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PROGRESS IN PHYTOCHEMISTRY

VOLUME 7

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This volume is respectfully dedicated to
Dr. E. C. Bate-Smith, C.B.E.
in celebration of his eightieth birthday

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FOREWORD

It is with pleasure and affection that we dedicate this volume of Progress in Phytochemistry to Dr. E. C. Bate-Smith, CBE, one of the leading pioneers of the subject, on the occasion of his eightieth birthday. An appreciation of Dr. Bate-Smith follows this Foreword. Many of the topics in this volume represent aspects of phytochemical research which he has encouraged in others or to which he has himself contributed. The first chapter, for example, is a contribution to chemotaxonomy, one of his major interests in the last 20 years, in that it considers in critical detail the contribution of isozyme electrophoresis to the understanding of plant variation at the population level. A key point made by L. D. Gottlieb, its author, is that studies of isozyme patterns must be linked to the genetic analysis of the plants from which the enzymes are obtained.

It is a continuing challenge to plant biochemists that many of the enzymes which vary electrophoretically are still 'looking for' a function; the precise roles of peroxidases, esterases, etc. in plant metabolism have yet to be fully defined. This is also true of carbonic anhydrase, the subject of our second chapter by M. L. Reed and D. Graham; although closely associated with the chloroplast and the photosynthetic process, this carboxylating enzyme has yet to have a definitive role assigned to it. The dynamic aspects of plant biochemistry are continued by a chapter on seed germination by A. M. Mayer and I. Marbach, who review key aspects of recent biochemical developments in this field. Plant hormones, particularly the gibberellins, have an essential role in the control of the germination process and they are considered here by R. Horgan in a chapter on hormone analysis. A multiplicity of new techniques, and especially high performance liquid chromatography, have recently been applied to the detection and estimation of each of the five main classes of endogenous plant regulators so that this up-to-date survey of the subject should be of interest and value to a wide audience.

The non-protein amino acids of plants were reviewed by L. Fowden in Volume 2 of this series. We are pleased to include here, therefore, a chapter by E. A. Bell covering progress in the study of these taxonomically and ecologically interesting molecules since that time. Of especial value is the listing of all new non-protein amino acids reported in higher plants over the last decade.

Various aspects of plant phenolics—another key area among Bate-Smith's interests—have been considered in earlier volumes of this series and in Volume 7 we include an account by J. Friend of their production in plants in response to microbial disease. Emphasis is given to the phenolic phytoalexins of which a very considerable number of different structures are now known. Structural diversity among natural products becomes the major theme of the following review by B. M. Howard and W. Fenical of the terpenoid variation

encountered within a single genus of marine algae, among species of *Laurencia*.

The final chapter represents a challenge to all phytochemists: it is an account by R. E. Schultes of those plants with hallucinogenic activity where the psychoactive principles have yet to be defined. There is clearly much to be learnt about the active chemical constituents of these remarkable plants; a rich field remains here for future investigation.

Reading, Jerusalem and Boston
July 1980

J. B. HARBORNE
L. REINHOLD
T. SWAIN

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E. C. Bate-Smith

E. C. BATE-SMITH: AN APPRECIATION ON HIS 80th BIRTHDAY

Edgar Charles Bate-Smith is and always will be E.C. to his numerous friends throughout the world. And they are numerous, for he has many interests outside of his work—painting, gardening, climbing, botanising, cricket—and his prestigious scientific papers published in muscle physiology (up to the early 1950's), phytochemistry (from 1948 on) and food science (from the late 1920's until the mid-1960's) have earned him a well deserved international reputation in all three fields. Besides all this, his period as Director of the Low Temperature Research Station (L.T.R.S.), (1947–1965) brought many new friends and his membership of the Council of the International Institute of Food Science and Technology and his founder Chairmanship of what is now the Phytochemical Society of Europe brought a whole host more. Above all he has been a Cantabrigian ever since the time he went to Gonville and Caius in the mid-20's, and the many lasting friendships he formed then are still as warm and solid as ever. He has always been a strong supporter of the Cambridge Philosophical Society (and was President, 1953–55), a habitué of Fenners (Cambridge University Cricket Club), and easily recognisable for years by the old-fashioned bicycle he rode to his laboratory each day. He is a good raconteur, especially over a pint of beer, has an erudite knowledge of many abstruse subjects (e.g. the lengths of railway tracks) takes a great delight in the countryside wherever it is and is an unusually kind, courteous, and thoughtful man.

His contributions to phytochemistry started in 1947 when he decided he could not continue as a full-time muscle physiologist and direct the L.T.R.S. at the same time. Impressed by Miles Partridge's demonstration that sugars could be separated by paper chromatography, E.C. decided to see if the method could be used for the flower pigments of the variously coloured phenotypes of *Dahlia variabilis* which he had been breeding over a number of years. He found to his delight he could and published the results in a note entitled "Paper Chromatography of Anthocyanins and Related Substances in Petal Extracts" in *Nature* (**161**: 835 (1948)). A succession of elegantly simple papers followed which pointed out many of the facts we now take for granted: for example, the relationships between structures and Rf values, the importance, identification and distribution of procyanidins, flavonols and hydroxycinnamic acids in the extracts of hydrolysed leaf tissues. These investigations culminated in E.C. demonstrating for the first time how common these constituents were in the land plants. He showed that their distribution could be used both for taxonomic purposes and to indicate evolutionary advancement. His survey of over 1500 species of angiosperms from nearly one half the known families for the ten "common" phenolic constituents (plus notes on the appearance and characteristics of uncommon

ones) was published in the Journal of the Linnean Society (Botany) in 1962 and 1968. In this survey, which has never been surpassed, he developed his ideas for distinguishing between primitive and advanced flavonoids based on complexity of their structural features. This approach forms the basis for more recent studies on many other groups of compounds.

Since he officially retired, 15 years ago, E.C. has continued to turn out a series of thought-provoking papers. Most of these have dealt with the identification and quantitative determination of the various procyanidins and prodelphinidins and of both gallo- and ellagitannins. His concept of relative astringency of these phenolic polymers and his simple method of determining it using a few drops of his own blood demonstrates the charming simplicity of approach which characterises his whole work. No wonder most workers in the field use the methods he has developed.

He has also made further contributions to the taxonomy of several plant groups, notably among species of *Acer*, *Geranium*, *Hydrangea*, *Ribes*, *Ulmus* and *Viburnum*. It seems obvious, from his recently published note on *Cosmos bipinnatus* (*Phytochemistry*, **19**, 982) and his papers still in the press that E.C.'s output will continue apace. He certainly will not allow his 80th birthday to get in the way! I pulled his leg when he reached his three quarters of a century by telling him that he had said he was only going to do ten years in the laboratory after he was 65 and then retire. "Why, he said, is my driving that bad?" (he travels 10 miles to the Institute of Animal Physiology outside Cambridge, to his lab 3–4 times a week). Well, E.C. your driving isn't at all bad: in fact if most of us at 60 can do what you are doing now we will be very satisfied indeed.

It has been a pleasure to write this short appreciation, even more to have been closely associated with you for over thirty years, and I look forward to celebrating your 90th! On behalf of all your colleagues, friends and admirers may I say "Floreat E.C. and for a long time".

TONY SWAIN

ELECTROPHORETIC EVIDENCE AND PLANT POPULATIONS

L. D. GOTTLIEB

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I. INTRODUCTION

The initial applications of gel electrophoresis of enzymes to studies of genetic variation in natural populations of plants and animals has had a remarkable impact on research in population genetics, evolution, and systematics.[1,2] This is because it became possible, for the first time, to identify large numbers of particular gene loci and to enumerate the proportion that showed variation or, as the geneticist says, are polymorphic in that they have more than one allele. Further, it became a simple matter to ascertain the proportion of gene loci that was heterozygous in single individuals.

Previously, the study of genetic variation was unsatisfactory because it depended on the discovery of rare recessive morphological mutants that, when homozygous, yielded visible changes or on infrequent morphological polymorphisms such as heterostyly or flower pigment variants. The problem is that such characters constitute only a very small proportion of genetic variation within natural populations. The vast majority of phenotypic characters are apparently controlled by many gene loci and are often also affected by the environment. The contributions of individual genes in such cases cannot be detected nor is it possible to determine if they vary among individuals. Consequently, the population geneticist in the past was forced to study either those rare characters controlled by one or two loci with major phenotypic effects or the much more common characters governed by many genes that are neither individually recognizable or distinguishable from environmental influence.

Electrophoretic evidence seemed to bypass these problems in a direct and simple way. For a given protein, the rate of migration through a gel is affected by its net electrostatic charge, a function of the relative number of amino acids with positive and negative charges on its surface, and its size and configuration. Thus, if a particular homologous enzyme extracted from two individuals migrates to different positions on the gel, the difference was initially assumed to be caused by changes in the amino acid sequence of the constituent polypeptide(s) and ultimately a mutation(s) in the coding structural gene(s). Alternatively, if the enzymes of different individuals have equivalent migrations on the gel, then it was assumed that the coding DNA sequences were the same. On these assumptions, electrophoretic evidence was thought to be a first analysis of a gene locus. For this reason and because of its simplicity, electrophoretic data became the preferred information for studies of many diverse genetic, population and taxononic problems.

While the applications of electrophoresis and the number of studies in plants and animals continue to multiply, the limitations and appropriateness of the method for certain uses have also been investigated and a number of warning signals have been hoisted. One important problem is that the pattern of enzyme bands has itself become the phenotype to be examined, yet the

biochemical meaning of differences or similarities in the electrophoretic patterns are frequently uncertain, and it is not agreed what contributions the electrophoretic evidence will make to studies of adaptive evolutionary change.[3,4] Problems of this type rarely arose when only morphological and physiological evidence were used.

In response to these new problems, studies are now being directed to determine the actual proportion of sequence variability at a structural gene locus that can be detected by electrophoresis of a protein product. Another question is whether loci sampled by electrophoresis are a random sample of the entire genome. A third question, the so-called neutralist-selectionist controversy, has been whether electrophoretically detected variants have different biochemical properties and are functionally distinct or whether the observed variation is physiologically irrelevant and the variants interchangeable. Also likely to emerge as an important problem in the future is the appropriateness of many current methodologies of analysis which treat the coding genes for different electrophoretic variants as if they were wholly equivalent. If answers to these questions cannot be obtained satisfactorily, then the unprecedented number of surveys of electrophoretic variation in natural populations and arguments about its significance may eventually be viewed as Sisyphean.

However, I should point out that electrophoretic data also provides an important source of single gene markers that can be used in a number of experimental programs without regard either for the metabolic functions of the enzyme products or for the representativeness of the genes. For example, electrophoretic gene markers can be used to estimate rates of outcrossing and other parameters of mating systems, to monitor changes in the level of heterozygosity brought about by forced inbreeding or other experimental manipulations, to seek evidence for the conservation of linkage groups in different species, and to identify cultivars or particular genotypes in studies of population structure. Several of these applications have been well reviewed elsewhere.[5,6]

In the present review, I analyze the results of electrophoretic studies in natural populations of plants. The application of electrophoretic techniques depends on correct genetic interpretations, so I will first review briefly the protocol of genetic analysis and present several examples. Since I believe that electrophoretic evidence needs to be evaluated from biochemical and physiological perspectives that take into account the catalytic activities of the enzymes as well as the metabolic pathways in which they operate, certain information about the enzymes is also presented. The review is based on reports published up to November 1979.

Other recent reviews of electrophoretic studies in plant populations have described the effect of mating systems on genotypic structure and population variability,[6] correlation between levels of variability and life form[7] and other

"life-history" traits,[8] and the application of electrophoretic data to certain problems in systematics and evolution.[9] Comprehensive reviews of the electrophoretic evidence for animal populations are also available.[10–12]

II. THE ELECTROPHORETIC TECHNIQUE

The primary evidence observed in studies of protein (enzyme) electrophoretic variation is a band(s) of color in a slab of starch or acrylamide gel. The colored band(s) which are produced by applying a variety of reagents to the developed gel marks the position(s) to which an enzyme or protein migrated during electrophoresis. In the study of natural populations, the question is whether a particular homologous enzyme extracted from different individuals has the same or different mobility.

In practice, a microliter amount of a crude tissue homogenate from each individual (leaves, stem, flower petals, endosperm, pollen) is extracted in appropriate buffer, often enriched with protective agents such as 2-mercapto-ethanol and polyvinylpolypyrrolidine,[13] loaded onto a paper wick, placed in adjacent slots cut in the buffered gel, which is then subjected to an electric potential difference. After a few hours, the electric current is turned off and the gel immersed in a solution that contains either a general protein dye or a substrate for the enzyme of interest, necessary cofactors such as NAD or NADP, and a reagent that couples specifically with a product of the reaction. This results in the formation of a colored band(s) at the position(s) on the gel occupied by the protein or enzyme(s). The combination of electrophoresis and staining specificity distinguishes particular enzymes among hundreds that may be present in the crude extract.

In such studies, starch or polyacrylamide slabs are preferred to small columns because extracts from as many as 20–25 individuals can be run at one time. These gels have the additional advantage that they can be poured in a mold of sufficient thickness to enable it to be sliced horizontally after electrophoresis to yield three or four thin layers that may each be assayed for different enzymes. In this way data can be readily accumulated on very large numbers of enzymes in hundreds of individuals.

III. GENETIC BASES OF ELECTROPHORETIC PATTERNS

The pattern or zymogram of enzyme bands on a gel following electrophoresis (number, spacing, relative intensity) depends on the particular enzyme assayed, its mode of inheritance, and the genotype and ploidy of the individual examined. The different molecular forms of an enzyme which catalyze the same reaction are called isozymes if they or their polypeptide constituents are coded by more than one gene locus, and allozymes when coded by different

alleles of the same locus. In plants, most enzymes routinely assayed have several isozyme forms often with specific subcellular locations, and the majority of isozymes have different allozymic variants.

The quaternary structure of the particular isozyme determines the number of bands displayed in a heterozygous individual. For example, a plant heterozygous at a locus specifying a monomeric enzyme will have only two allozymes. If the enzyme is dimeric, the electrophoretic pattern will be comprised of three bands: two homodimers and a heterodimer usually with intermediate mobility and usually more intense staining. This increased staining of the heteromer occurs when random association of A and B subunits produces twice as many AB molecules as AA or BB. Tetrameric enzymes show five bands (AAAA, AAAB, AABB, ABBB and BBBB) when the coding gene is heterozygous.

The important determinant of electrophoretic patterns in population surveys is the amount of genetic variability within the sampled population: the proportion of the isozyme gene loci that are polymorphic, the number and relative frequencies of alleles at these loci, and the proportion of gene loci heterozygous per individual. Ploidy level influences the number of isozyme bands because polyploids have more genes than diploids and often these code for electrophoretically distinct isozymes (see Section X). Occasionally, different gene loci specify the subunits of isozymes that "overlap" on the gel because they have the same mobility in the particular electrophoretic condition applied.[14] The number of isozymes observed is also reduced when a locus is homozygous for a "null" allele (an absence of activity that is allelic to active forms).[15] Artefacts that result from procedures of extraction or the electrophoresis itself can also change band number.[16–18]

The presence of so many factors which influence the appearance of the electrophoretic pattern means that such data usually cannot be evaluated just by direct inspection. Simple band counting and mobility comparisons are biochemically faulty and nullify several of the important advantages of electrophoresis, particularly the potential inference of enzyme homology and the precise and quantifiable genetic basis of the evidence. The frequent complexity of electrophoretic patterns in plants means that a genetic analysis is often necessary. Several methods are now available to do this. The standard classical approach is to cross individuals with different banding patterns for a given system and score the number of individuals in each progeny class. Such analyses are reasonably straightforward in most cases because the isozyme bands generally show codominant inheritance and segregate as single Mendelian factors. An example of such a genetic analysis of the glutamate–oxaloacetate transaminase isozymes in *Stephanomeria exigua* is presented in Fig. 1.

Recently a technique has been developed on the basis of the observation that many of the enzymes examined in leaf or stem tissue are also expressed in

pollen (isozyme identity is assumed when the electrophoretic mobility of the enzyme activity in extracts from different tissues is the same; of interest is that lack of electrophoretic identity of an isozyme in different tissues is not necessarily evidence for different isozymes,[19]). The procedure is simply to compare the electrophoretic pattern of the extracts from somatic tissues such as leaf or stem with that from pollen.[14] Since pollen is haploid, the grains contain only one allele of each locus and not both. Thus, the pollen of heterozygotes produces one or the other subunit of dimeric isozymes with the important result that allozymic heterodimers or hybrid enzymes which are present in the diploid somatic tissues, are not formed. [14]Consequently the side-by-side comparison of zymograms of extracts from the pollen with those from somatic tissues quickly reveals which enzyme band is missing from the pollen; its absence identifies it as an allozyme coded by different alleles.[14] In contrast, the isozymic heteromers are produced in the individual grains and their zymograms from pollen and somatic extracts are the same. The procedure is particularly useful for analysis of complex electrophoretic patterns specified by two or more gene loci (Fig. 2).

Genetic studies based on haploid tissue are widely used to analyze electrophoretic patterns in conifers.[20–23] Unlike the microscopic female gametophyte of angiosperms, the female gametophyte of gymnosperms is a large multicellular structure that both carries the gametes and serves as a food storage tissue. Nevertheless, it is haploid and meiotically derived by a complex process from a single megaspore. The megagametophytes of a single tree are expected to show a 1:1 segregation at heterozygous loci. In practise, the embryo is excised from the seeds (megagametophyte) and the remaining tissue used as the enzyme source. In addition to its advantage for genetic studies, the seed tissue does not have high concentrations of protein-binding phenolic and other compounds characteristic of conifer leaf tissue. However, new extraction techniques have been developed which make it possible to assay many different enzymes for electrophoresis from juniper leaves[13] and from mature ponderosa pine needles.[24]

IV. ENZYMES FREQUENTLY STUDIED IN PLANT POPULATIONS

Table 1 lists the enzymes examined by electrophoresis in plant populations and the number of species in which each was studied in the sample of 38 diploid species analyzed in this review. In this section, I describe the catalysis, quaternary structure, subcellular location, and mode of inheritance of the most frequently assayed enzymes. The citations were selected to facilitate rapid entry into the relevant literature for each enzyme system.