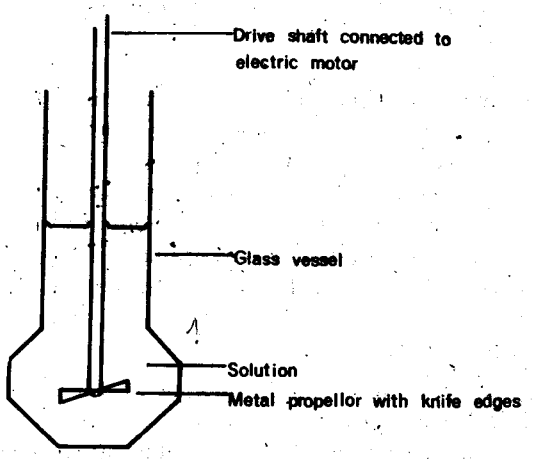

FIG. 2.1. High speed blender.

TABLE 2.2
Applicability of extraction procedures

Procedure	Organic solvents	Aqueous solvents	Disadvantages	Advantages
Mechanical shaking with solvent	Yes	Yes	Suitable only for dried and milled material	Simple and straightforward
Pestle and mortar	No	Yes	Difficult to reproduce and some cells may not be disrupted	Inexpensive apparatus and convenient to carry out
Power-driven cone pestle and mortar	Some	Yes	Suitable only for small samples	High shearing forces and good cell breakage
Waring blender	Yes	Yes	May cause frothing of solvent	Suitable for large samples
Reflux of solvent in presence of sample	Yes	No	Not suitable for fresh material	Very efficient

procedure, which is applicable only to organic solvents, usually involves the use of the *Soxhlet apparatus*. These procedures, together with comments concerning their applicability, are listed in Table 2.2.

2.3.1 *Aqueous solvents*

Occasionally, water itself is used as an extraction medium without the addition of any solutes. For example, the extraction of nitrate from dried plant material is usually accomplished by shaking a weighed sample with distilled water. However, some attempt is often made to control the pH and tonicity of the solvent, particularly when extracting enzyme proteins from fresh material. The problems associated with the extraction of enzymes are discussed in section 5.2.4. When the intention is to remove as much as possible of a particular enzyme from the tissue, a low molarity buffer likely to promote the osmotic lysis of subcellular organelles is normally employed. Tris buffer (0·04 M; pH 7·8) would be appropriate as a starting point in the development of a suitable extraction medium in these circumstances. On the other hand, if there is a requirement to maintain the structural integrity of the subcellular organelles, the osmotic strength of the solution must be increased by the addition of sucrose at a concentration of around 0·4 M. Other additives which

may be employed as a means of preserving subcellular structures include certain commercial products such as 'Ficoll', which is synthesized by the co-polymerization of sucrose and epichlorohydrin. These compounds are uncharged and of high molecular weight, and give solutions of low osmotic strength at a given concentration. They act as stabilizing agents in the extraction of proteins and in the preparation of subcellular fractions (Honda, Hogladorum, and Laties 1966).

It is often advisable to carry out extractions at low temperature in order to minimize enzyme activity. Buffers, glassware, and other containers should therefore be stored in the refrigerator before use. Certain electrically powered homogenizers have an outer container and this can be packed with an ice–water mixture, so cooling the slurry or brei as it is broken—up by the rotating blades.

One disadvantage of power driven homogenizers is a tendency for the mixture under treatment to froth at its surface, with a resultant deleterious effect on proteins in solution. If denaturation occurs as a result of frothing this inevitably leads to a loss of enzyme activity.

2.3.2. *Organic solvents*

Organic solvents differ in their polarity and thus in their suitability for the extraction of different components. Harborne (1973) recommends ethanol as a good all-purpose solvent in preliminary studies of phytochemical constituents. Mixtures of ethanol and water are suitable for the extraction of many different classes of compounds. The amount of water in the mixture can be modified, according to the water content of the sample, in order to arrive at an appropriate final concentration of ethanol. Light petroleum (b.p. 40–60°C) is useful for extracting non-polar lipids but mixtures of chloroform and methanol are more suited to the removal of phospho- and glycolipids. A mixture of $CHCl_3$:MeOH (2:1) has the advantage of remaining as a single-phase system even in the presence of small amounts of water derived from fresh samples.

Further specific recommendations relating to the use of organic solvents are given in Chapter 5.

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