DISTRIBUTION OF ENDOGENOUS GIBBERELLINS

IN

ETIOLATED BROAD BEAN (*VICIA FABA*) SEEDLINGS

by

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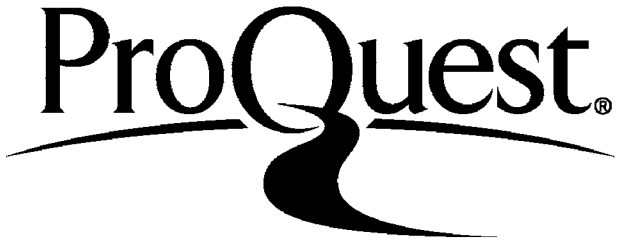

ABSTRACT

The work involved here was undertaken as an attempt to extract, purify, fractionate, separate, and identify the endogenous gibberellins in the etiolated broad bean (Vicia faba) seedling. Following a series of preliminary experiments, it was decided to extract the gibberellins in each organ of the seedling separately by ice-cold absolute methanol and fractionate this solvent extract into three fractions, for rough separation of the gibberellins, as follows:

i. Free gibberellins or acidic ethyl acetate-soluble fraction.

ii. Conjugated-gibberellins or acidic butanol-soluble fraction.

iii. Bound-gibberellins or water-soluble fraction.

Both the conjugated-gibberellins and the bound-gibberellins were released by acid hydrolysis.

The term bound-gibberellin is used here for those gibberellins which appear neither in the acidic ethyl acetate-soluble fraction nor in the acidic butanol-soluble fraction and which are extracted by ethyl acetate at low pH upon hydrolysis. The term does not imply that I have any proof of binding to specific molecules or cell particles.

The studies carried out in this thesis revealed that PVP slurries, PVP column chromatography followed by TLC of the gibberellins recovered from the columns effluents are satisfactory tools for the purification and separation of the endogenous gibberellins in the different organs of the etiolated Vicia seedling. During the study, use has been made of both bioassay and GLC following TLC.

Peaks with retention time corresponding to the methyl esters of gibberellins A_7, A_9, A_{13}, A_{15}, A_{17}, and A_{24} were detected in the acidic ethyl acetate-soluble fraction obtained from the radicle tissue extracts after methylation; that obtained from the plumule tissue extracts gave

peaks with retention time corresponding to the methyl esters of gibberellins A_{12}, A_{13}, iso-A_{13}, A_{14}, A_{20}, and A_{24}; while the acidic ethyl acetate-soluble fraction of the cotyledons tissue extracts gave peaks with retention time corresponding to the methyl esters of gibberellins A_3, A_5, A_6, A_7, A_{12}, A_{13}, iso-A_{13}, A_{14} and A_{15}.

The free gibberellins released by acid hydrolysis of the conjugated-gibberellins in radicle extracts after methylation gave peaks with retention time corresponding to the methyl esters of gibberellins A_9, A_{12}, A_{13}, A_{14}, A_{20}, and A_{24}; those released from the conjugated-gibberellins of the plumule gave peaks with retention time corresponding to the methyl esters of gibberellins A_8 and A_{15}; while the free gibberellins released in the case of the cotyledons tissue extracts gave peaks with retention time corresponding to the methyl esters of gibberellins A_{13}, and A_{14}.

On the other hand, acid hydrolysis of the bound-gibberellins in the radicle tissue extracts released gibberellins which after methylation gave peaks with retention time corresponding to the methyl esters of gibberellins A_7, A_9, A_{17}, and A_{24}; those released from extracts of the plumule tissue gave peaks with retention time corresponding to the methyl esters of gibberellins A_7, A_8, A_9, A_{12}, and A_{24}; while the free gibberellins released by acid hydrolysis of the cotyledons bound-gibberellins gave peak with retention time corresponding to the methyl ester of gibberellin A_{13}.

The cotyledons which are the main storage organs in broad bean contain a number of gibberellins in a "bound" and/or a conjugated form. These gibberellins appear to release free gibberellins on germination. The possible sites of gibberellin metabolism is discussed on the basis of the results obtained.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Prof. L.J. Audus, M.A., Ph.D., Sc.D. Cantab., F.L.S., F.I.Biol., for his guidance throughout this investigation and his helpful criticism and advice during the course of writing this thesis.

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I am also indebted to the Egyptian Government for the award of a Studentship and other financial supports.

I must apologise to my daughter, Rasha, and to my son, Nezar, to whom I present this thesis, for missing me some times during the course of my study and hope they will forgive me.

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The Problem:-

Separation and characterisation of endogenous gibberellins in higher plants is essential for the evaluation of the roles of gibberellins in various physiological phenomena. It is certainly known that the content of endogenous gibberellins in higher plants changes and its distribution varies dramatically during seed development, maturation and germination (Skene & Carr, 1961; Murakami, 1961a; Ogawa, 1963a, 1963b; Hashimoto & Rappaport, 1966a, 1966b; Gotoh, 1970; Durley, MacMillan and Pryce, 1971; Hiraga et al., 1972; Sembdner et al., 1972; Hiraga et al., 1974b). However, little is known about chemical changes of this group of hormones as we shall see later. For these reasons, the distribution of gibberellins in the different organs of etiolated broad bean (Vicia faba) seedlings was investigated in this thesis.

Two main reasons lay behind the choice of broad bean as an experimental plant: firstly because it is one of the main crops in Egypt, and secondly because the naturally occurring gibberellins of broad bean plants have received less attention than those of other plants, and very little work has been done on the endogenous gibberellin-like substances in broad bean.

The reason behind the choice of etiolated seedlings as a plant material for the work involved in this thesis was the limitation of available place to grow the plants in the light, and the requirement of a considerable number of seedlings to obtain sufficient fresh weight of tissue for analysis, as the endogenous gibberellin-like substances of vegetative tissues are of the order of a few micrograms per Kgm fresh weight (Knapp, 1963; Köhler & Lang, 1963; Crozier et al., 1969).

CHAPTER I

INTRODUCTION

1. Historical Introduction

One group of the hormones that control many fundamental processes of growth and development in flowering plants is the gibberellins (Stowe & Yamaki, 1957, 1959; Phinney & West, 1960; Paleg, 1965; Lang, 1970; Paleg & West, 1972). Since the potentialities of auxins, the first characterised growth regulators from higher plants, were first widely recognised, no group of plant hormones has excited as much botanical and horticultural interest as have the gibberellins.

The manner in which the gibberellins came to be discovered is a fascinating story which, so far as written records go, started over one and a half centuries ago. It originated in the study of the "Bakanae" disease of rice (Oryza sativa L.) and the examination of the unusual symptoms of the disease. Ito and Kimura (1929, 1931) attribute the earliest known description of the disease to Konishi (1828), but the first scientific descriptions of the disease with its causal fungus are attributed to Hori (1898). There are other detailed Japanese descriptions but the most complete monographs are those of Ito and Kimura (1929, 1931) which cover aspects of the history, symptoms, methods of infection, treatment, and recommended agricultural practices to reduce its incidence. The most characteristic symptom of the disease is the appearance of tall thin plants, markedly over-topping their uninfected neighbours, thus the name "Bakanae", "foolish seedling", is generally in use.

2. Investigation of Gibberellins

The first suggestion that the bakanae effect might be due to a substance produced by the fungus causing the disease appeared in 1912 by Sawada. In the early 1920's Kurosawa and Sawada worked together on the control of the "Bakanae" disease, and Kurosawa took it on himself to isolate and, if possible, to determine the nature of the substance excreted by the fungus. In 1926, Kurosawa showed that the rice "Bakanae" fungus secretes a type of active principle, he called a toxin, which accelerates rice growth and plays a vital role in differentiating the fungus from similar fungi.

The first serious attempt to isolate the active principle was by Ito and Shimada (1931). They concluded that the substance was thermostable, that it was neither volatile nor enzymic in nature, and that it was diffusible through a semipermeable membrane.

In the early 1930's a group of investigators at the University of Tokyo initiated an extensive programme to isolate the growth stimulant and by 1935, Yabuta announced the isolation of the growth stimulant as a fairly pure solid, although it was not yet pure enough for analysis. The substance was named "gibberellin" after the name of the fungus, Gibberella fujikuroi, and this appears to be the first use of the term in the scientific literature. By 1938, Yabuta and Sumiki reported the isolation of two crystalline "gibberellins" -

i) Gibberellin "A" which was shown later by Cross (1954) to be the physiologically inactive allogibberic acid, contaminated with a little gibberellin, and

ii) Gibberellin "B" which has been shown by Takahashi et al. (1955) to be a mixture of at least three gibberellins.

The pioneering work on gibberellins by the Japanese scientists did not attract much attention outside of the Orient until the early

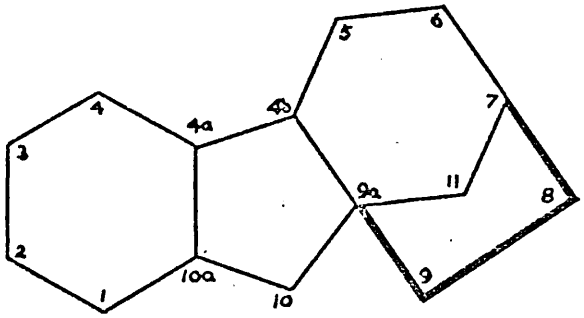
1950's. The forbidding nature of the Japanese language, delayed abstracting, inaccessibility of journals, and the intervening war, all contributed their part to the neglect of the subject in the West.

The first exploratory work on the gibberellins in the United States was performed in 1950 by Mitchell and Angel; they cultured the fungus and obtained taller bean plants using the fungal culture medium. Their work prompted a group at the United States Department of Agriculture (U.S.D.A.), headed by Stodola to undertake the purification of the active principle. Apparently almost simultaneously, scientists at Imperial Chemical Industries (I.C.I.) in England also began large scale preparation of the material. Both the American and the British work resulted in the isolation of an entirely new compound from their fermentations, named "gibberellic acid" by the I.C.I. group (Curtis & Cross, 1954), and "gibberellin-X" by the U.S.D.A. team (Stodola et al., 1955), the first name has now been accepted universally. Both these substances were found to be identical (Cross, 1954; Stodola, 1958), and contain a gibbane skeleton (Cross et al., 1961) (Figure 1).

3. Allocation of Gibberellin "A" Numbers

All naturally occurring compounds which possess a gibbane skeleton and the requisite biological properties are given the generic name gibberellin; these have been designated as GA_1, GA_2, GA_3, GA_4 ..... etc. The symbols A_1, A_2, A_3, and A_4 were originally used by Takahashi et al. (1955, 1957) to denote the first four gibberellins isolated from the fungus Gibberella fujikuroi; this convenient trivial nomenclature was continued by MacMillan and his group (MacMillan et al., 1960, 1962; Pryce & MacMillan, 1967), Cross and his colleagues (Cross et al., 1962; Cross & Norton, 1965; Cross, 1966), Hanson (1966, 1967), and Galt (1965), to include gibberellins A_5 to A_{17}. On the other hand, Tamura and his

Figure 1



The Gibbane Skeleton

group, Mitsui and his colleagues, and Crozier and Audus, used provisional names for their recently discovered plant gibberellins based on their source of discovery, namely Bamboo-gibberellin (Murofushi et al., 1966; Tamura et al., 1966), Pharbitis-gibberellin (Takahashi et al., 1967a), Canavalia gibberellin-I and Canavalia gibberellin-II (Takahashi et al., 1967b; Tamura et al., 1967), Lupinus gibberellin-I and Lupinus gibberellin-II (Koshimizu et al., 1966; Koshimizu & Mitsui, 1968), and Phaseolus gibberellin-I and Phaseolus gibberellin-II (Crozier & Audus, 1968a, 1968b), but the names based on the original source of isolation have obvious disadvantages, for example Bamboo-gibberellin has been found in the seeds of Phaseolus multiflorus (Pryce et al., 1967).

In 1968, MacMillan and Takahashi reported that the gibberellin A_1 ... A_n system of trivial names be extended to include the Bamboo-gibberellin, Pharbitis-gibberellin, Canavalia-gibberellin, ... etc; they further proposed a procedure for the allocation of gibberellin "A" numbers to future gibberellins. These "A" numbers should be used only for naturally occurring, fully characterised compounds which possess the gibbane skeleton and the appropriate biological properties but do not necessarily follow the order of discovery, for example gibberellin A_3 was the first characterised gibberellin, and gibberellin A_{17} was isolated after gibberellins A_{18} to A_{22}.

4. The Structure of Gibberellins

Gibberellins occur naturally in three chemical forms, two of which are chemically defined and the third one is hypothetical (e.g. Moore & Ecklund, 1975); these forms are (i) "free gibberellins", (ii) "conjugated-gibberellins", and (iii) other water-soluble or "bound-gibberellins". The name "conjugated-gibberellins" was first given to gibberellin-

glucosides by Sembdner et al. (1968) and it is likely to be adopted for the glucosides, glucosyl-esters, and other conjugated forms of gibberellins that may be found (Lang, 1970).

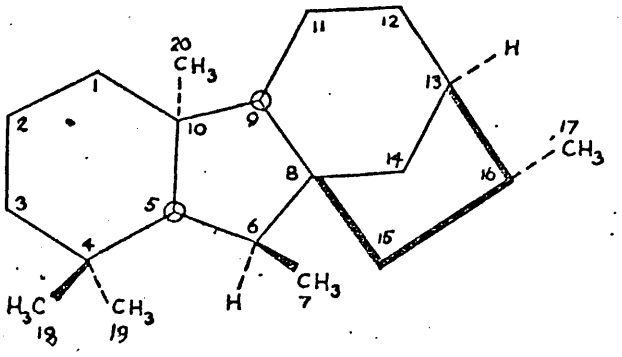
At the time of writing, forty-four free gibberellins, seven gibberellin-glucosides, and four gibberellin glucosyl-esters have been isolated from fungi and higher plants, and their structures have been firmly established. All the gibberellins are closely related modified diterpenoids containing the gibbane skeleton (Fig. 1) and differing in substitution pattern and degree of unsaturation.

In 1968, Rowe suggested that the gibberellane skeleton (Fig. 2) which has a numbering system corresponding to that of other cyclic diterpenes, especially that of kaurene (the crucial intermediary in gibberellins biogenesis), should be regarded as the parent skeleton of gibberellins. The gibberellins would all be derived from the enantiomer of gibberellane (ent-gibberellane, Fig. 2), the kaurene intermediary would be ent-kaurene (previously known as (-)-kaurene), while the corresponding "typical" enantiomer, i.e. gibberellane itself, is not represented by any naturally occurring compounds.

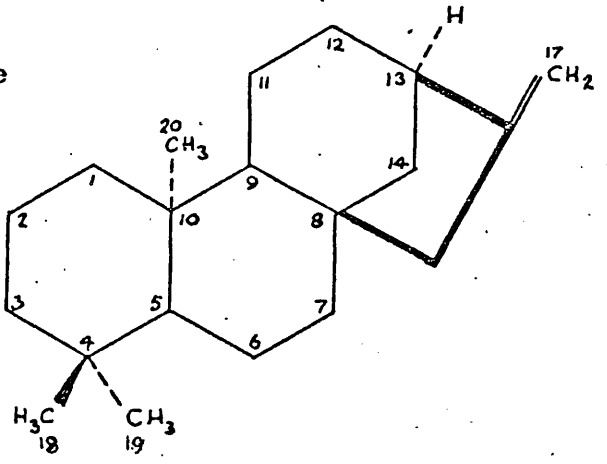
The structural formulae of gibberellic acid, the most accessible of the gibberellins, and of the other free gibberellins up to GA_{42}, seven gibberellin-glucosides; and four gibberellin glucosyl-esters are shown in Figures 3A, 3B and 3C respectively (adapted from Yokota et al., 1971c; Takahashi, 1974).

The two major differences in the structure of the gibberellins lie in the possession of 19 or 20-carbon atoms, and the presence or absence of hydroxyl groups in positions 3 and 13. The 20-C gibberellins have carboxyl groups in positions 7 and 18 and some also in position 20 (others have an aldehyde group in the latter position), while the 19-C gibberellins are all monocarboxylic acids with the carboxyl group in

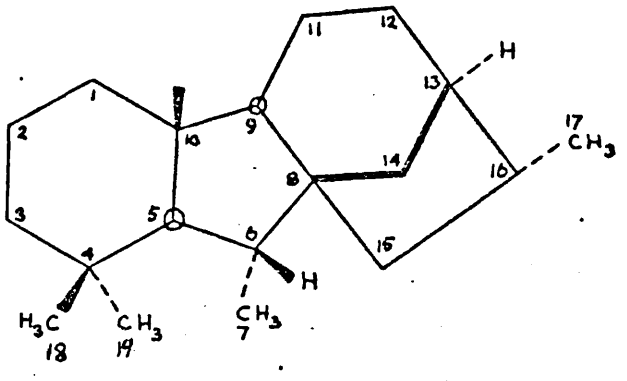
Figure 2



ent-Gibberellane



ent-Kaurene [(-)-Kaurene]



Gibberellane

The structures of Gibberellane, ent-Gibberellane and ent-Kaurene [(-)-Kaurene], heavy lines or wedges indicate bonds lying above the plane of the ring system, while broken lines indicate bonds lying below this plane (adapted from Lang, 1970).

position 7 for which reason the derivatives prepared for characterisation of gibberellins are frequently esters. The 19-C gibberellins have a lactone configuration in the "A" ring which arises with the loss of the "extra" carbon atom. The presence or absence of hydroxyl groups in positions 3 and 13 seems to characterise the difference between those gibberellins that occur only in the fungus Gibberella fujikuroi and those which occur both in the fungus and in higher plants or only in higher plants. Most of the 19-C and 20-C gibberellins have a methylene group which is always found at position 17 (Fig. 3A).

Most of the conjugated-gibberellins identified are glucosides of the gibberellins. These have been found to have the glucose in the pyranose form, bonded by the anomeric carbon atom to a hydroxyl group of the gibberellin (Fig. 3B). Acid or enzyme hydrolysis of gibberellin-glucosides liberates glucose and the free gibberellin or its nor-ketone. A small number of the conjugated-gibberellins isolated have been shown to be glucose-esters in which the glucose is attached to C-7 carbonyl group by an ester linkage (Fig. 3C). These esters are neutral compounds and hydrolyse by acid, alkali, or enzymes to produce glucose and the free gibberellins.

The term "bound-gibberellins" continues to be used for as yet unidentified gibberellin-like substances more polar than the known gibberellins (Lang, 1970). Since free gibberellins are released by hydrolysis of extracts containing unidentified "bound-gibberellins", some authors have suggested the existence of protein-bound gibberellins (McComb, 1961; Hayashi & Rappaport, 1962; Jones, D.F., 1964; Pegg, 1966; Reinhard & Sacher, 1967; Jones, R.L., 1968), but the reality of the natural occurrence of gibberellins in protein-bound states remains uncertain (Lang, 1970). Whereas glucosyl-gibberellins have been isolated from tissues following treatment with free gibberellins (Murakami, 1961b;

Figure 3

Structural Formulae of

A. Free gibberellins up to GA_{42} (from Takahashi,1974)

B. Seven gibberellin-glucosides (from Yokota et al.,1971c)

C. Four gibberellin glucosyl-esters (from Takahashi,1974)

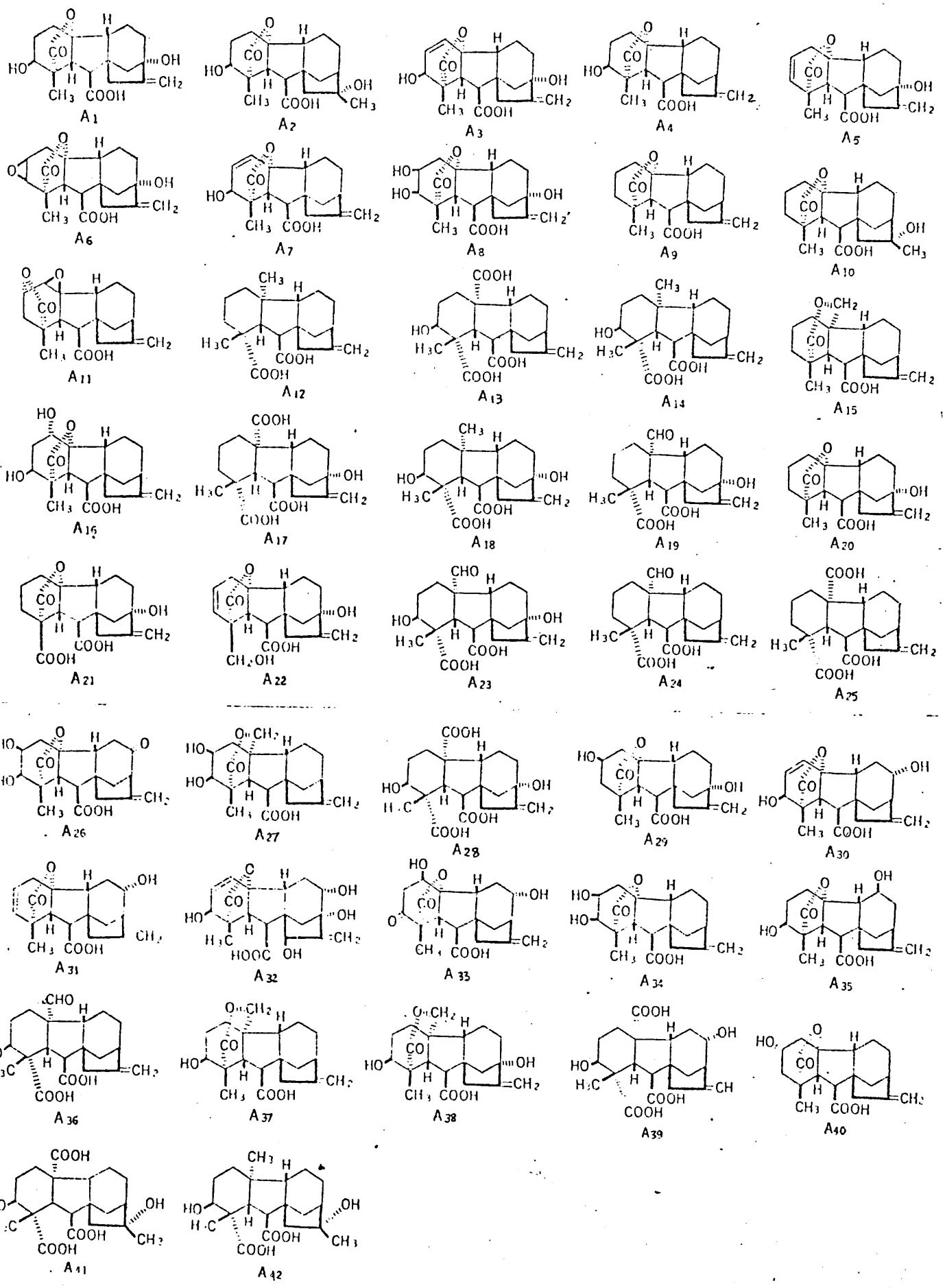

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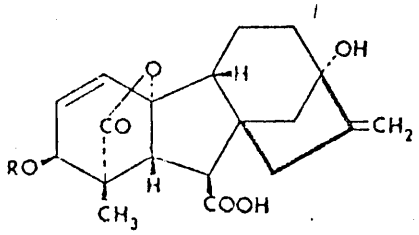
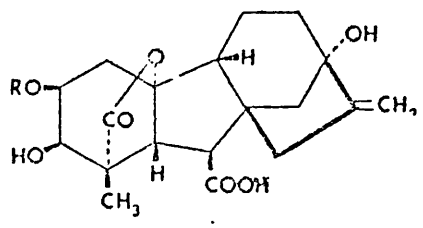
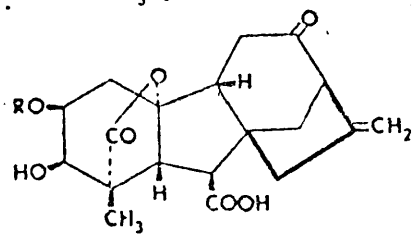
Figure 3A



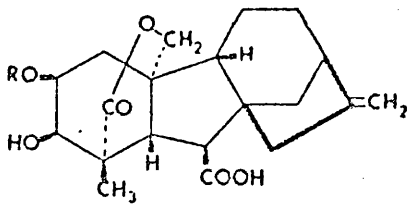
GA_3-glucoside



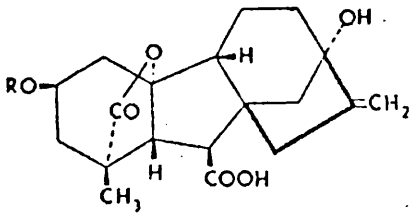
GA_8-glucoside



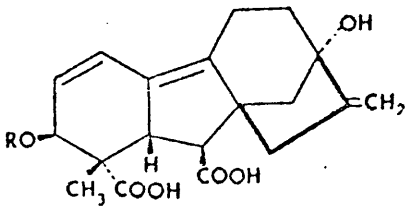
GA_{26}-glucoside



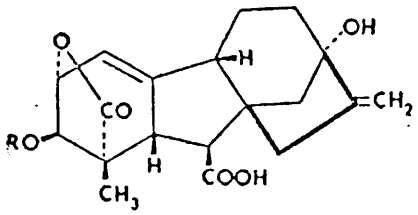
GA_{27}-glucoside



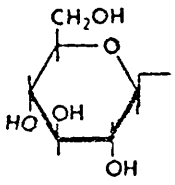
GA_{29}-glucoside



Gibberellenic acid glucoside

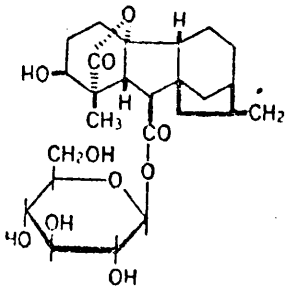


Iso-GA_3-glucoside

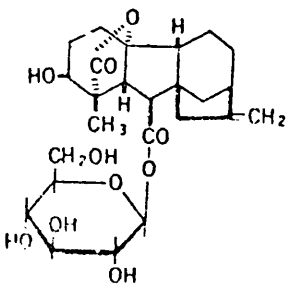


R = β-D-glucose

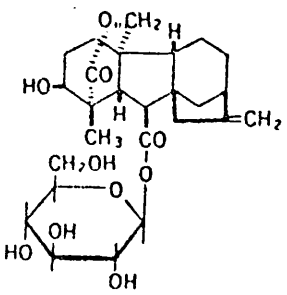
Figure 3B



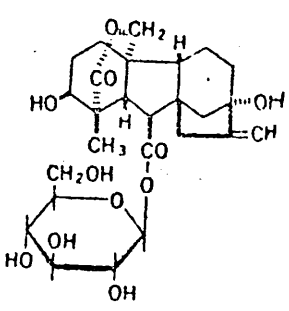
A_1 glucosyl ester



A_4 glucosyl ester



A_{37} glucosyl ester



A_{38} glucosyl ester

Figure 3C

Sembdner et al., 1968; Barendse, 1971), no such evidence for the formation of gibberellin-protein complexes has been reported.

On the other hand, certain other naturally occurring substances, the chemical structures of which are not yet established, have the ability to induce normal growth in certain single gene mutants of Zea mays L., and are termed gibberellin-like substances (West & Phinney, 1956; Phinney & West, 1957, 1961; Phinney et al., 1957). These mutants were known to exhibit a normal type of growth response to the fungal gibberellins and no growth response to numerous other kinds of plant growth regulators.

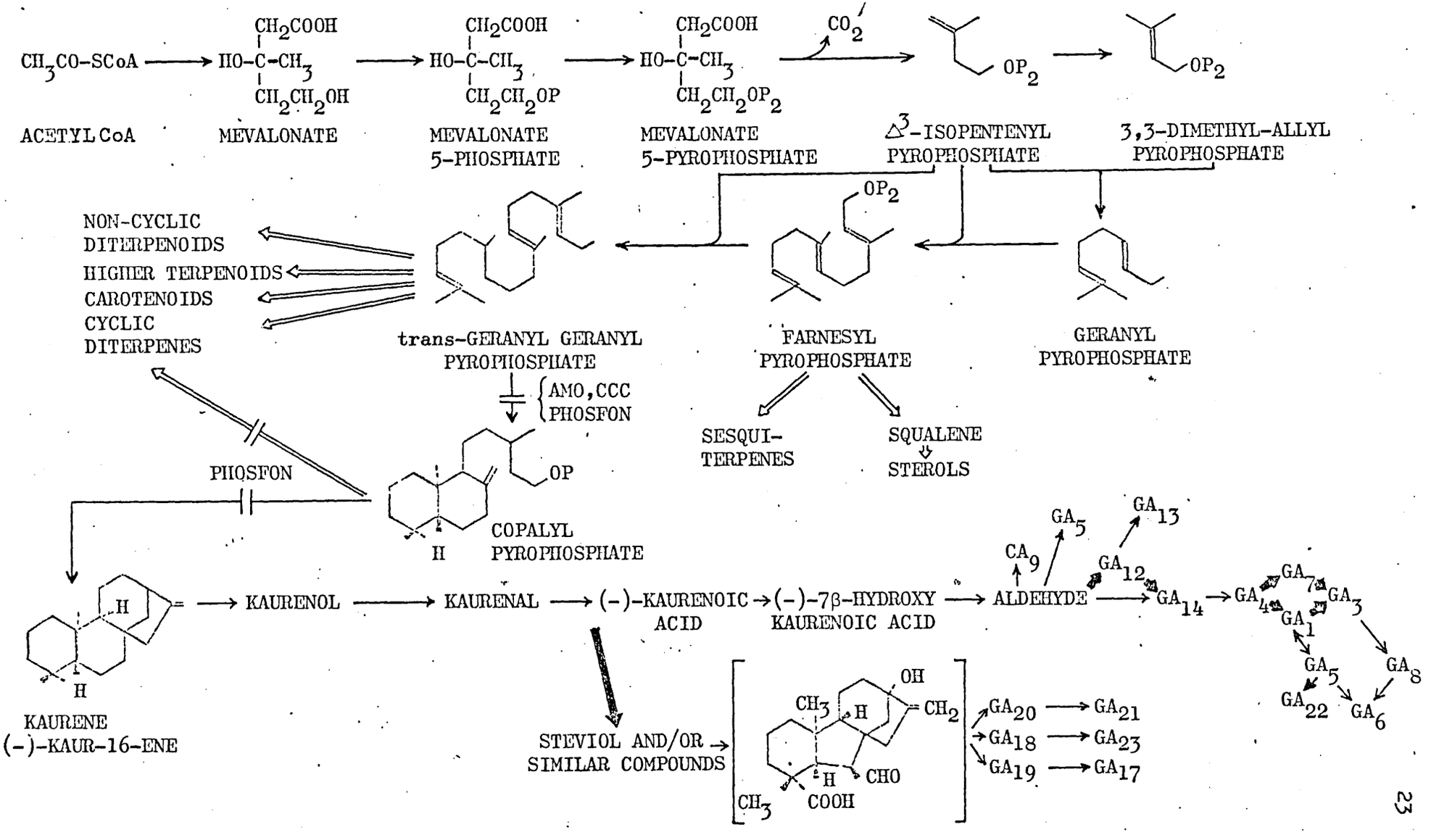
5. Gibberellins Biosynthesis

The observations to date suggest that the pathway of gibberellin biosynthesis in higher plants follows the same scheme as found in Gibberella fujikuroi. The biosynthetic sequence originally postulated by Birch et al. (1958) for the fungus showed acetate and mevalonate as the starting points of the biosynthetic pathway. Since then West and his co-workers have investigated the process in higher plants using enzyme systems isolated from the endosperm of Echinocystis macrocarpa Greene. Radioactive mevalonate has been converted to farnesyl pyrophosphate along the general biosynthetic pathway for terpenes, sterols, ... etc. This has been then enzymatically converted to geranyl-geranyl pyrophosphate, from which (-)-kaurene, the first complete cyclic compound, has subsequently been formed. The cell-free enzyme system is then able to cause the formation of a number of kaurene derivatives, through successive oxidation, of which kaurenoic acid is suggested as the precursor of the gibberellin compounds. The reactions up to these first cyclic compounds have been elucidated by Graebe et al. (1965), Dennis and West (1967), Upper and West (1967), West et al. (1967), Graebe (1968)

Figure 4

The biogenetic pathways from acetyl-CoA and mevalonate to the gibberellins, based mainly on the work of West and co-workers and after Katsumi and Phinney as shown by Lang (1970)

Figure 4


ACETYL CoA
MEVALONATE
MEVALONATE 5-PHOSPHATE
MEVALONATE 5-PYROPHOSPHATE
Δ3-ISOPENTENYL PYROPHOSPHATE
3,3-DIMETHYL-ALLYL PYROPHOSPHATE
NON-CYCLIC DITERPENOIDS
HIGHER TERPENOIDS
CAROTENOIDS
CYCLIC DITERPENES
trans-GERANYL GERANYL PYROPHOSPHATE
FARNESYL PYROPHOSPHATE
GERANYL PYROPHOSPHATE
AMO,CCC
PHOSFON
SESQUI-TERPENES
SQUALENE
STEROLS
PHOSFON
COPALYL PYROPHOSPHATE
KAURENE
(-)-KAUR-16-ENE
KAURENOL
KAURENAL
(-)-KAURENOIC ACID
(-)-7β-HYDROXY KAURENOIC ACID
ALDEHYDE
STEVIOL AND/OR SIMILAR COMPOUNDS

and Oster and West (1968). Figure 4 is taken from Lang (1970) and shows postulated inter-relationships of some of the known gibberellins.

6. Occurrence of Gibberellins in Higher Plants

6.1. Gibberellins and Dwarfism

Although the occurrence of gibberellin-like substances in immature bean seeds was shown by Mitchell et al. in 1951, the first chemically pure gibberellin (GA_1) from a flowering plant, Phaseolus multiflorus L., was isolated by MacMillan and Suter in 1958. The idea that the gibberellins might be naturally occurring in organisms other than the fungus, came from the appearance of certain specific examples of gibberellin-induced growth in flowering plants; the examples involved contrasting growth habits, i.e. cases where both tall and dwarf habits of growth were known within the same species of plant. Addition of gibberellin to the dwarf types resulted in shoot elongation such that the treated plants appeared similar, if not identical, to the non-treated tall types (Brian & Hemming, 1955; Lang, 1956a, 1956b; Phinney, 1956). The nature of these responses would immediately lead one to suspect that the gibberellins might be native plant growth regulators of flowering plants, and that their level or availability in the plant could be responsible for the contrasting type of growth (Brian, 1957; Phinney & West, 1957).

Various observations were reported on the content of gibberellin-like substances in relation to dwarfism. Radley (1956) using pea, french bean, broad bean, and maize plants, Phinney (1961) using maize seedlings, Ogawa (1962) using morning glory seedlings, Köhler (1965) using pea plants, Proano and Greene (1968) using red beans, Suge and Murakami (1968) using rice seedlings, Gotoh (1970) using kidney beans, Gotoh and Esashi (1973) using bean seedlings and Jindal et al. (1974)

using apple shoots, all reported a greater amount of gibberellin-like substances in tall plants as compared to dwarfs. On the other hand, Jones, R.L. (1968) failed to obtain differences in gibberellin-like substances between tall and dwarf peas while Radley (1970) reported that dwarf cultivars of wheat contain more endogenous gibberellin-like substances than those of tall cultivars and suggested that dwarf cultivars have a block to the utilisation of gibberellin in the shoot. In 1971, Higgins and Bonner reported consistent differences between the levels of abscisic acid-like inhibitor in dwarf and normal pea plants, dwarfs having at least 10 times more inhibitor; and also Yadava and Dayton (1972) demonstrated higher levels of abscisic acid in the extracts of dwarfing root stock.

6.2. Gibberellins in the Plant Kingdom

Following the initial reports concerning the presence of substances in flowering plants having biological properties similar to the gibberellins isolated from the fungus (Phinney et al., 1957; Radley, 1958; Phinney & West, 1960), information rapidly accumulated for the widespread occurrence of gibberellin-like substances in the plant kingdom. Murakami (1957, 1959a) employed rice seedling assay to obtain evidence for gibberellin-like substances from chromatographed extracts obtained from 15 species of the family Leguminoseae; several workers had studied gibberellins in cereals. As examples, Radley and co-workers (Jones, D.F., MacMillan & Radley, 1963; Radley, 1966, 1968) have characterised GA_1 and GA_3-like substances in barley, Faull et al. (1974) reached similar conclusions for barley gibberellins using different techniques, Murphy and Briggs (1973) identified gibberellins A_3 and A_7 in germinating barley, Nicholls (1974) found very high levels of gibberellin in barley inflorescence and Kaufman et al. (1976) found that gibberellic acid forms the major native gibberellin in Avena inflorescence, nodes, inter-nodes, and roots.

Gibberellins as well as gibberellin-like substances are present in various organs of the plant, such substances having been reported from whole shoots as well as various parts of the shoot system (Radley, 1956, 1958; Simpson, 1958; McComb & Carr, 1958; Harada & Nitsch, 1959; Lang, 1960; Hill & Selman, 1966; Murofushi et al., 1966; Jackson, 1967; Perez & Lachman, 1971; Railton & Reed, 1974; Lance et al., 1976; Wakmloo, 1976; Zieslin & Halevy, 1976); from roots and root apices (Nickell, 1958; Takahashi et al., 1958; Sircar & Kundu, 1959; Butcher, 1963; Sitton et al., 1967; Murakami, 1968; Crozier & Reid, 1971); from developing seeds as well as immature and mature seeds (MacMillan, Seaton & Suter, 1959, 1960, 1961; Murakami, 1959a, 1959b; Skene & Carr, 1961; Corcoran & Phinney, 1962; Jones, D.F. et al., 1963; Ogawa, 1963a, 1963b; Jones, D.F., 1964; Dennis & Nitsch, 1966; Hashimoto & Rappaport, 1966a, 1966b; Koshimizu et al., 1966; Pegg, 1966; Jackson, 1967; Krugman, 1967; MacMillan et al., 1967; Ogawa, 1967; Pryce & MacMillan, 1967; Banerjee, 1968; Koshimizu et al., 1968; MacMillan & Pryce, 1968b; Murofushi et al., 1968, 1970; Radley, 1968; Yokota et al., 1969a; Skene, 1970; Yamaguchi et al., 1970; Durley, MacMillan & Pryce, 1971; Ross & Bradbeer, 1971; Yamane et al., 1971; Yokota et al., 1971b; Coombe & Tate, 1972; Hiraga et al., 1972; Eeuwens, Gaskin & MacMillan, 1973; Frydman & MacMillan, 1973; Gotoh & Esashi, 1973; Paul et al., 1973; Raja Rao & Nagar, 1973; Sinska et al., 1973; Frydman et al., 1974; Hiraga et al., 1974b; Williams et al., 1974; Yamaguchi et al., 1975b; Radley, 1976); from bulbs, flower buds, flowers, fruits and tubers (Radley & Dear, 1958; Porlingis & Boynton, 1961; Hayashi & Rappaport, 1962, 1966; Hayashi et al., 1962; Banerjee & Radforth, 1967; Aung & DeHertogh, 1967, 1968; Harada & Nitsch, 1967; Smith & Rappaport, 1969; Bhalla, 1970; Goren & Goldschmidt, 1970; Kender & Desrochers, 1970; Ramsay & Martin, 1970; Sircar et al., 1970; Aung et al., 1971; Fukui et al., 1971; Hemphill et al., 1972; Jeffcoat

& Cockshull, 1972; Browning, 1973; Aung, 1974; Alpi et al., 1976); and from embryos, pollen grains, and ovules (Wiltbook & Krezdorn, 1969; Kamienska & Pharis, 1975; Shindy & Smith, 1975).

The endogenous gibberellins and/or gibberellin-like substances of the germinated seedlings has been shown by several investigators; as examples, Crozier and Audus (1968b) reported the isolation of two gibberellin-like compounds from etiolated Phaseolus seedlings; Jones, R.L. and Lang (1968) reported extractable and diffusible gibberellins from light and dark grown pea seedlings; Radley (1968) reported production of gibberellin-like substances in berley seedlings; Crozier et al. (1971), employing gas-liquid chromatography and combined gas chromatography-mass spectrometry detected endogenous gibberellins from dark grown Phaseolus coccineus seedlings; and Faull et al. (1974). extracted and characterised gibberellins in Hordeum vulgare L. seedlings.

Gibberellin-like substances have also been detected in some coniferous species by many workers; as examples, Kato et al. (1962), Murakami (1966), Ruddat et al. (1968), Crozier et al. (1970a), Dunberg (1973), (1974), Lorenzi, Horgan and Heald (1975), and Dunberg (1976).

In addition to the fungus Gibberella fujikuroi and higher plants, gibberellin-like substances have been found in certain other fungi (see Stowe & Yamaki, 1957; Hagimoto & Konishi, 1959, 1960; Gruen, 1963; Hagimoto, 1963a, 1963b; Pegg, 1973), several species of algae including unicellular algae, two species of Fucus and a unialgal culture of a unicellular green algae (Radley, 1961; Mowat, 1963; Banergee & Horsley, 1969).

On the other hand, conjugated-gibberellins (gibberellin-glucosides and glucosyl-esters) have been isolated particularly from immature seeds which appear to be a rich source of such polar gibberellins. From immature seeds of Pharbitis nil, Tamura et al. (1968a) and Yokota et al. (1969b) isolated GA_3-glucoside. GA_8-glucoside has been found in immature

fruits of Phaseolus cocceineus by Schreiber et al. (1967, 1970), in mature and immature seeds of Phaseolus vulgaris by Hiraga et al. (1974b), in immature seeds of Pharbitis nil by Yokota et al. (1969b), and in shoot apices of Althaea rosea by Harada and Yokota (1970). The glucoside-esters of gibberellins A_{26}, A_{27} and A_{29} all have been isolated from the immature seeds of Pharbitis nil by Yokota et al. (1969a, 1970), and the glucoside-ester of GA_{35} has been isolated from immature seeds of yellow broom by Yamane et al. (1971).

The glucosyl-esters of gibberellins A_1, A_4, A_{37} and A_{38}, in which the glucose is connected to the C-7 carboxyl group by ester linkage, were isolated from the mature seeds of Phaseolus vulgaris by Hiraga et al. (1972, 1974b).

The presence of unidentified bound-gibberellins has been indicated in several organs of different plants, for example potato tubers (Hayashi et al., 1962; Hayashi & Rappaport, 1962), shoot tips of tobacco (Sembdner & Schreiber, 1965), tomato seeds and seedlings (Pegg, 1966), and tulip bulbs (Aung et al., 1969).

From the literature cited it is quite clear that the occurrence of gibberellins and gibberellin-like substances in the plant kingdom is very widespread.

7. Extraction, Fractionation and Separation of Gibberellins

7.1. Extraction of the Gibberellins

Since the first reports on the presence of gibberellin-like substances in higher plants by Murakami (1956), Radley (1956), and West and Phinney (1956), most investigations on endogenous gibberellins have been conducted using solvent extraction techniques. There are almost as many extraction methods as there are research workers. The first step includes extraction of the tissue under investigation

with methanol preferably very rapidly by using a blender (Kende, 1967; Crozier et al., 1969). Some workers have used other different solvents for this first step of extraction (see West & Phinney, 1959; MacMillan et al., 1960; Tamura et al., 1967; Skene, 1970); other workers have used water (Tamura et al., 1966), or buffer solutions (Jones, R.L., 1968).

7.2. Fractionation of the Gibberellins

i. Acidic Ethyl Acetate-Soluble Gibberellins (Free Gibberellins)

The organic solvent is usually removed under reduced pressure and the aqueous concentrate partitioned against ethyl acetate at low pH, most of the free gibberellins are extracted in the acidic ethyl acetate fraction (see Hayashi & Rappaport, 1962; Hashimoto & Rappaport, 1966a; Crozier et al., 1969; Hiraga et al., 1972).

ii. Acidic Butanol-Soluble (Water-Soluble) Gibberellins

There is growing evidence for the occurrence of water-soluble or bound-gibberellins in higher plants. The existence of these substances was suggested by Radley (1958) by analogy with "bound" growth substances of the auxin type. McComb (1961) following Radley's suggestion, hydrolysed a buffer preparation of bean seeds using crude proteolytic enzyme preparation and detected more free acidic gibberellin-like substances in the hydrolysed extracts. Similar results were also obtained by Murakami (1961b) who showed that water-soluble gibberellins could be removed from the water phase with ethyl acetate, after acid or enzymatic hydrolysis. Later studies showed that such polar, water-soluble substances could be removed from the water phase with n-butanol at low pH values (e.g. Hashimoto & Rappaport, 1966b). Later on, the identity of such water and butanol-soluble substances has been established (Tamura et al., 1968a; Yokota et al., 1969a, 1971c; Hiraga et al., 1972, 1974b).

7.3. Separation of Gibberellins

i. By Paper Chromatography

Paper chromatography of the gibberellins has been investigated by many investigators but no comprehensive study of all the gibberellins has been reported (e.g. Takahashi et al., 1955; Phinney et al., 1957; Murakami, 1959a; MacMillan et al., 1961; Ikekawa et al., 1963a).

Attempts were made to identify the compounds in crude extracts of many plant species which have been found to possess biological properties similar to the gibberellins by paper chromatographic comparison with known gibberellins (Murakami, 1959a; Radley, 1959; Phinney & West, 1960; Murakami, 1961a). A disadvantage of this technique is that biologically inactive derivatives of the gibberellins cannot readily be detected.

ii. By Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) (Kutácek et al., 1962; Sembdner et al., 1962; Kagawa et al., 1963; MacMillan & Suter, 1963) combined with a number of bioassay tests provides a more convenient and sensitive method for separating and identifying gibberellins in higher plants extract and in culture filtrate of Gibberella fujikuroi, particularly when extended to include the chromatography of derivatives (Jones, D.F., MacMillan & Radley, 1963; Elson et al., 1964; Dennis & Nitsch, 1966; Geissman et al., 1966). However, for a decisive identification of gibberellins by this method, the appropriate authentic specimens are required because the R_f values are not reproducible and cannot be standardised in terms of reference compounds (Cavell et al., 1967). The need for reference specimens of the scarce gibberellins is a serious disadvantage.

iii. By Gas-Liquid Chromatography (GLC) and Combined Gas Chromatography-Mass Spectrometry (GC-MS)

In seeking methods without these disadvantages, attention has been turned to gas-liquid chromatography (GLC). The feasibility of GLC for the separation and identification of pure gibberellins was first reported by Ikekawa et al. (1963b) for gibberellins A_1-A_9 as their methyl esters. Later, the GLC technique was extended by MacMillan and his co-workers (e.g. Cavell et al., 1967); this group have since extended this technique by using GC-MS (e.g. MacMillan, 1968; MacMillan & Pryce, 1968a, 1968b; Binks, MacMillan & Pryce, 1969; MacMillan, 1972).

Scope and Limitation of GLC

The main requirement for GLC is that the growth substance, or suitable derivatives can be gas chromatographed which is a severe limitation and precludes the direct investigation of many conjugates, such as glucosides, although they can be examined indirectly after hydrolysis. Another hazard is the possibility of decomposition during gas chromatography especially for derivatives with longer retention times. The use of pure solvents for extraction and purification steps is essential to avoid contamination with plasticisers (Binks et al., 1970), also the use of parafilm should be avoided at any time (Gaskin et al., 1971).

8. Biological Assays for Gibberellins

Since the discovery of gibberellins, biological assays for these substances have been a vital research tool. Much of the early Japanese work on gibberellins was carried out using tests depending on the measurement of the height of a treated rice seedling and comparing it with that of a control (Kurosawa, 1926; Ito & Shimada, 1931; Yabuta & Hayashi, 1939).

When the work on gibberellins began in the West in the early 1950's, an increasing range of biological tests has been used to detect and quantify amounts of pure gibberellins and of extracted substances with gibberellin-like biological properties. Some of the many biological assays that have been developed by different workers have come to be very widely used and have been modified in various ways from time to time. There are many reviews that have discussed gibberellin bioassay to some extent, for example Phinney and West (1960), Bentley (1962) and Paleg (1965), but the most extensive review is that of Bailiss and Hill (1971) in which they catalogued 33 test systems available for the study of biological activities of gibberellins in qualitative and quantitative terms. More recently, five bioassays have been described in detail with respect to practical use and theoretical aspects by Reeve and Crozier (1975).

One of the most common assay tests for gibberellins involves the measurement of hypocotyl growth of plants with epigeal germination e.g. the lettuce hypocotyl assay developed by Frankland and Wareing (1960). It should be pointed out that this test may react strongly to inhibitors in plant extracts (Frankland & Wareing, 1962, 1966). This disadvantage may be overcome by repeated purification of plant extracts (Dennis & Nitsch, 1966) or choosing chromatographic solvent system which well separates growth promoters and inhibitors.

Another biological assay for the gibberellins is that which depends on gibberellin induced α-amylase production. This phenomena was first reported by Hayashi (1940) but its use as a bioassay for gibberellins was studied by Paleg (1960) using barley endosperm and measuring the subsequent release of reducing sugars into the ambient medium. This work was the basis of the test described by Nicholls and Paleg (1963).

A problem noted in the recent years is the tendency of certain solvents to contain residues with gibberellin-like activity in this test (Briggs, 1966; Coombe et al., 1967a) but this can be overcome by redistilling the solvents and by using an appropriate control (Hartley et al., 1969). In 1967, Jones, R.L. and Varner described a barley half-seed bioassay in which the activity of the α-amylase produced as a result of gibberellin treatment of the de-embryonated grains is measured directly using soluble starch and staining with iodine, and they found that this assay was not affected by solvent residues (Briggs, 1966) or by substances other than the gibberellins present in crude plant extracts.

9. Specificity and Relative Activity of the Gibberellins in Bioassays

The gibberellins as a group, comprise molecules based on the gibberellane skeleton (Fig. 2) in which slight alterations in substitutions may alter the spectrum of activity. Each gibberellin type has a characteristic range of biological activity and in turn each bioassay responds to a distinct spectrum of gibberellins (Crozier et al., 1970b).

Data on the biological activities of gibberellins A_1-A_9 have been reported by many workers (see Hashimoto & Yamaki, 1960; Brian et al., 1962, 1964; Paleg et al., 1964; Bentley-Mowat, 1966; Hayashi & Rappaport, 1966; Ogawa, 1967; Tamura, 1969; Crozier et al., 1970b). On the other hand, the biological activities of gibberellins A_{10}-A_{34} have been examined less thoroughly and in fewer bioassay systems (Brian et al., 1967; Hoad, 1969). The activities of gibberellins A_1-A_{26} in nine bioassay systems have been described by Crozier et al. (1970b), the activity of gibberellin A_{18} in comparison with GA_3 was studied by Katsumi et al. in 1968; the activity of GA_{32} in eight bioassays has been reported by Coombe (1971) and by Coombe and Tate (1972), and the biological activities of gibberellins A_{30}-A_{35} have been tested in seven bioassay tests by Yamane et al. in 1973.

On the other hand, the biological activities of conjugated-gibberellins have been studied in few bioassay systems. Yokota et al. (1971a) reported that gibberellin-glucosides possess less activities than their free gibberellins and they concluded that the glucosides do not appear to be active in growth regulation; Hiraga et al. (1974a) found that the synthesised glucosyl-esters of gibberellins A_1, A_3, A_4 and A_{37} were as active as their free acids in the rice seedling assay. Recently, Sembdner et al. (1976) on studying the biological activities of several gibberellin-conjugates, of which some were synthesised chemically and some were known to occur as endogenous plant products, found in four bioassay systems that the gibberellin-conjugates possess different relative activities depending on the chemical structure and the bioassay system. They concluded that the activity of gibberellin-conjugates as measured in different bioassays is based upon the ability of plant enzymes and possibly of certain microorganisms to hydrolyse glucosidic, glucosyl-esters and amide-like linkages to liberate the free acid (see structural formulae of conjugated-gibberellins, Figs. 3B and 3C).

The relative activities of the characterised free gibberellins (up to GA_{38}) in five bioassay tests namely barley α-amylase, dwarf pea, lettuce hypocotyl, Tan-ginbozu dwarf rice microdrop, and cucumber hypocotyl bioassays are presented in Table 1, taken from Reeve and Crozier (1975).

In the present study, use has been made of both bioassay and GLC following TLC.

Table 1 Relative Activities of Gibberellins in Five Bioassay Systems*

Gibberellin	Barley aleurone	Dwarf pea	Lettuce hypocotyl	Dwarf rice	Cucumber hypocotyl
A_1	++++	+++	+++	+++	++
A_2	++++	++	++	+++	++
A_3	++++	++++	+++	++++	++
A_4	+++	+++	++	++	+++
A_5	++	+++	++	+++	+
deoxy A_5		+++		+++	++
A_6	++	++	++	+++	+
A_7	+++	+++	++++	+++	++++
A_8	+	+	+	+	0
A_9	+	++	+++	++	+++
A_{10}	+	0	0	+++	++
A_{11}	+	0	0	+	+
A_{12}	0	0	0	+	+
A_{12} aldehyde	0	0	0	+	0
A_{13}	+	0	+	+	0
A_{14}	0	0	0	+	0
A_{15}	0	+	++	++	++
A_{16}	+	++	+	+	+
A_{17}	0	0	0	+	0
A_{18}	0	++	+	+++	0
A_{19}	0	0	+	+++	0
A_{20}	+	+	+++	+++	0
A_{21}	0	0	+	0	0
A_{22}	+++	+++	++	+++	0
A_{23}	++	++	+	+++	0
A_{24}	0	+	0	+++	+++
A_{25}	0	0	0	0	+
A_{26}	0	0	0	0	0
A_{27}	0	+	0	+	0
A_{28}	0	0	0	+	0
A_{29}	+	0	0	+	0

cont'd..

Table 1 cont'd.

Gibberellin	Barley aleurone	Dwarf pea	Lettuce hypocotyl	Dwarf rice	Cucumber hypocotyl
A_{30}	+++	+++	+	++	+
A_{31}	+	++	0	++	0
A_{32}	+++	+++	++	++++	+++
A_{33}	+	+	+	+	0
A_{34}	0	+	0	+	0
A_{35}	+++	++	+	++	+++
A_{36}	++	++	+	+++	+++
A_{37}	++	++	++	+++	+++
A_{38}	+	+++	0	+	+

Relative activities:

++++ very high
+++ high
++ moderate
+ low
0 very low-inactive

* Based on data of Crozier et al. (1970b), Yokota et al. (1971), Fukui et al. (1972), Yamane et al. (1973), Hoad (unpublished) and Pharis (unpublished). Quoted from Reeve & Crozier (1975).

CHAPTER II

MATERIALS AND METHODS

1. Plant Material and Growing Conditions

Uniformly sized broad bean (Vicia faba) seeds cv. Green Sleeves (purchased from Dickson, Brown and Tait Ltd., Timperley, Altrincham, Cheshire) or cv. Green Windsor (purchased from Hurst Gunson Cooper Taber Ltd., Avenue Road, Witham, Essex, England, see Experimental Section), were soaked in aerated running tap water at approximately 25°C for about 40 hours and the turgid seeds were selected for uniformity by choosing those with radicle protrusion of 1-2 mm in length. These uniformly germinated seeds were planted in rows in Ward "Half" seed trays (7" x 8.5" x 2" height, at a density of 20 seeds/tray) half-filled with sterile sand moistened with distilled water in the porporation of 4:1 v/v sand/water, and the seeds were covered with a $\frac{1}{2}$" layer of sterile moistened sand.

The seedlings were grown in complete darkness at a temperature of about 25°C (± 1°C) for seven or ten days and watered with distilled water every other day. At the end of the germination period, the seedlings were harvested, washed with tap water to free them from sand particles and rinsed with distilled water. The testas were discarded, and the seedlings were either used whole or separated with a sharp blade into radicle, plumule, and cotyledons. The fresh weight of the whole seedling or of each organ was determined (see Experimental Section).

To ensure uniformity in all samples, selection was confined to those seedlings whose plumule height was 15-20 cm or 25-30 cm for the

seven and the ten days old seedlings respectively, and where the first leaf was not yet unfolded. Seedlings showing any signs of abnormality or malformation were rejected.

The tissue was immediately frozen with dry ice and kept in a deep freezer at -20°C until used for extraction. All extractions were completed within three weeks after the date of harvesting to avoid chemical transformations of gibberellins similar to those reported by Rappaport (1972).

2. Solvents and Chemicals

The solvents used in extraction and purification procedures were either laboratory or Analar grade. The chemicals were of Analar grade (unless otherwise stated), and were supplied by British Drug Houses.

All the solvents were redistilled; ethyl acetate was redistilled as near to the time of use as possible and, immediately prior to extractions, was deacidified by shaking with 20 ml/L of 5% aqueous sodium bicarbonate and washed with three changes of distilled water (cf. Hartley et al., 1969). These conditions ensure that the solvents did not possess any significant biological activity.

3. Standard Gibberellins

Stock aqueous solutions of a British Drug Houses gibberellic acid (GA_3) were prepared in a range of concentrations from 0.001 µg/ml to 100 µg/ml for the standardisation of the bioassay methods involved in this thesis. Other gibberellins ($GA_{4/7}$, GA_{13} and GA_{14}), which were used in derivative form for GLC, were gifts from Dr. A. Crozier of the Department of Botany, University of Glasgow and Dr. J. MacMillan of the Department of Chemistry, University of Bristol.

Precautions Against Contaminations

During experimental work, stringent precautions were taken at all times to preclude contamination of glassware and solvents with authentic gibberellins. All glassware was soaked in a solution of 5% "decon" for 24 hours, washed repeatedly in hot water, rinsed in distilled water and dried in a hot oven.

4. Extraction and Fractionation of Endogenous Gibberellins

4.1. Extraction Procedure

The frozen tissue of the plumule and the radicle was macerated separately in a Waring Blender with redistilled ice-cold absolute methanol at the ratio of 2 ml of methanol per gram fresh weight of tissue. After satisfactory tissue disintegration had been achieved (ca five minutes), the extract was filtered in a Büchner funnel through two layers of Whatman No.1 filter paper, and the residue was stirred for 24 hours on a magnetic stirrer in a cold room at 4°C with a fresh amount of 80% aqueous methanol and then filtered as before. The insoluble tissue residue was washed with ice-cold absolute methanol until the latter was colourless. The methanolic extracts were combined and the cell debris discarded.

The cotyledons were too hard to blend in the liquidiser, and too wet to mill; they were therefore passed first through the coarse screen of a mincer which effectively broke up the tissue into coarse fragments, which were further macerated in the blender and treated thereafter as previously described for the plumule.

The combined methanolic extracts of each organ were reduced separately to the aqueous phase under vacuum at 35°C and the aqueous phase was further reduced in volume. After freezing overnight at -20°C in a deep freezer, the extract was thawed at room temperature and

centrifuged for 60 minutes at 15,000 R.P.M. and 0°C, using stainless steel centrifuge tubes, to remove precipitated pigments and protein residues. The supernatant was adjusted to pH 8.0 with 1N KOH and the gibberellins were then removed from the aqueous extracts employing methods which have also been used by other workers (see Hayashi & Rappaport, 1962, 1966; Kende, 1967; Crozier & Reid, 1971; Yokota et al., 1971b; Williams et al., 1974).

The aqueous phase at pH 8.0 was partitioned against equal volumes of redistilled petroleum ether (B.P. 60-80°C) and the partition was continued until no more colour was extracted (Crozier & Reid, 1971), pigmented material migrated into the petroleum ether phase leaving the ionised acids in the aqueous layer. Petroleum ether was used in preference to ethyl acetate because it has been found that ethyl acetate will remove some non-polar gibberellins such as GA_9, GA_4 and GA_7 from aqueous solutions at pH 8.0 (Crozier et al., 1969).

4.2. Fractionation of the Gibberellins

i. Acidic Ethyl Acetate-Soluble Fraction (Free Gibberellins)

After partitioning with petroleum ether at pH 8.0, the aqueous phase was next adjusted to pH 2.5 with 5N HCl, and the undissociated acids were removed by partitioning four times against equal volumes of redistilled deacidified ethyl acetate. The ethyl acetate extracts were combined, reduced in volume under vacuum at 35°C, and back extracted three times with one third of its volume of 1% aqueous sodium bicarbonate in order to remove acidic compounds from the organic phases. The bicarbonate phase was then adjusted to pH 2.5 with 1N HCl and the acidic compounds were back extracted into redistilled deacidified ethyl acetate by partitioning four times with a ratio of two volumes of bicarbonate to one volume of ethyl acetate. The ethyl acetate extracts were combined together and dried by freezing out the water overnight at -20°C. The

ice crystals were filtered off rapidly through glass wool in a pre-chilled Büchner funnel and the filtrate evaporated to dryness on a rotary film evaporator at 35°C. The residue contained the free gibberellins. (see Flow Diagram of Extraction Technique and Subsequent Purification, Fig. 5).

ii. Acidic n-Butanol-Soluble Fraction (Gibberellin-Glucosides)

It has been extensively shown in the literature that gibberellin-glucosides, previously referred to as bound-gibberellins, which are water-soluble and more polar than the gibberellins known prior to 1970, can be efficiently extracted from aqueous extracts with n-butanol, but not with ethyl acetate, at low pH (Hashimoto & Rappaport, 1966b; Sembdner et al., 1968; Tamura et al., 1968a; Yokota et al., 1969a, 1971a; Hiraga et al., 1972, 1974b). This fraction includes also the most polar free gibberellin, GA_{32}, together with a part of GA_{28}, when these are present in the extracts (Takahashi, 1974). The method used here for extraction of acidic butanol-soluble gibberellins was the same as that reported by Yokota et al. (1971c) with slight modification; in its final form the method was as follows:

The aqueous phase, previously partitioned against ethyl acetate at pH 2.5 was freed of remaining traces of organic solvent by vacuum and exhaustively partitioned three times against equal volumes of re-distilled n-butanol at pH 2.5. The acidic butanol phase was reduced in volume under vacuum at 40°C and then extracted three times with four-fifths its volume of saturated sodium bicarbonate solution. The bicarbonate phase was acidified to pH 2.5 with 1N H_2SO_4 and re-extracted three times with half its volume of redistilled n-butanol. The butanol phase was next adjusted to pH 5.0 with diluted ammonium hydroxide solution to neutralise sulphuric acid and then it was evaporated to dryness under vacuum at 40°C. The gummy solid was

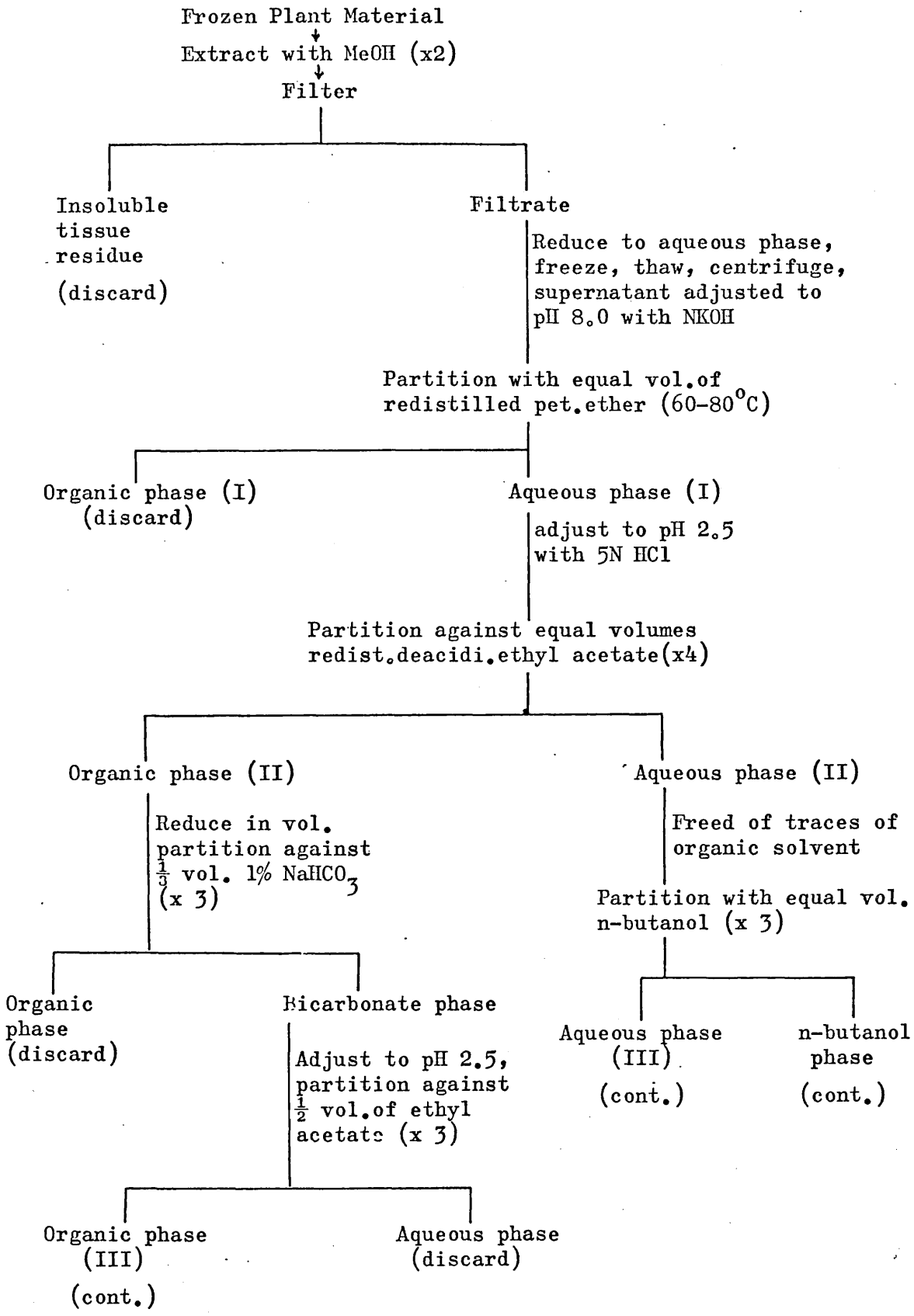


Figure 5 Flow Diagram of Extraction and Purification Technique

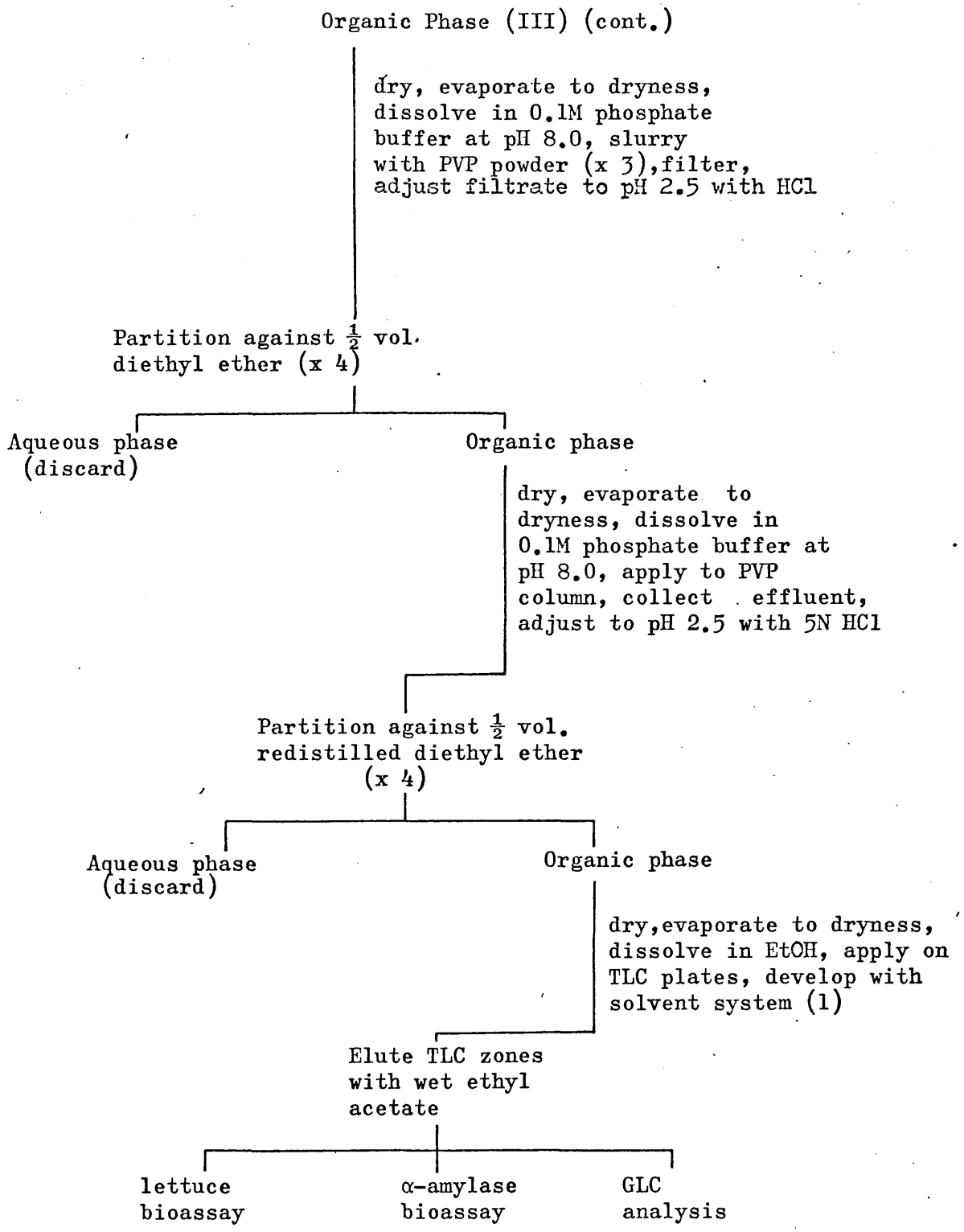


Figure 5 cont'd.

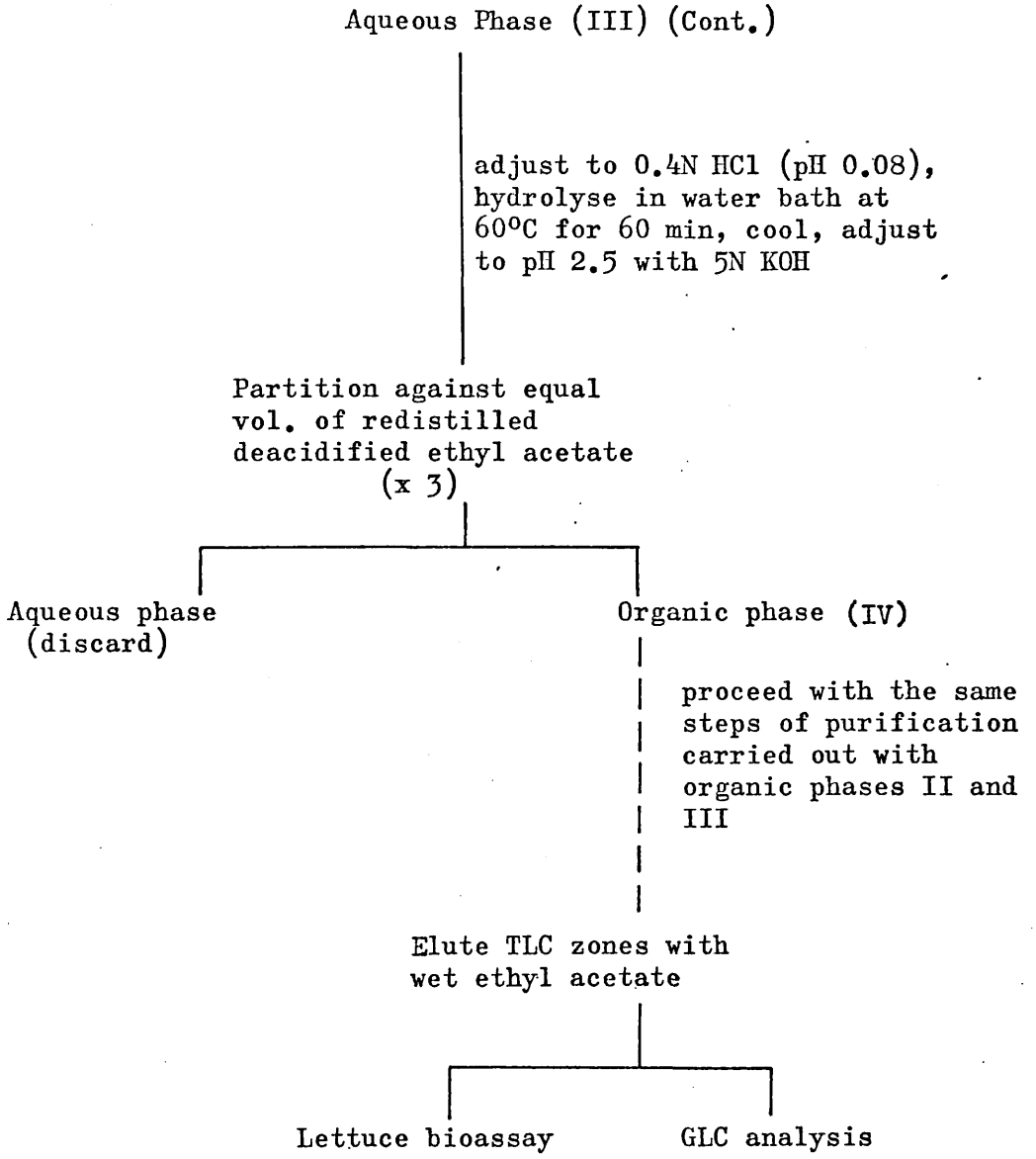


Figure 5 cont'd.

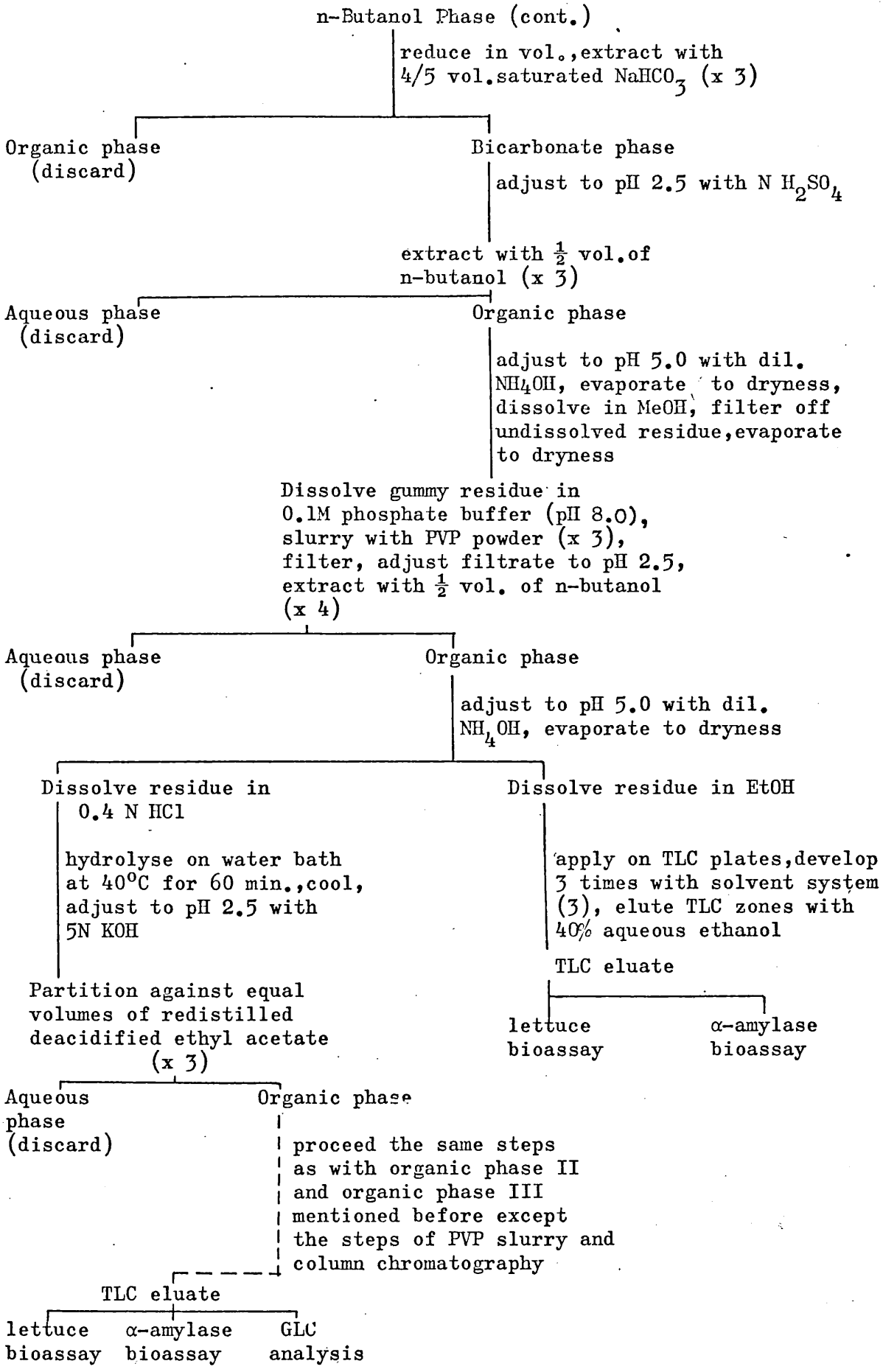


Figure 5 cont'd.

dissolved in redistilled absolute methyl alcohol, the insoluble matter filtered off through a sintered glass funnel and the filtrate was concentrated to yield a butanol-soluble acidic gum containing the gibberellin-glucosides.

Acid-Hydrolysis of Gibberellin-Glucosides

The acidic gummy solid containing gibberellin-glucosides was purified by PVP slurries, as will be explained later. Then the gibberellin-glucosides recovered were hydrolysed with 0.4N HCl in a water bath at 40°C for 60 minutes (Aung & DeHertogh, 1968). After cooling, the pH of the hydrolysate was adjusted to 2.5 with 5N KOH and the free gibberellins released were extracted from the aqueous phase by partitioning three times against equal volumes of redistilled deacidified ethyl acetate. The three ethyl acetate extracts were combined into one, reduced in volume under vacuum at 35°C, then partitioned three times against one third of its volume of 1% aqueous sodium bicarbonate. The bicarbonate phase was adjusted to pH 2.5 with 1N HCl and re-extracted three times with redistilled deacidified ethyl acetate in a ratio of 2:1 v/v bicarbonate to ethyl acetate.

The ethyl acetate extracts were combined together, dried by freezing out the water as previously described and evaporated to dryness under vacuum at 35°C. The residue obtained, containing hydrolysed gibberellin-glucosides, was dissolved in 0.1 ml of redistilled absolute ethyl alcohol and stored in 10 ml pear-shaped flasks in a deep freezer until required for further work (see Flow Diagram of Extraction, Fig. 5).

iii. Acid-Hydrolysis and Extraction of Water-Soluble or Other Bound-Gibberellins

The treatment of the aqueous fractions of a variety of tissues with dilute mineral acids has been successfully employed to release "bound" gibberellin-like substances in a less polar form (see McCombe,

1961; Murakami, 1961b, 1962; Ogawa, 1963b; Aung & DeHertogh, 1968; Barendse, 1971). Such substances behave exactly like free gibberellins and can be readily extracted from the aqueous phase with ethyl acetate at low pH.

The aqueous phase remaining after extraction of acidic butanol-soluble gibberellins was hydrolysed following the procedure described by Barendse, Kende and Lang (1968) as follows:

A sufficient amount of 5N HCl was added to the aqueous phase (after partitioning with n-butanol) to make the final solution 0.4N (pH 0.08) and the fractions were held on a water bath at 60°C for one hour. The hydrolysate was then cooled, adjusted to pH 2.5 with 5N KOH and the free gibberellins released by acid hydrolysis of water-soluble or other bound-gibberellins were extracted with redistilled deacidified ethyl acetate. The ethyl acetate extracts were combined and treated in exactly the same way as described for the free gibberellins released by acid hydrolysis of gibberellin-glucosides. The residue containing hydrolysed water-soluble or bound-gibberellins was dissolved in 0.05 ml of redistilled absolute ethanol and stored in 10 ml pear-shaped flasks in a deep freezer at -20°C until required for further work.

These three fractions (acidic ethyl acetate-soluble, acidic butanol-soluble, and water-soluble fraction) may be considered to represent the total gibberellin content of the tissue. The only gibberellins not accounted for would be any left in the tissue residue not extractable with methanol, or in the aqueous phase but not released by acid hydrolysis, or any gibberellins not active in the bioassay methods used.

5. Purification of Endogenous Gibberellins

Detection, characterisation and quantitation of gibberellins in extracts of vegetative tissue is generally very difficult due to the presence of only microgram amounts of the hormones and excessive quantities of impurities which interfere with gibberellin-bioassay (Knapp, 1963; Köhler & Lang, 1963; Crozier et al., 1969). The major source of impurities and possible inhibitory substances would seem to be phenolic compounds which in plants are widespread and frequently occur in high concentrations (Loomis & Battaile, 1966), so it is often disirable to separate the phenol fraction from plant tissue extracts. Lead acetate has been widely used as a precipitant for ortho-hydroxy phenols, but a disadvantage is that it does not precipitate some plant phenols, and may co-precipitate other compounds (Seikel, 1964).

More recently, polyclar AT, an insoluble form of the polymer, poly-N-vinyl pyrolidone (PVP), has been shown to be reasonably specific in separating a phenolic fraction from plant tissue extracts by hydrogen bond formation. In 1968, Anderson and Sowers worked out the optimum conditions for bonding of plant phenols to this polymer and in 1972, Glenn et al. recommended the use of PVP as a safe and efficient method for purification of plant extracts containing a broad spectrum of gibberellins and/or abscisic acid (ABA) by selective removal of phenolic and perhaps other organic acid impurities.

Free gibberellins and the free gibberellins released by acid hydrolysis of bound-gibberellins were purified by means of PVP slurries, PVP column chromatography and thin-layer chromatography (TLC), while acidic butanol-soluble gibberellins and the free gibberellins released by acid hydrolysis of the glucosides were purified by means of PVP slurries and thin-layer chromatography.

The Use of Non-Soluble Poly-N-Vinyl Pyrolidone Powder for Slurries and Chromatography Columns

In preparation for use in slurries or column chromatography, non-soluble polyclar AT powder (purchased from GAF, Great Britain Ltd., Chemical Division) with particles of 60-120 mesh size range was mixed thoroughly with at least five times its volume of distilled water and the remaining fines decanted after a settling period of about 10-15 minutes; fine mesh size particles required longer settling times. Following 6-8 decantations, the slurry was either filtered and air dried to a constant weight at room temperature to be used in slurries, or slurried once with 0.1M phosphate buffer at pH 8.0 and any fines from the buffer slurry decanted. The slurry was then used to set up 1.9 x 30 cm columns.

A. The Use of PVP in Slurries

The crude residue of acidic ethyl acetate-soluble (or free) gibberellins, that of the free gibberellins released by acid hydrolysis of gibberellin-glucosides as well as other bound-gibberellins, and/or the gummy residue of acidic butanol-soluble gibberellins were dissolved separately in 0.1M phosphate buffer at pH 8.0; PVP powder (treated as previously described and air dried to a constant weight at room temperature) at concentrations ranging from 50-100 mg/ml was added and slurried thoroughly with constant shaking for 30 minutes (Anderson & Sowers, 1968). The PVP was filtered off by vacuum filtration and the residue washed with successive aliquots of buffer. Fresh PVP powder was added to the filtrate and the shaking and filtration procedures repeated for a total of three times.

Recovery of free gibberellins from filtrates was effected by acidifying the solutions to pH 2.5 with 5N HCl and partitioning four times against redistilled diethyl ether in the ratio of 2:1 v/v buffer/

/ether (Glenn et al., 1972), while recovery of acidic butanol-soluble gibberellins was achieved by partitioning against redistilled n-butanol in the same ratio and at the same pH as with free gibberellins.

The combined ether extracts of each fraction were dried by freezing out the water as previously described and evaporated to dryness under vacuum at 30°C. The residues obtained with free gibberellins and with the free gibberellins released by acid hydrolysis of bound-gibberellins were dissolved separately in 5 ml of 0.1M phosphate buffer at pH 8.0 and applied by means of a long Pasteur pipette to the top of 1.9 x 30 cm columns packed with PVP, while the residue obtained with the free gibberellins released by acid hydrolysis of gibberellin-glucosides was dissolved in 0.05 ml of redistilled absolute ethanol and stored in 10 ml pear-shaped flask in a deep freezer at -20°C until required for TLC analysis.

In the case of acidic butanol-soluble gibberellins on the other hand, the combined butanol extracts were adjusted to pH 5.0 with diluted ammonium hydroxide solution to neutralise the acid, evaporated to dryness under vacuum at 40°C, and the residue obtained was dissolved in the least possible amount of redistilled absolute ethanol and stored in 10 ml pear-shaped flask in a deep freezer at -20°C until required for further work.

To check the recovery of the standard gibberellin from PVP, gibberellic acid in the concentration of 10 µg/ml (in phosphate buffer) was slurried with PVP powder with continuous shaking for 30 minutes. After filtration, the filtrate was adjusted to pH 2.5 and rigorously partitioned against redistilled diethyl ether. After evaporating the ether, the residue was taken up in 5 ml of distilled water and assayed using the lettuce hypocotyl bioassay. With the chemicals used and under the conditions employed, recovery of about 56% of gibberellic acid was achieved.

B. The Use of PVP in Column Chromatography

In preparation for use in column chromatography, PVP, after being slurried with distilled water 6-8 times and with phosphate buffer once as previously described, was poured into 1.9 x 45 cm columns, employing glass wool overlain by silver sand as a support and allowed to pack with gravity flow. The columns were packed to a height of 30 cm and equilibrated with the eluant prior to use in chromatography. Elution was carried out with 0.1M phosphate buffer at pH 8.0 which was delivered from reservoirs 20-30 cm above the column at room temperature.

The first 50 ml of the effluent were discarded unless otherwise stated, and the following 200 ml, 400 ml, or 600 ml, were collected for further processing (see Experimental Section). Recovery of the gibberellins from the column effluents was achieved as described in the case of PVP slurries and the dry residue, after evaporating the organic phase, was taken up in 0.05 ml of redistilled absolute ethanol and stored in 10 ml pear-shaped flasks in a deep freezer until required for thin-layer chromatography.

6. Thin-Layer Chromatography

The residues of acidic ethyl acetate-soluble gibberellins and/or the free gibberellins released by acid hydrolysis of water-soluble (or bound) gibberellins, which were recovered from PVP columns effluent, and the residues of acidic butanol-soluble gibberellins and/or the free gibberellins released by acid hydrolysis of gibberellin-glucosides, which were recovered from PVP slurries as described earlier, were dissolved separately in redistilled absolute ethanol. They were then streaked quantitatively on the starting line of 5 x 20 cm or 20 x 20 cm thin-layer plates coated with an 0.25 mm thick layer of Keiselgel 60 F_{254} (Merck)(see Experimental Section), which in the case of free gibberellins and the free gibberellins released by acid hydrolysis of conjugated and/

/or bound-gibberellins, had been pre-run in redistilled ethyl acetate and activated at 105°C for one hour (Williams et al., 1973). The plates were then developed with solvent system (1). In the case of acidic butanol-soluble gibberellins, the plates were developed three times with solvent system (3). On one occasion, the radicle's free gibberellins after being resolved by TLC, eluates of the zones at R_f 0.0-0.5 were mixed and re-chromatographed on thin-layer plates which were developed with solvent system (2) (see Experimental Section for full details).

All the TLC were developed in the dark at room temperature in Shandon chromatographic tanks lined with Whatman No. 1 filter sheet to ensure saturation of the tanks with the solvent system vapours.

Solvent Systems for Thin-Layer Chromatography

(1) Ethyl acetate-chloroform-acetic acid (15:5:1, v/v; Cavell et al., 1967).

(2) Benzene-n-butanol-acetic acid (70:25:5, v/v; Kagawa et al., 1963).

(3) Acetone-acetic acid (97:3, v/v; Yokota et al., 1971c; develope three times).

The term "developed three times" means that after developing the chromatograms in the normal manner, it was air dried for about half an hour in a fume cupboard at room temperature and re-chromatographed in the same solvent system and in the same direction. After drying again, it was re-chromatographed for the third time in the same manner. The mean R_f values of the available authentic gibberellins in solvent systems (1) and (2) mentioned above and under the conditions employed are shown in Table 2.

Table 2 Thin-Layer Chromatography of Standard Gibberellins on Plates of Keiselgel 60 F_{254}

Gibberellin	Mean R_f in solvent systems	
	(1)	(2)
A_3	0.33	0.52
$A_{4/7}$	0.60	0.79
A_{13}	0.34	0.52
A_{14}	0.61	0.81

The solvent front was allowed to travel exactly 15 cm from the starting line; the plates were then removed and air dried in a fume cupboard at room temperature for 2-3 hours. The developed chromatograms were divided with a sharp seeker into ten equal zones between the starting line and the solvent front.

In the case of the 5 x 20 cm plates, each zone was scraped off into an individual 4" diameter crystallising dish, over the powder was placed a disc of Whatman No. 1 filter paper before the addition of 5 ml of 0.05% Tween "20" (polyoxyethylene sorbitan monolaurate) or of distilled water (see Experimental Section) and ten lettuce seedlings to test the activity of the different zones with the lettuce hypocotyl bioassay test.

In the case of the 20 x 20 cm plates, each zone was scraped off into a 0.8 x 10 cm glass centrifuge tube and eluted with a known volume of wet redistilled ethyl acetate (in the case of free gibberellins and the free gibberellins released by acid hydrolysis of conjugated and/or bound-gibberellins), or a known volume of 40% aqueous ethanol (in the case of acidic butanol-soluble gibberellins) over-night at 2°C (Aung & DeHertogh, 1968). Elution of the silica gel here resulted in about 30% extraction of the gibberellins as indicated by biological assay

(Fig. 6), which is the same as the extraction percentage reported by Ross (1970). After a brief centrifugation at 10,000 x g to pellet the chromatographic substrate, the supernatant was transferred into 1.5 x 5 cm specimen tubes with screw caps lined with aluminium foil and the tubes were stored in a deep freezer at -20°C until required for further use.

Amounts of the thin-layer eluates ranging from 0.5-1.0 ml were evaporated separately in 4" diameter crystallising dishes, the residue was dissolved in 5 ml of distilled water and used for the lettuce hypocotyl bioassay test; and amount of the thin-layer eluates ranging from 0.02-0.2 ml were evaporated separately in test tubes, the residue was suspended in 1.0 ml of distilled water and used for the α-amylase bioassay test. Eluates of the TLC zones that showed considerable activity in the bioassay tests were derivatised for GLC analysis. On some occasions, the TLC eluates were tested biologically with the lettuce hypocotyl bioassay test only (see Experimental Section).

A blank thin-layer plate (5 x 20 or 20 x 20 cm) was chromatographed, divided into ten equal zones which were treated in exactly the same way as described for the zones of the thin-layer plates loaded with extracts and assayed with each set of bioassay so as to determine an accurate blank value and the confidence limits could be calculated (P = 0.05) by the method of Coombe et al. (1967b), using the following equation:

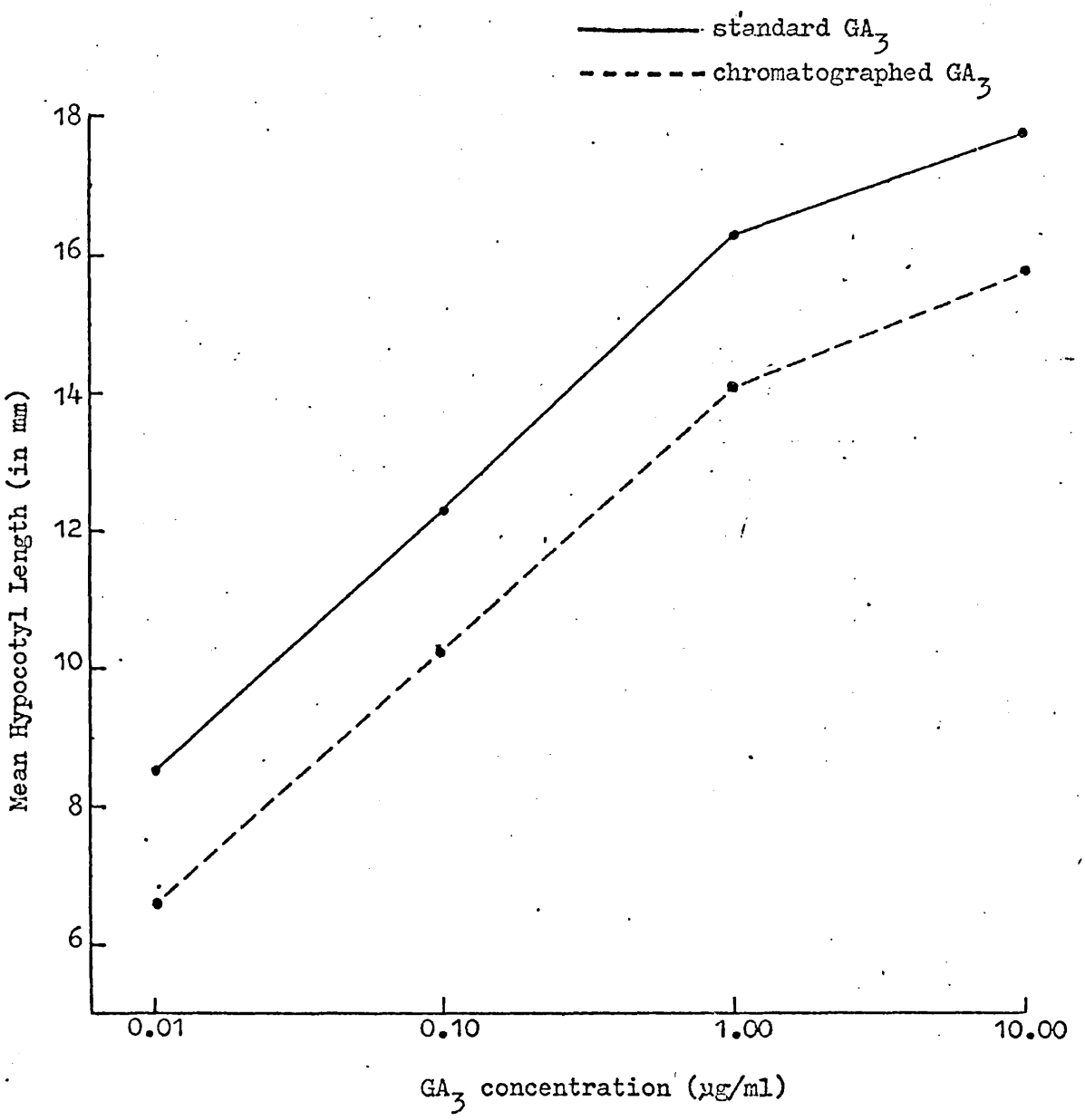
$$\text{L.S.D.} = \frac{\sigma}{\sqrt{n}} \times 2.228 \qquad \text{where}$$

n = number of blank readings, and

$$\sigma = \sqrt{\frac{1}{n-1}\left[\Sigma x^2 - \frac{1}{n}(\Sigma x)^2\right]} \qquad \text{where}$$

x = the individual blank values.

Figure 6



The mean response of lettuce hypocotyl bioassay to both standard GA_3 solutions and to chromatographed GA_3 (chromatographed on thin-layer plates of Keiselgel 60 F_{254} which were developed with solvent system (1) and the TLC zones were eluted with wet ethyl acetate overnight at 2°C).

7. Chromogenic Sprays

Gibberellins were located on thin-layer chromatograms by using ethanol/sulphuric acid spray mixture in the ratio of 95:5 v/v alcohol/ /acid (MacMillan & Suter, 1963; Jones, D.F. et al., 1963). After heating in an oven at 120°C, standard gibberellins appear at the appropriate R_f as fluorescent spots visible under UV light as shown in Table 3, adapted from Jones, D.F. et al. (1963).

Table 3 Effect of Heat on the Induction of Fluorescent Spots of Gibberellins on TLC after Spraying with Ethanol/ /Sulphuric Acid (95:5) (Jones, D.F. et al., 1963)

Gibberellin	Fluorescence induction period in min at 120°C	Colour of spot
A_1	30 - 40	blue
A_2	4 - 8	purple
A_3	1 - 3	green blue
A_4	4 - 8	purple
A_5	10 - 20	blue
A_6	10 - 20	blue
A_7	1 - 2	bright yellow
A_8	10 - 20	blue
A_9	4 - 8	purple

Attempts to detect similar fluorescent spots on thin-layer chromatograms of plant tissue extracts after spraying with ethanol/sulphuric acid followed by heating were unfortunately unsuccessful, probably because the extracts did not contain quantities of gibberellins sufficient to permit visual identification.

8. Gas-Liquid Chromatography

8.1. Apparatus and Mode of Operation

This was carried out on a Pye Series 104 dual column gas chromatograph (Model 24) fitted with a flame ionization detector head, and a desk type Servoscribe Potentiometric Recorder Model 15 operating at a chart speed of one cm/minute. The column used was a glass one (1.5 m long x 6 mm external diameter x 4 mm internal diameter) with glass to metal seal. The inert packing was 80-100 mesh Diatomite CT-AW-DMCS, coated with 2% QF-1 (Methyl fluoralkyl siloxane) as an active phase, selective for configurational isomers of alcohols, ketones, and esters, but not for carbon-carbon unsaturation. A glass wool plug was situated at the column inlet 3-4" below the injection septum.

Conditioning of the column before use was carried out to remove volatile contaminants and also to effect a rearrangement of the polymers which is essential for their full efficiency (Kuksis, 1966). This pre-treatment was achieved by heating overnight at 250°C accompanied by flushing Argon gas at a flow rate of about 30 ml/minute.

The chromatograms were normally run isothermally at 220°C with Argon carrier gas at an inlet pressure of about 1.8 Kg/cm^2 which gave a flow rate of 60 ml/minute. All injections were of 2-5 µl and were made with a Hamilton 10 µl syringe (701 R S N), fitted with 4½" removable needle. Injections were made directly into the column, just under the glass wool plug to avoid disturbance of the packing or blocking of the needle (P. Gaskin, personal communication). Soft silicon rubber septa were used and changed after 10-15 injections.

The amplifier sensitivity was adjusted as necessary but the usual settings were between x500 and x50, far enough from the bottom end of the amplifier range to avoid difficulties with excessive instrument noise. Typical running time for a complete chromatogram under the conditions used was between 30 and 45 minutes.

8.2. Preparation of Derivatives for GLC

For the gas-liquid chromatography of biological compounds it is often necessary to form derivatives which are both volatile and stable. The most common and useful of these derivatives are the methyl esters, and the trimethyl silyl ethers of the methyl esters (TMS ethers). All reactions involved in the preparation of derivatives were carried out in 1.5 x $\frac{1}{2}$" specimen tubes, sealed with screw lids lined with aluminium foil and in which the sample remained for storage and use. In the preparation of derivatives it is necessary to reduce the extract to dryness many times. This was done by immersing the specimen tube in a water bath at 40°C and blowing on dry oxygen-free nitrogen.

It is important to avoid contact of the extracts with any plastics or rubbers since common plasticisers interfere with the GLC of gibberellin derivatives (Gaskin et al., 1971). Dryness is also of great importance in the preparation and storage of the derivatives, especially the TMS ethers, as they are readily hydrolysed in contact with any moisture.

i. Preparation of Methyl Esters

Methyl esters of the extracts were prepared by taking a suitable volume of the TLC eluates (0.5 - 1.5 ml) that showed considerable activity in the bioassay tests. These volumes were carefully dried in specimen tubes, as described above, before being taken up in 0.01 ml of redistilled absolute methanol which works as a proven catalyst for the reaction (Dalgliesh et al., 1966). Standard gibberellins were directly dissolved in the least possible amount of redistilled absolute methanol.

An excess of ethereal diazomethane was added into the tubes; the yellow reagent caused effervescence, and the reaction, which is recorded as instantaneous (Dalgliesh et al., 1966), was taken as complete when the effervescence ceased. Although the reaction is believed to be

complete within one minute, the reaction mixture was usually allowed to stand at room temperature for 20 minutes before the excess diazomethane and solvents were removed under a stream of oxygen-free nitrogen (Kuksis, 1966) and the methylated products were redissolved in a known volume of redistilled ethyl acetate ranging from 20 to 50 µl.

ii. Preparation of Trimethyl Silyl Ethers (TMS Ethers)

These were prepared according to the method described by Dalgliesh et al. (1966) as follows:-

A portion of the methyl ester was evaporated to dryness under a stream of oxygen-free nitrogen and the residue was taken up in 0.25 ml of GLC grade dry pyridine. To this solution was added 0.15 ml hexamethyl disilizane and 0.05 ml trimethyl chlorosilane, the latter giving a white precipitate of ammonium chloride. The tube was sealed and left overnight at room temperature in a desiccator containing KOH pellets. After brief centrifugation, the supernatant solution was used for injection. These derivatives are only stable in the absence of moisture. They can be stored for a period up to a month in a desiccator containing KOH pellets. If the solutions become dry, pyridine could be added.

During the development of the GLC, TMS ethers proved to be difficult and unsuccessful (see Experimental Section for full details) although the TMS ethers of standard gibberellins were satisfactorily prepared and run (Table 4 and Fig. 8).

8.3. Preparation of Diazomethane

Diazomethane was prepared from the reagent "Diazald" (N-Methyl-N-nitroso-p-toluene 4-sulphonamide) according to the method described in Vogel "Elementary Practical Organic Chemistry" as follows:

In a round-bottomed flask were placed 14.0 gm KOH, 65 ml of 95% ethyl alcohol, and 65-70 ml of distilled water. An ethereal solution of Diazald (43.0 gm in 250 ml ether) was carefully dropped into this

flask which was held in a water bath at 65-70°C. The flask was fitted with an efficient condenser and collecting conical flask containing 20 ml dry ether and held in an ice box. After the addition of the last drop of the ethereal Diazald solution and the termination of the reaction, the collecting flask was sealed with aluminium foil-covered cork stopper and stored in a deep freezer. As the reaction is explosive, ground joints were avoided and the apparatus was connected by means of cork stoppers wrapped with aluminium foil.

8.4. Identification of Gibberellins via GLC Analysis

Individual gibberellin derivatives could be provisionally identified from their retention times in relation to chromatographed standards with the column used. Absolute retention times are not reproducible from one laboratory to another owing to the differences in the GLC apparatus and in the column specifications, as well as in the conditions under which the GLC is operated. However, it is possible to derive times using the available gibberellins as standards, and hence calculating relative retention times for gibberellins of which no standards are available (Cavell et al., 1967).

Table 4 is calculated from a table published by Macmillan and Pryce (1968a), of retention times for gibberellins A_1-A_{24}, according to our retention time of gibberellin A_{14}. The possibility of day to day variations makes it necessary to chromatograph a number of standards during each series of analysis.

Figures 7 and 8 show chromatograms of standard methyl esters and TMS ethers chromatographed on 2% QF-1 column.

The method employed to identify the endogenous gibberellins in the different organs of the etiolated broad bean seedling extracts was the comparison of the retention times of the various GLC peaks of the samples with the retention times of Table 4.

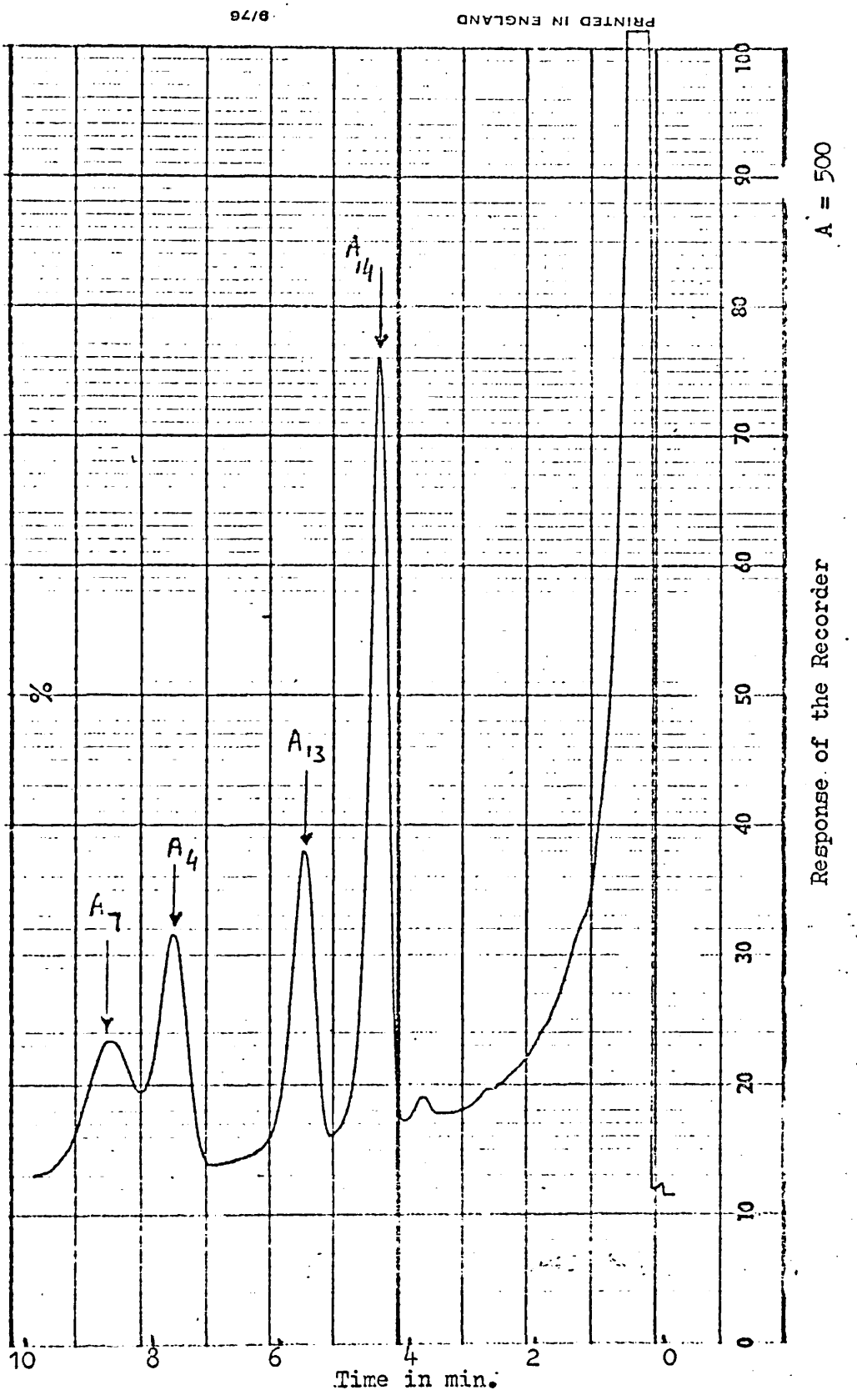
Table 4 Retention Times of Gibberellins A_1 to A_{24} as Methyl Esters and TMS Ethers of the Methyl Esters on 2% QF-1 Column. (Calculated from MacMillan & Pryce,1968a and based on our value of GA_{14} = 4.45 (methyl ester) and 3.10 (TMS ether)

Gibberellin	Methyl Ester	TMS Ether
GA_1	18.26	11.3
GA_2	19.19	16.0
GA_3	19.09	13.2
GA_4	7.79	7.7
GA_5	9.64	7.8
GA_6	15.85	13.2
GA_7	8.44	8.9
GA_8	34.12	14.4
GA_9	3.99	-
GA_{10}	9.64	11.1
GA_{11}	6.40	-
GA_{12}	2.04	-
GA_{13}	5.75	4.3
GA_{14}	4.45	3.1
GA_{15}	13.54	-
GA_{16}	15.48	12.0
GA_{17}	6.03	4.4
GA_{18}	9.92	4.8
GA_{19}	8.06	6.8
GA_{20}	9.08	7.5
GA_{21}	23.27	20.3
GA_{22}	17.24	11.8
GA_{23}	18.26	8.9
GA_{24}	2.87	2.0

Figure 7

Gas chromatogram of a mixture of standard gibberellin methyl esters on 2% QF-1 at 220°C, carrier gas at a flow rate of 60 ml/min, injection volume 1 µl, attenuation x 500

Figure 7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Response of the Recorder
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100
90
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70
60
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40
30
20
10
0
10
8
6
4
2
0
Time in min.

Figure 8

Gas chromatogram of trimethyl silyl ethers of the methyl esters of gibberellin standards chromatographed on 2% QF-1 at 220°C and carrier gas at flow rate of 60 ml/min injection volume 1 µl, attenuation x 500

Figure 8

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9. Bioassay of Gibberellins

9.1. Lettuce Hypocotyl Bioassay

The assay is based on the fact that increasing the concentration of gibberellin will increase the stimulation of lettuce hypocotyl growth. The technique used was based on the method described by Frankland and Wareing (1960) with the exception that lettuce seeds, cultivar Arctic King (purchased from Carters Tested Seed Ltd., Raynes Park, London SW20) were used as it has been recorded that this variety offers a significant increase in sensitivity, at least 10 times, over other lettuce varieties commonly used for this assay (Jones, R.L., 1968). The lower limit of sensitivity of the lettuce bioassay was found to be 0.01 µg/ml gibberellic acid.

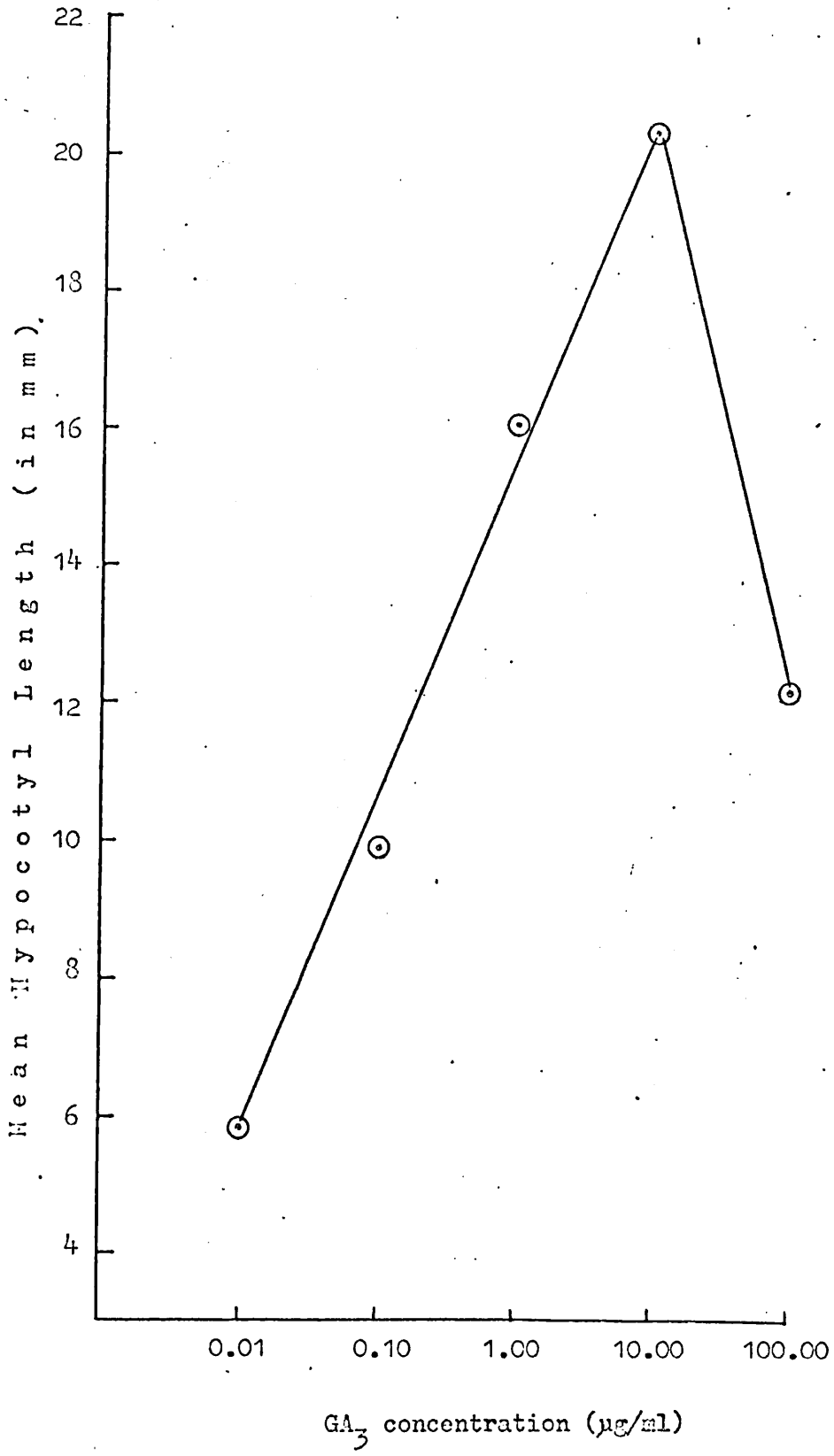
Table 5 shows a typical dose response of the lettuce hypocotyl (variety Grand Rapid, used by Frankland & Wareing, 1960; and variety Arctic King, used by Jones, R.L., 1968) to increasing concentrations of gibberellic acid.

Table 5 Response of Lettuce Hypocotyl to Standard Concentrations of Gibberellic Acid

GA_3 treatment (µg/ml)	Mean Lettuce Hypocotyl Length (± S.E.)* (mm)	
	var. Grand Rapid	var. Arctic King
0.00 (cont.)	3.70 ± 0.14	4.90 ± 0.17
0.01	4.40 ± 0.21	5.80 ± 0.24
0.10	6.30 ± 0.28	9.90 ± 0.61
1.00	11.00 ± 0.37	16.10 ± 0.85
10.00	13.60 ± 0.68	20.40 ± 0.43
100.00	8.10 ± 0.30	12.20 ± 0.63

* S.E. = Standard Error

Figure 9



The dose:response curve of increasing gibberellic acid concentration on the growth of lettuce hypocotyl.

In its final form, the assay was carried out as follows:

Lettuce seeds were sown on Whatman No. 1 filter paper moistened with 2.5 ml of distilled water in 9 cm diameter disposable sterile plastic Petri dishes at a density of 50-60 seed /dish. The dishes were then placed in the dark at 25°C (± 1°C) for 30-35 hours after which time seedlings with uniform radicles, and in which the hypocotyl had not started elongation were selected. In individual 4" diameter crystallising dishes, a known volume of the TLC eluates (see Experimental Section) was evaporated and the residue was dissolved in 5 ml of distilled water. The dishes were lined with Whatman No. 1 filter paper and the selected lettuce seedlings were planted on the filter paper at a density of 10 seedling /dish. The dishes were then incubated at 28°C (± 2°F) and continuous illumination of white "day light" fluorescent tubes (450 f.c.) in Sherer growth chambers, Model CEL. 25-7. Hypocotyl length was recorded after 72 hours, each hypocotyl being measured to the nearest mm. Under these conditions, the relationship between log-dose and response is linear in the dosage range 0.01-10.0 µg/ml GA_3 (Fig. 9).

9.2. Barley Half Seed Alpha-Amylase Bioassay

In a detailed survey of some solvents commonly used in the extraction of gibberellin-like substances from plants, Briggs (1966) showed that a number of these solvents contained impurities active in the barley (Hordium sativum L.) endosperm bioassay for gibberellins described by Nicholls and Paleg (1963) which is based on the release of reducing sugars from embryoless barley seeds in response to gibberellin. Subsequently, Jones, R.L. and Varner (1967) developed an assay based on the measurement of the α-amylase released from barley grains deprived of their embryos and reported this to be insensitive to residues from

ethyl acetate and methyl alcohol. The procedure used here is a modification of the method described by Jones, R.L. and Varner (1967).

Sensitivity of the barley endosperm bioassay for gibberellins exhibits considerable variability (Jackson, 1971). In part, the effectiveness of the bioassay changes with the variation in the variety of barley to be used, the season and the location in which seeds were grown, as well as the age of the seeds. Attempts to purchase the American variety Himalaya, which seems to be the most sensitive and popular variety for this bioassay were unsuccessful, so it was decided to use one of the local barley varieties.

A preliminary investigation of eight native barley varieties, namely: Clermont, Lofa-abed, Mazurka, Midas, Sabarlis, Sultan, Vada and Zephyr, was carried out and the sensitivities of the tested varieties were compared to that of Himalaya. The results are presented in Table 6 which shows a typical α-amylase response to dose of gibberellic acid for the eight tested varieties as well as variety Himalaya.

Table 6 Response of Different Varieties of Barley to Gibberellic Acid (data expressed in μg α-amylase per flask)

Variety	Concentration of GA_3 (μg/ml)				
	0	0.01	0.100	1.000	10.000
Himalaya	66.30	453.09	667.12	777.48	725.40
Clermont	1.52	21.92	83.43	203.24	292.64
Lofa-Abed	4.12	47.86	172.86	253.46	292.02
Mazurka	2.08	55.98	115.85	133.49	98.70
Midas	1.75	17.14	177.94	222.46	237.09
Sabarlis	2.52	40.92	159.49	237.67	255.44
Sultan	5.53	105.01	208.10	312.78	414.94
Vada	3.97	142.23	198.15	278.09	357.86
Zephyr	2.00	64.46	160.70	217.00	270.84

In general, all the varieties tested were found to be less sensitive to the assay than the American variety; this is probably due to the conditions of low moisture and high temperatures during ripening of the American seeds. Among the local varieties, Sultan seemed to be most sensitive to the assay, while Mazurka was found to be the least sensitive. The sensitivities of the other six varieties were more or less similar (Table 6). For this reason, variety Sultan was chosen for the assay. Unfortunately, after some time, this variety became unobtainable from the same or other sources, as the production of barley is subjected to change from one season to another. The work was therefore continued using variety Zephyr. This was not the variety nearest in sensitivity to variety Sultan, but it was chosen because its supply is not normally susceptible to changes. Figure 10 shows a typical dose response curve of the two barley varieties used.

Reagents

(1) 0.01M sodium acetate-acetic acid buffer pH 4.8.

(2) 0.1M calcium chloride solution.

(3) Chloramphenical solution (0.5 mg/ml).

(4) Suitable gibberellic acid standard.

(5) Starch solution, prepared as follows:

150 mg of natave (non-solubilised) potato starch, 600 mg of KH_2PO_4 and 2 ml of solution No. 2 (above) in a total volume of 100 ml of distilled water. The mixture was boiled for one minute, cooled, then centrifuged for 10 minutes at 3000 x g. The supernatant was decanted and the pellet was discarded. The supernatant was stored in a refrigerator and fresh solution was prepared for each assay.

(6) Iodine reagent; this consisted of two solutions:

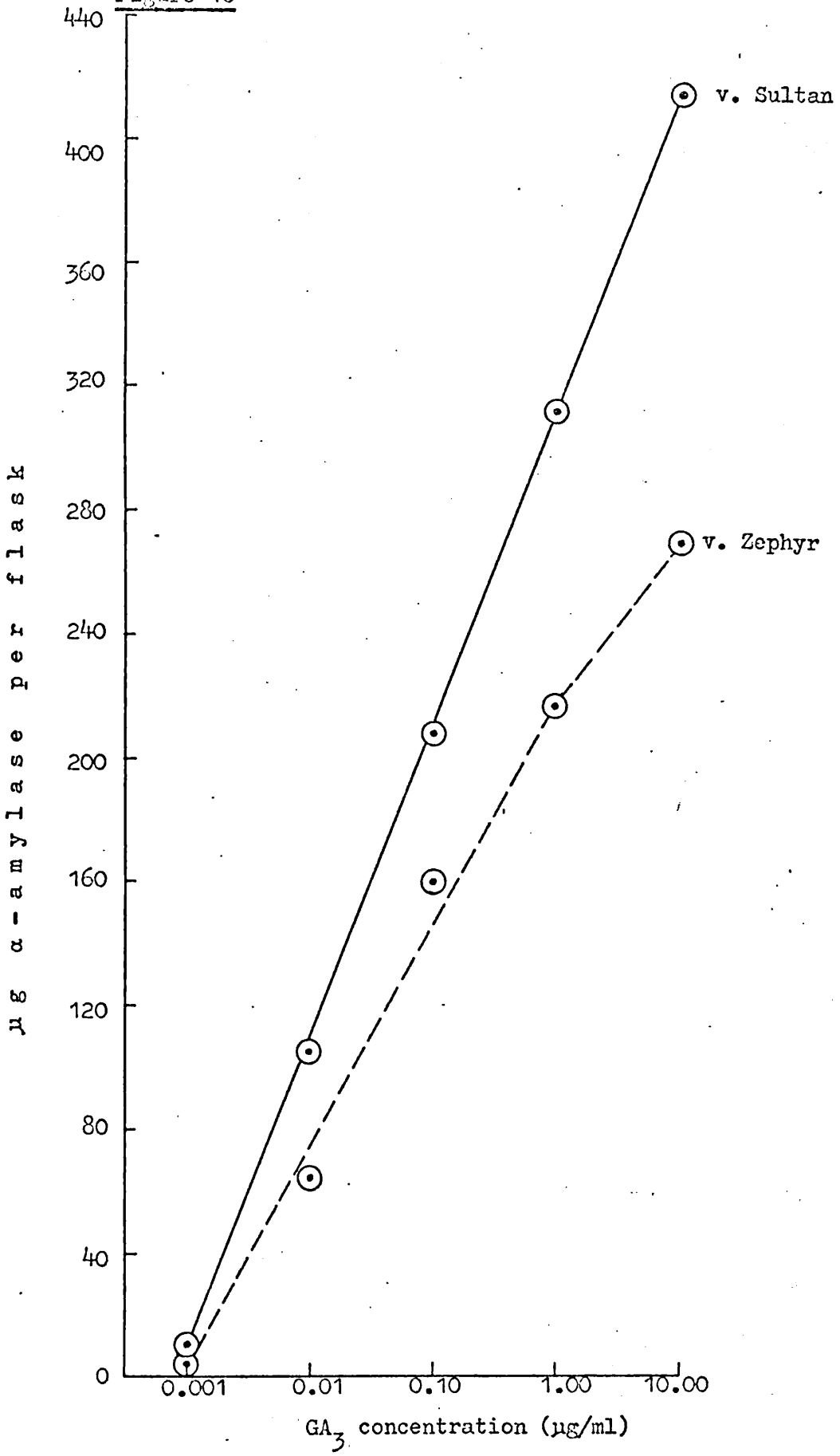
a - iodine stock solution which was prepared by mixing 6 gm of potassium iodide and 600 mg of iodine in 100 ml of distilled water.

b - 0.05N HCl.

Figure 10

The dose:response curve of increasing gibberellic acid concentration on the α-amylase released from barley grains var. Sultan and var. Zephyr. Data expressed in ug α-amylase per flask.

Figure 10


440
400
360
320
280
240
200
160
120
80
40
0
µg α-amylase per flask
v. Sultan
v. Zephyr
0.001
0.01
0.10
1.00
10.00
GA3 concentration (µg/ml)

Two ml of the stock solution were added to 100 ml of the acid. The solution does not keep and must be prepared freshly for each assay.

Setting up the Assay

Barley seeds (Hordeum vulgare), cultivar Sultan or Zephyr, were soaked in 50% (by volume) sulphuric acid for three hours at room temperature then washed at least 15-20 times with generous volumes of autoclaved distilled water with vigorous shaking to dislodge husks. The dehusked seeds were cut in half transversely with a sharp razor blade and the embryo portions were discarded. The endosperm halves were sterilised for 20 minutes in 2% sodium hypochlorite solution (commercial bleach) with stirring at 5 minutes intervals, then washed at least three times with generous volumes of sterile distilled water. The seeds were plated out on sterile paper circles (Whatman No. 1) in sterile 9 cm diameter Petri dishes with sufficient sterile distilled water to saturate but not to flood. The Petri dishes were wrapped in aluminium foil which had the advantage that it prevented the filter paper from drying out. The half seeds were allowed to imbibe for 48 hours at room temperature with a daily check that the filter paper was moist. Sterile distilled water was added where necessary with extreme care to maintain the sterility of the system which was achieved in the assay by carrying out all manipulations in a sterile room. The imbibition time is not critical and eight hours either way does not affect the assay.

Incubation

In 25 ml conical flasks was added 0.5 ml of 0.01M sodium acetate buffer pH 4.8, and 0.5 ml of 0.1M calcium chloride solution. The flasks were stoppered with cotton-wool, sealed with aluminium foil and autoclaved for 20 minutes at 15 lb pressure (121°C). Under sterile conditions 1.0 ml of aqueous test solution, one drop of 0.5 mg/ml chloramphenicol

(20 ug/ml final concentration, Wilson, 1966) and ten barley half grains were added to each flask and the stopper was replaced. The flasks were incubated for 24 hours at 25°C on a water-bath shaker (50 oscillations/ /minute).

Measurement of Released α-Amylase

The medium was decanted from the incubation flasks into 0.8 x 10 cm glass centrifuge tubes, the grains were rinsed with 2.0 ml of deionised water and the rinsings were added to the original incubate. The tubes were centrifuged at 2000 x g for 5 minutes, and the supernatant was used as enzyme solution.

An aliquot in the range of 0.02-0.20 ml was placed in a suitable test tube (1.5 x 10 cm). To the test tube 1.0 ml of starch substrate was added and timing was commenced. The reaction was allowed to proceed for a suitable time period (in the range of 1-20 minutes) then it was terminated by the addition of 1.0 ml iodine/HCl solution. To this final reaction mixture 5 ml of distilled water were added, mixed, and the optical density measured at 620 nm.

A starch blank consisting of 1.0 ml of starch substrate, 1.0 ml of iodine/HCl solution, and 5 ml of distilled water was prepared and its optical density was recorded. The decrease in optical density at 620 nm is directly proportional to the quantity of α-amylase present in the reaction mixture.

Optical density values were converted to micrograms α-amylase released using a factor which is obtained for each particular starch sample by incubation with a pure α-amylase preparation. The conversion factor for the starch sample used was 5.58. α-Amylase keeps well in a deep freeze and loses little activity after 3-5 days.

Calculation of Activity

By subtracting each reading from the starch blank value we obtain Δ.O.D.

$$\mu g\ \alpha\text{-amylase} = \frac{\Delta.O.D. \times Tv \times C.F.}{t \times v}$$ where,

Tv = volume of supernatant

Δ.O.D. = O.D. of zero time control - O.D. of sample

C.F. = conversion factor for the starch sample

t = time of incubation with starch

v = volume of supernatant taken for incubation reaction.

Silica-Gel Partition Column Chromatography Techniques (Stepwise Elution) (see Experimental Section)

Silica gel: Silicic acid (Bio-Sil A) special for column chromatography, with particle size 100-200 mesh (purchased from BIO-RAD Laboratories,Richmond) was washed with 3N HCl and then repeatedly washed with distilled water to remove all traces of acidity. Although the silicic acid was purchased as 100-200 mesh size, many finer particles were also present and these could alter the elution pattern of known gibberellins placed on the column. Therefore it was necessary to grade the silica gel by shaking it in distilled water for one minute and discarding the supernatant liquid after one hour. This was repeated with consecutive sedimentation periods of 45-, 30-, 15-, 10- and 5-minutes respectively, each time discarding the supernatant solution (Kozel & Tukey,1968). The silica gel was then dried and activated to constant weight at 110°C and stored in a desiccator until required for use.

Formic acid: 0.5M (21.2 ml of stock solution per litre) was standardised against 0.5M NaOH, the end point of the titration being pH 8.4.

Slurrying: Eight or sixty-four gm of silica gel were hydrated with 5 ml or 40 ml of 0.5M formic acid respectively and mixed until a free-flowing powder was obtained (Powell & Tautvydas,1967).

Chromatographic column: The hydrated silica gel was slurried with n-hexane saturated with 0.5M formic acid, and poured into a 15 mm or a 26 mm inner-diameter column with a stopcock in one end. The columns were packed with air pressure from a hand squeeze bulb while excess hexane was drained from the column.

Residues containing gibberellins may be applied to the top of the column by various means (Powell,1960,1964) but the method used here consisted of dissolving the residue of the tissue extracts in the least

possible amount of redistilled absolute ethanol, then mixing it with a small sample of silica gel which had previously been hydrated with formic acid. The alcohol was carefully evaporated from the sample with as little loss of the hydrating liquid as possible, then it was placed on the top of the column. The column was eluted stepwise with increasing concentrations of ethyl acetate in n-hexane (Powell & Tautvydas,1967), all solvents being saturated with 0.5M formic acid. Twenty-five fractions 25 ml each or 27 fractions 40 ml each (see Experimental Section) were collected as shown in Tables 7 and 8 respectively.

It should be mentioned here that Powell & Tautvydas (1967) and Crozier et al (1971) found that as a general rule, gibberellins are eluted from the silica gel column in a reverse order of polarity, with gibberellin A_9 in fraction 4, GA_4 and GA_7 in fractions 7-8, GA_5 in fraction 10, GA_6 in fraction 13, GA_1 and GA_3 in fractions 21-22, GA_2 in fractions 23-24, and GA_8 in fraction 30 in the case of the 15 mm inner diameter column (Powell & Tautvydas,1967). With the 26 mm inner diameter column, on the other hand, Crozier et al (1971) found that GA_9 was eluted in fraction 2, GA_4 and GA_7 in fraction 4, GA_5 in fractions 6-7, GA_6 in fraction 8, GA_{13} in fraction 11, GA_1 and GA_3 in fractions 13-14, and GA_8 in fraction 18. They mentioned also that with extracts, slight variations in this elution pattern may occur particularly with the more polar gibberellins.

Fractions from the silica gel columns were evaporated separately to dryness under vacuum at 35°C in 1.5 x 10 cm glass test tubes using a Buchler portable rotary evapomix and the residue obtained was dissolved in 0.5 or 1.0 ml of redistilled absolute ethanol (see Experimental Section). A known volume of the ethanol extract from each of the silicic acid column fractions was used to test their gibberellin activity with the lettuce hypocotyl assay and another known volume was used to test the

gibberellin activity of the column fractions with the α-amylase bioassay test as described above.

Table 7 Percentage of Ethyl Acetate in n-Hexane and the Volume Taken of n-Hexane for Stepwise Elution of Extracts from 15 mm Inner Diameter Silica Gel Partition Column

Col. fracn.	% EtoAC in n-hexane	cc taken of n-hexane	Col. Fracn.	% EtoAC in n-hexane	cc taken of n-hexane
1	0.0	25.00	14	25.5	18.62
2	1.0	24.75	15	27.0	18.25
3	3.0	24.25	16	30.0	17.50
4	6.0	23.50	17	33.0	16.75
5	9.0	22.75	18	36.0	16.00
6	12.0	22.00	19	39.0	15.25
7	15.0	21.25	20	40.5	14.88
8	16.5	20.88	21	42.0	14.50
9	18.0	20.50	22	43.5	14.12
10	19.5	20.12	23	45.0	13.75
11	21.0	19.75	24	46.0	13.50
12	22.5	19.38	25	48.0	13.00
13	24.0	19.00			

Table 8 Percentage of Ethyl Acetate in n-Hexane and the Volume Taken of n-Hexane for Stepwise Elution of Extracts from 26 mm Inner Diameter Silica Gel Partition Column

Col. Fracn.	% EtoAC in n-hexane	cc taken of n-hexane	Col. Fracn.	% EtoAC in n-hexane	cc taken of n-hexane
1	0.0	40.00	15	27.0	29.20
2	1.0	39.60	16	30.0	28.00
3	3.0	38.80	17	33.0	26.80
4	6.0	37.60	18	36.0	25.60
5	9.0	36.40	19	39.0	24.40
6	12.0	35.20	20	40.5	23.80
7	15.0	34.00	21	42.0	23.20
8	16.5	33.40	22	43.5	22.60
9	18.0	32.80	23	45.0	22.00
10	19.5	32.20	24	46.0	21.60
11	21.0	31.60	25	48.0	20.80
12	22.5	31.00	26	51.0	19.60
13	24.0	30.40	27	54.0	18.40
14	25.5	29.80			

EXPERIMENTAL SECTION

CHAPTERS III - V

CHAPTER III

1. Preliminary Studies of the Gibberellin Content of Etiolated Broad Bean (Vicia faba) Seedlings

The results of several workers who studied the gibberellin content of many plant tissues and organs have been extensively discussed in the introduction but very little of the literature cited has been found to deal with endogenous gibberellins in broad bean plants, for which reason the present investigation has involved the performance of a considerable number of preliminary experiments with the following objectives:

a. To detect and identify endogenous gibberellins in the etiolated broad bean seedlings.

b. To locate the distribution of these gibberellins in the different organs of the seedling.

c. To devise a convenient method for the purification and separation of these gibberellins.

In these experiments, seven-days old etiolated broad bean seedlings (cv. Green Sleeves) were harvested and the selected ones were treated as described in the Materials and Methods Section. The seed coat was discarded, the fresh weight of ten seedlings was determined (ca 50 gm) and they were immediately frozen with dry ice. The endogenous gibberellin-like substances of the seedlings were extracted and the extracts fractionated into an acidic ethyl acetate-soluble fraction, and a non-acidic ethyl acetate-soluble (or neutral) fraction in exactly the same way as described by Crozier et al. (1969) as follows:

The frozen tissue was crushed and homogenised in a blender with 75% ice-cold redistilled aqueous methanol at 1°C using 10 ml of methanol/gm fresh weight of tissue. The methanol was filtered through two layers of Whatman No. 1 filter paper in a Büchner funnel and the residue was stirred with a fresh amount of ice-cold 75% aqueous methanol followed by filtration. The residue was next stirred for 24 hours with another fresh amount of ice-cold 75% aqueous methanol in a cold room at 4°C then it was filtered as before. The tissue residue was discarded, the three methanolic extracts were combined and reduced to the aqueous phase under vacuum at 35°C. An equal volume of 0.5M phosphate buffer at pH 8.0 was added to the aqueous extracts and the pH was adjusted to 8.0 with 1N KOH. The aqueous phase was then partitioned eight times against one-third volume of redistilled deacidified ethyl acetate and the ethyl acetate extracts were combined together.

The aqueous phase was next adjusted to pH 2.5 with 5N HCl and partitioned five times against two-fifths of its volume of redistilled deacidified ethyl acetate. The acidic ethyl acetate extracts were combined, evaporated to dryness under vacuum at 35°C and the residue obtained (referring to the acidic ethyl acetate-soluble fraction) was dissolved in 0.5 ml of redistilled absolute ethyl alcohol and stored in 10 ml pear-shaped flask in a deep freezer at -20°C until required for chromatographic work.

The ethyl acetate extracts collected at pH 8.0 were evaporated to dryness under vacuum at 35°C and the residue obtained was dissolved in 0.5M phosphate buffer at pH 8.0. The pH of the buffer was next adjusted to 2.5 with 5N HCl and partitioned three times against equal volumes of redistilled deacidified ethyl acetate. The ethyl acetate extracts were combined, evaporated to dryness under the conditions described before and the residue obtained (referring to the nonacidic ethyl acetate-

-soluble fraction) was treated and stored as mentioned above until required for chromatographic work. For simplicity, the above mentioned fractions will be referred to as the acidic fraction and the nonacidic (or neutral) fraction. An aliquot of each fraction was used separately for paper chromatography/bioassay and thin-layer chromatography/bioassay.

1.1. Paper Chromatography/Bioassay of Extracts from Etiolated Broad Bean (Vicia faba) Seedlings

An amount of each of the acidic and nonacidic fractions of the etiolated broad bean seedling extracts equivalent to 5 gm fresh weight of tissue was streak loaded separately on the starting line of 3 cm wide Whatman No. 1 paper strips which were developed in the dark at room temperature by the descending chromatographic method using one of the following solvent systems:

a - Chloroform-ethanol-water-formic acid
(20:4:2:1, v/v; Radley, 1956).

b - Ethanol-3N ammonium hydroxide
(4:1, v/v; Phinney & West, 1960).

The solvent front was allowed to travel 19 cm from the starting line.

After drying the chromatograms for 2-3 hours at room temperature in a fume cupboard, each paper strip was cut into 10 equal transverse sections which were placed individually in crystallising dishes 4" diameter containing 5 ml of distilled water and their gibberellin-like activity was assayed by the lettuce hypocotyl bioassay test, as described in the Materials and Methods Section, with the exception that the lettuce seedlings were incubated in a constant temperature room at 25°C (± 1°C) and a photoperiod of 16 hours at 450 f.c. light intensity (the only available conditions at that time).

A blank paper strip was chromatograpaed, divided into 10 segments, each of which was assayed for the determination of an accurate blank value and confidence limits. The results of the bioassay are represented in histogram form in Figures 11 and 12 for solvent systems (a) and (b) respectively.

With solvent system (a), the acidic fraction was resolved into two separate zones which showed activity with the lettuce hypocotyl bioassay test; one zone at R_f 0.1-0.4 with maximum activity at R_f 0.2-0.3, and the other zone at R_f 0.7-1.0 with maximum activity at R_f 0.8-0.9 (Fig. 11A). The nonacidic (or neutral) fraction of the same extract was resolved into a major zone of activity at R_f 0.8-1.0 which showed maximum activity at R_f 0.8-0.9, and a minor zone of activity at R_f 0.1--0.3 with maximum activity at R_f 0.2-0.3 (Fig. 11B). The R_f of GA_3, the only available standard at that time, was 0.88.

With solvent system (b) on the other hand, the acidic fraction was resolved into three separate zones of activity as follows (Fig. 12A):

i. A major zone at R_f 0.5-0.8 with maximum activity at R_f 0.7-0.8,

ii. A next zone at R_f 0.2-0.4 with maximum activity at R_f 0.2-0.3, and

iii. A minor zone at R_f 0.0-0.1.

The nonacidic fraction was resolved into a major zone of activity at R_f 0.6-0.9 with maximum activity at R_f 0.8-0.9, and a minor zone of activity at R_f 0.3-0.5 (Fig. 12B). The R_f of GA_3 in this solvent system was 0.67.

Whether each of the above-mentioned zones was composed of a single biologically active substance, or several of these substances, was not possible to say at this stage.

Figure 11

Lettuce hypocotyl bioassay of paper chromatogram loaded with the extracts from 7-day old etiolated broad bean seedling.

A - Acidic fraction

B - Nonacidic (or neutral) fraction

Five gms fresh weight equivalent were chromatographed on 3 cm wide paper strips Whatman No. 1 with the solvent system:

Chloroform-ethanol-water-formic acid

(20:4:2:1 v/v)

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 11

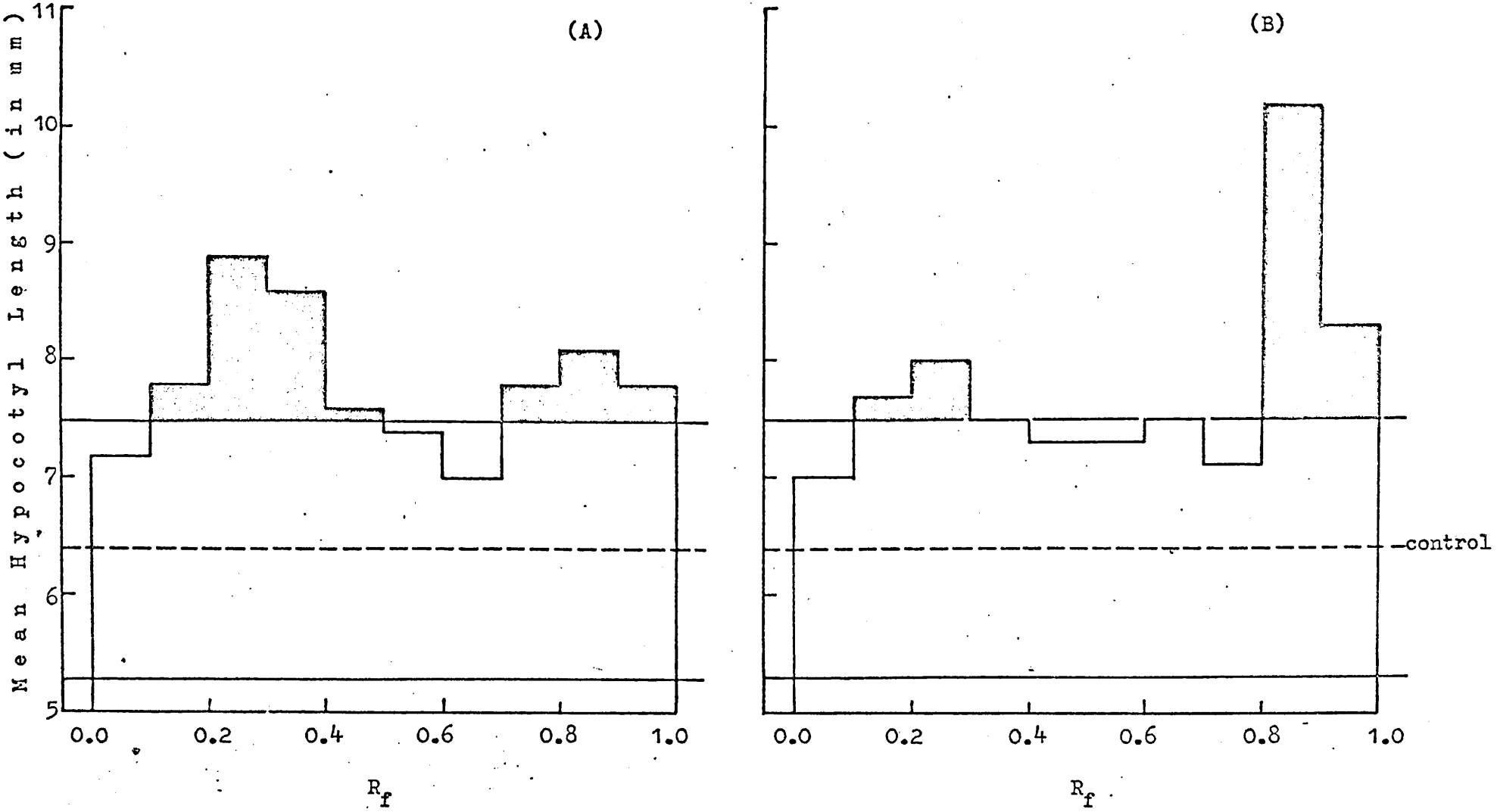

(A)
(B)
Mean Hypocotyl Length (in mm)
11
10
9
8
7
6
5
0.0
0.2
0.4
0.6
0.8
1.0
R_f
control

Figure 12

Lettuce hypocotyl bioassay of paper chromatogram loaded with the extracts from 7-day old etiolated broad bean seedling.

A - Acidic fraction

B - Monacidic (or neutral) fraction

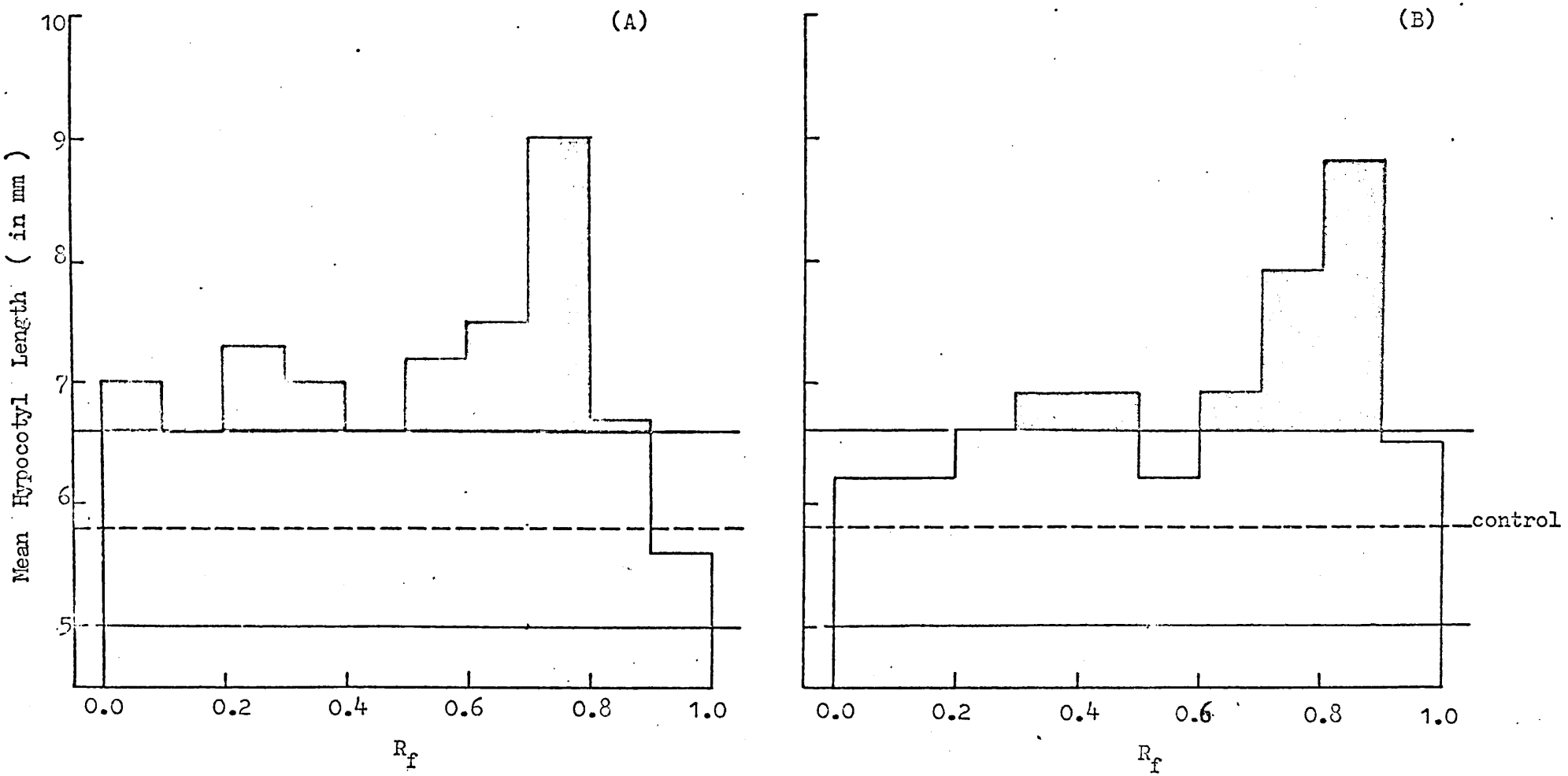
Five gms fresh weight equivalent were chromatographed on 3 cm wide paper strips Whatman No. 1 with the solvent system:

Ethanol-3N ammonium hydroxide

(4:1 v/v)

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 12


(A)
(B)
Mean Hypocotyl Length (in mm)
10
9
8
7
6
5
0.0
0.2
0.4
0.6
0.8
1.0
R_f
control

1.2. Thin-Layer Chromatography/Bioassay of Extracts from Etiolated Broad Bean (Vicia faba) Seedling

A volume equivalent to 10.0 gm fresh weight of tissue of each of the acidic and the nonacidic fractions of the etiolated broad bean seedling extracts was streak loaded separately on the starting line of 5 x 20 cm thin-layer plates coated with 0.25 mm thick layer of Kieselgel 60 F_{254} (Merck), and the plates were developed under the conditions described in the Materials and Methods Section with one of the following solvent systems:

i. Di-isopropyl ether-acetic acid

(95:5, v/v; Cavell et al., 1967).

ii. n-Butanol-1.5N ammonium hydroxide

(1:1, v/v; organic phase, Pitel et al. 1971).

Once the solvent had migrated the desired distance from the starting line (15 cm exactly), the plates were removed from the tank, air dried for 2-3 hours in a fume cupboard at room temperature and divided into 10 x 1.5 cm zones which were treated as described in the Materials and Methods Section and their gibberellin content was assayed by the growth of the lettuce hypocotyl after 72 hours under the conditions described in the previous experiments.

A blank thin-layer plate was chromatographed and treated in exactly the same way as described for the plates loaded with the extracts to determine an accurate blank value and the confidence limits. The results are represented in histogram form in Figures 13 and 14 for solvent systems (i) and (ii) respectively.

Using solvent system (i), the acidic fraction was resolved into three separate zones showing gibberellin-like activity (Fig. 13A), while the nonacidic fraction was resolved into at least four zones with gibberellin-like activity (Fig. 13B). It was not possible to state

whether each of these zones was composed of a single biologically active substance or several such substances, so they were designated peaks. Peak (I) at R_f 0.0-0.2, peak (II) at R_f 0.3-0.6, peak (III) at R_f 0.5-0.7, and peak (IV) at R_f 0.7-1.0 (Figs. 13A and 13B).

Peak (I) behaved as a fairly polar component and was barely mobile in this solvent system under the conditions employed. On the other hand, peak (IV) behaved as the least polar gibberellin-like substance present, migrating with the solvent front. The R_f of GA_3 was 0.05.

With solvent system (ii) on the other hand, the acidic fraction was resolved into three separate peaks as follows (Fig. 14A):

a. A major peak at R_f 0.5-0.7 with maximum activity at R_f 0.5-0.6 (peak III),

b. A second peak at R_f 0.8-1.0 (peak IV), and

c. A minor peak at R_f 0.0-0.2 with maximum activity at R_f 0.1-0.2 (peak I).

The nonacidic fraction was resolved into at least four peaks showing gibberellin-like activity (Fig. 14B). The R_f of GA_3 was 0.17.

The results of these experiments prompted the extention of the work to deal with each organ of the seedling separately and to include the determination of water-soluble or "bound" gibberellin-like substances.

Figure 13

Lettuce hypocotyl bioassay of TLC loaded with the extracts from 7-day old etiolated broad bean seedling.

A - Acidic fraction

B - Nonacidic (or neutral) fraction

Ten gms fresh weight equivalent were chromatographed on 5 x 20 cm thin-layer plates of Keiselgel 60 F_{254} with the solvent system:

Di-isopropyl ether-acetic acid

(95:5 v/v)

and eluted with 0.05% Tween "20".

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 13

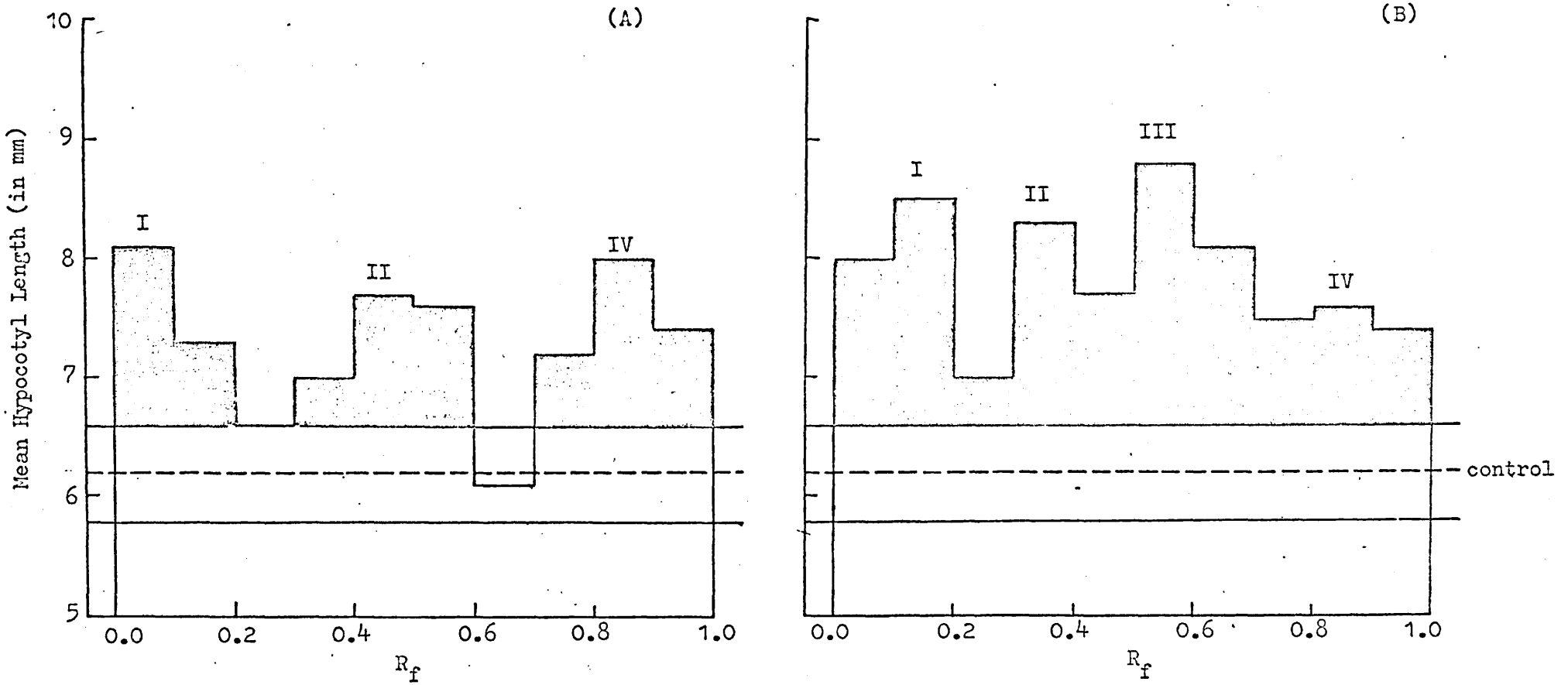

(A)
(B)
Mean Hypocotyl Length (in mm)
10
9
8
7
6
5
I
II
III
IV
control
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 14

Lettuce hypocotyl bioassay of TLC loaded with the extracts from 7-day old etiolated broad bean seedling.

A - Acidic fraction

B - Nonacidic (or neutral) fraction

Ten gms fresh weight equivalent were chromatographed on 5 x 20 cm thin-layer plates of Keiselgel 60 F_{254} with the solvent system:

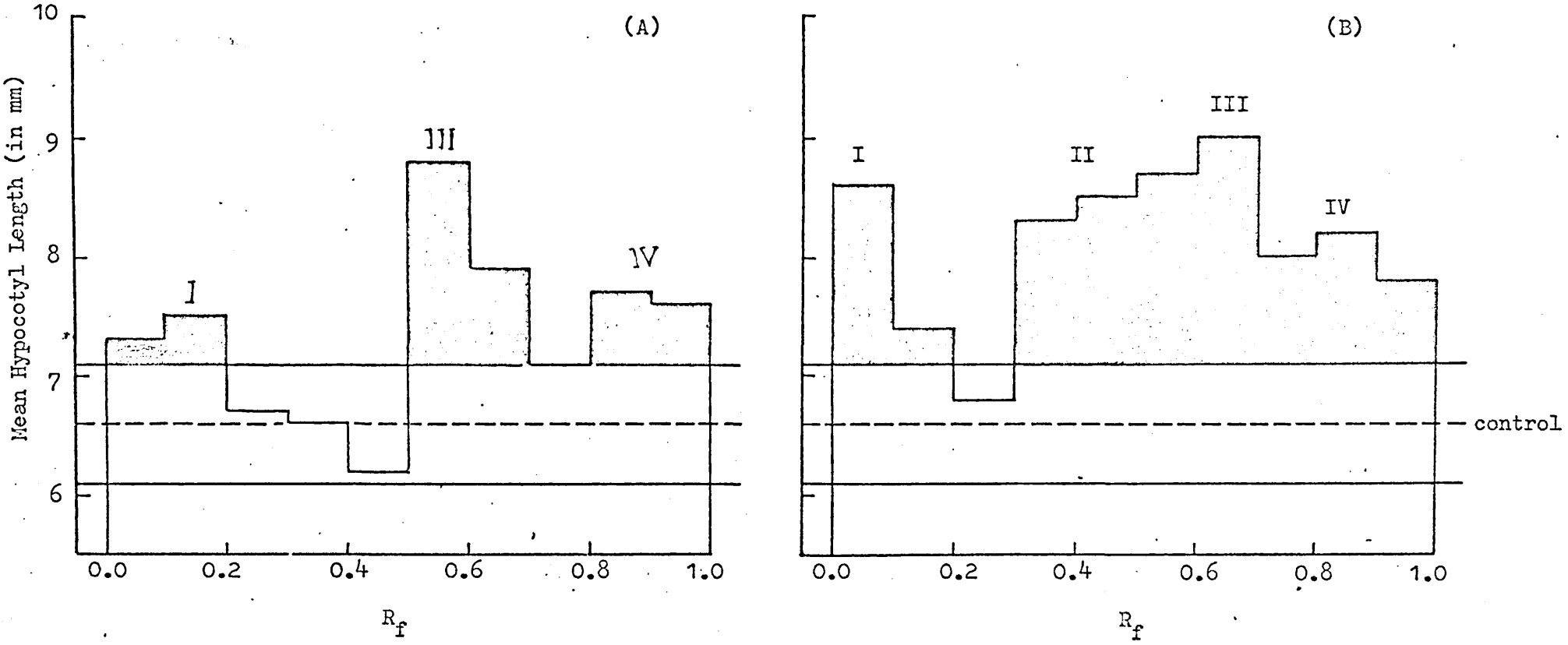
n-butanol-1.5N ammonium hydroxide

(1:1 v/v organic phase)

and eluted with 0.05% Tween "20".

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 14


(A)
(B)
Mean Hypocotyl Length (in mm)
10
9
8
7
6
I
II
III
IV
control
0.0
0.2
0.4
0.6
0.8
1.0
R_f

2. Distribution of Free and "Bound" Gibberellin-Like Substances in Etiolated Broad Bean Seedlings as Determined by TLC/Bioassay

A number of experiments was carried out to locate free (acidic ethyl acetate-soluble), as well as "bound" (water-soluble) gibberellin--like substances in the different organs of etiolated broad bean seedling. In these experiments, seven-days old etiolated seedlings were harvested, selected and treated as described in the Materials and Methods Section. The testas were discarded, the fresh weight of each organ from 20 seedlings was determined (radicle ca 25 gms, plumule ca 40 gms, and cotyledons ca 35 gms) and the tissue was frozen immediately with dry ice.

The native gibberellin-like substances of the different organs were extracted separately and fractionated into an acidic and a nonacidic ethyl acetate-soluble fraction as described in the previous experiments. The aqueous phase remaining (containing bound-gibberellins) was adjusted to 0.4N HCl (pH 0.08) and the bound-gibberellins were released free and extracted with redistilled deacidified ethyl acetate at pH 2.5, as described in the Materials and Methods Section.

The ethyl acetate extracts of each fraction were combined, evaporated to dryness under vacuum at 35°C, and the residue obtained in each case was dissolved in 0.5 ml of redistilled absolute ethanol and stored in 10 ml pear-shaped flask in a deep freezer at -20°C until required for further work.

A volume of each of the three fractions equivalent to 10 gms fresh weight of tissue of each organ of the etiolated seedling (0.20 ml in the case of radicle extracts, 0.12 ml in the case of plumule extracts, and 0.14 ml in the case of cotyledon extracts) was streaked separately on the starting line of 5 x 20 cm thin-layer plates of Kieselgel 60 F_{254} (Merck) and developed with solvent system (i). The plates were air dried as described before, divided into 10 equal parts, which were treated and tested for gibberellin-like activity with the lettuce hypocotyl assay.

The results of the bioassay are represented in histogram form in Figures 15, 16, and 17 for the radicle extracts, the plumule extracts, and the cotyledon extracts respectively.

The Figures show that in the case of the acidic fraction, gibberellin-like substances of peaks (I) and (II) of Figure 13A were extracted from the plumule and the cotyledon tissue but not from the radicle tissue, while the gibberellin-like substance or substances of peak (IV) of the same Figure were extracted from the radicle tissue mainly, and to some extent from the cotyledon tissue (see Figs. 15A, 16A, and 17A for comparison with Fig. 13A).

In the case of the nonacidic fraction, on the other hand, the gibberellin-like substances of peaks (I) and (II) of Figure 13B were extracted from the cotyledon and the radicle tissue mainly, while the gibberellin-like substances of peak (IV) of the same Figure were extracted from the plumule as well as the cotyledon tissue (see Figs. 15B, 16B, and 17B for comparison with Fig. 13B).

The results of the bioassay of the free gibberellins released by acid hydrolysis of the "bound" gibberellin-like substances show that those of the radicle extracts were resolved into three main peaks and a just significant peak with gibberellin-like biological activity (Fig. 15C), those of the plumule extracts were resolved into two major peaks and a small one with gibberellin-like biological activity (Fig. 16C), while the free gibberellins released by acid hydrolysis of the cotyledon bound-gibberellins were resolved into at least three peaks of activity (Fig. 17C).

Figure 15

Lettuce hypocotyl bioassay of TLC loaded with extracts from the radicle tissue of 7-day old etiolated broad bean seedlings.

A - Acidic fraction

B - Nonacidic (or neutral) fraction

C - Acid hydrolysed fraction

Ten gms fresh weight equivalent were chromatographed on 5 x 20 cm thin-layer plates of Keiselgel 60 F_{254} with the solvent system:

Di-isopropyl ether-acetic acid

(95:5 v/v)

and eluted with 0.05% Tween "20".

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 15

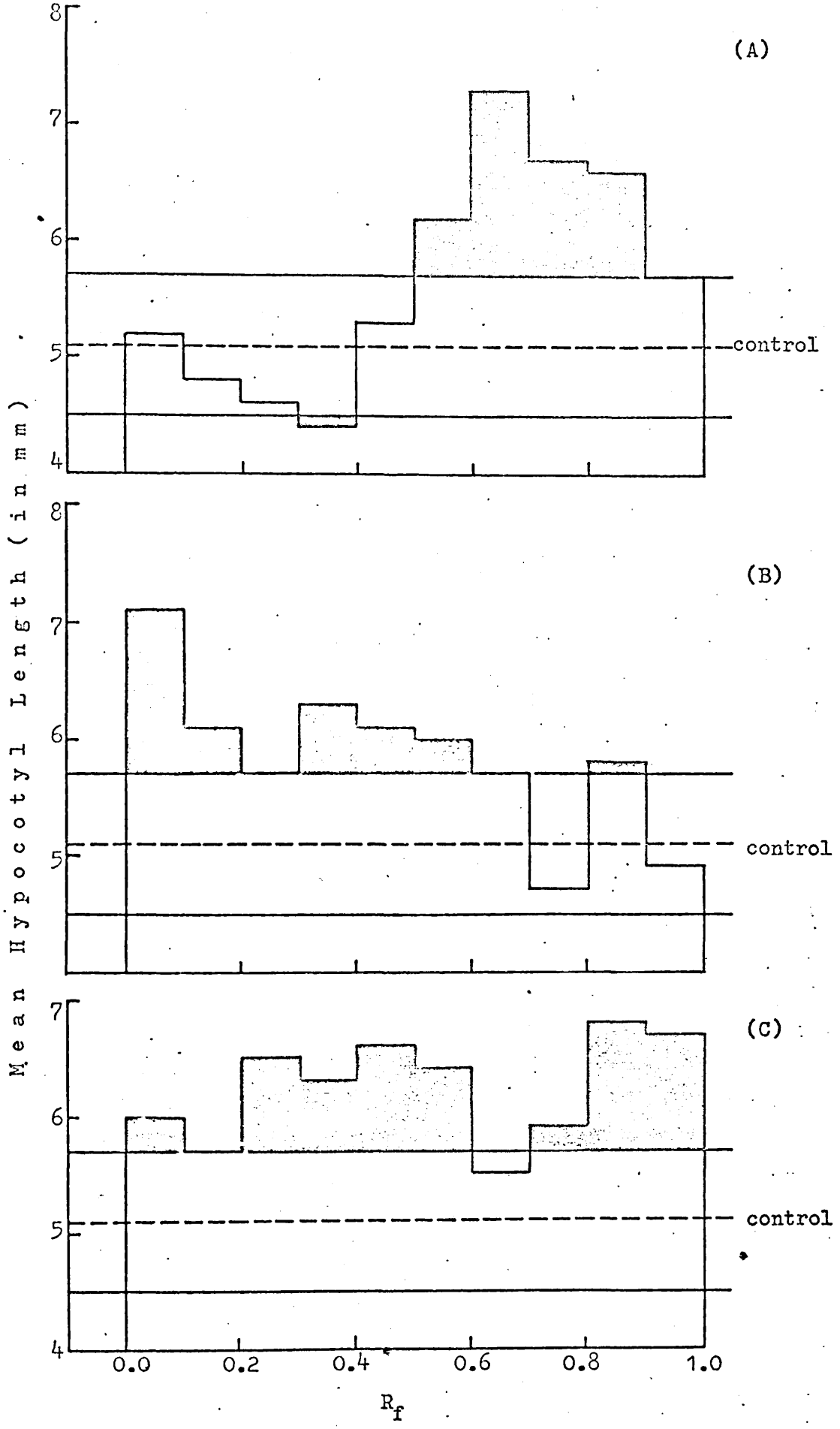

(A)
(B)
(C)
control
control
control
Mean Hypocotyl Length (in mm)
8
7
6
5
4
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 16

Lettuce hypocotyl bioassay of TLC loaded with extracts from the plumule tissue of 7-day old etiolated broad bean seedling.

A - Acidic fraction

B - Nonacidic (or neutral) fraction

C - Acid hydrolysed fraction

Ten gms fresh weight equivalent were chromatographed on TLC plates of Keiselgel 60 F_{254} with the solvent system:

Di-isopropyl ether-acetic acid

(95:5 v/v)

and eluted with 0.05% Tween "20".

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 16

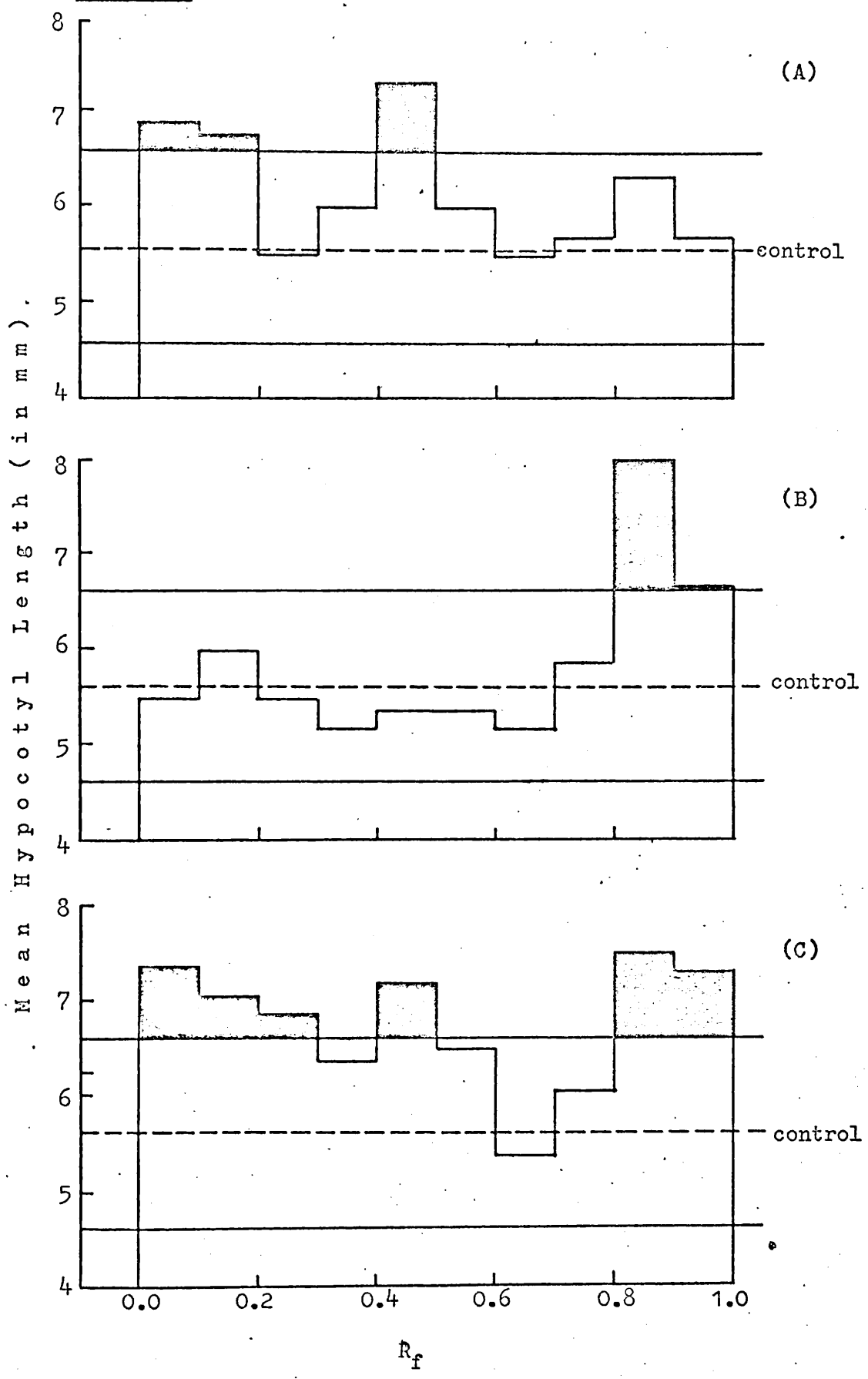

Mean Hypocotyl Length (in mm).
8
7
6
5
4
(A)
control
(B)
control
(C)
control
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 17

Lettuce hypocotyl bioassay of TLC loaded with extracts from the cotyledon tissue of 7-day old etiolated broad bean seedling.

A - Acidic fraction

B - Nonacidic (or neutral) fraction

C - Acid hydrolysed fraction

Ten gms fresh weight equivalent were chromatographed on 5 x 20 cm thin-layer plates of Keiselgel 60 F_{254} with the solvent system:

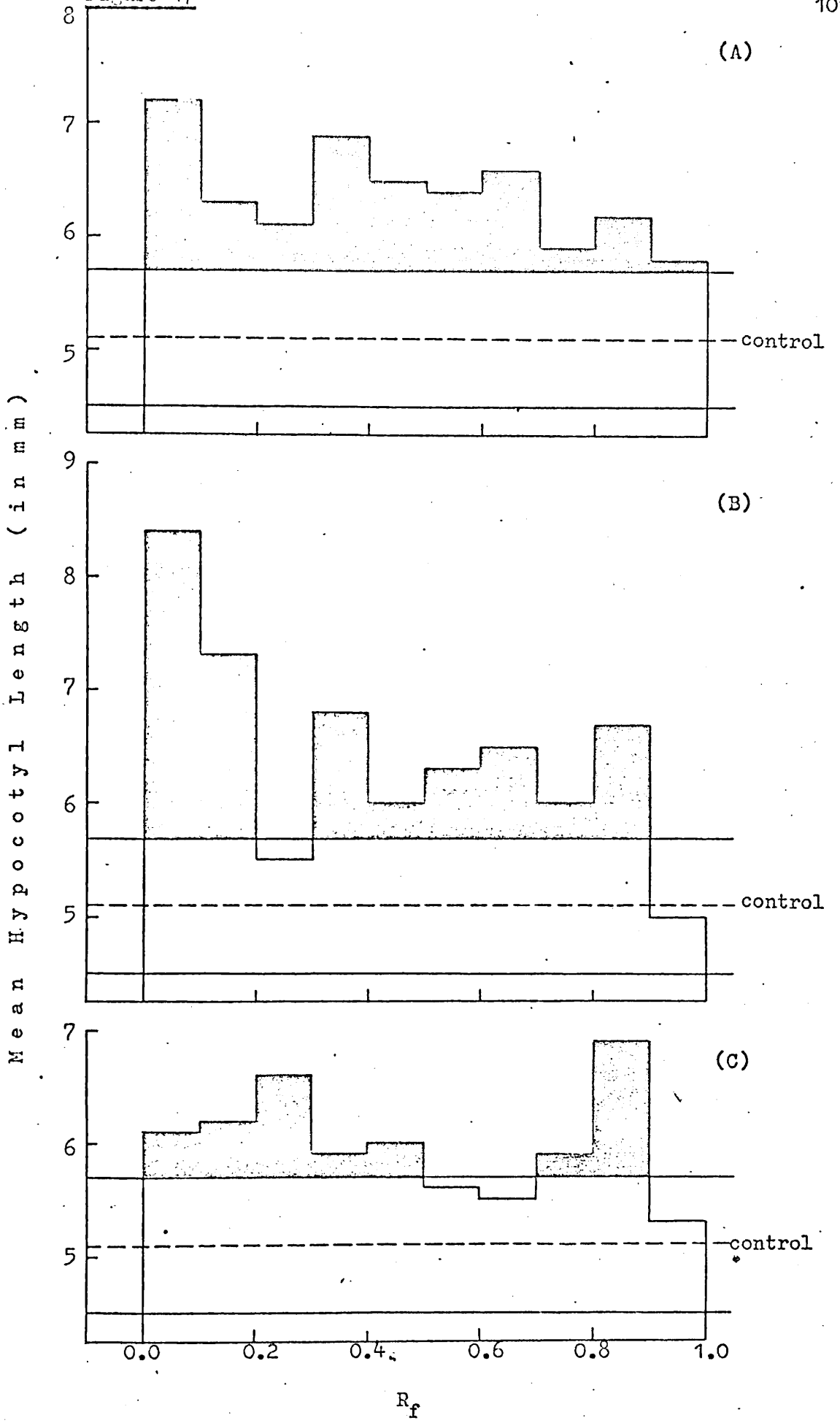
Di-isopropyl ether-acetic acid

(95:5 v/v)

and eluted with 0.05% Tween "20".

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 17


(A)
(B)
(C)
control
control
control
Mean Hypocotyl Length (in mm)
8
7
6
5
9
8
7
6
5
7
6
5
0.0
0.2
0.4
0.6
0.8
1.0
R_f

3. Silicic Acid Partition Column Chromatography of Endogenous Gibberellins in the Different Organs of Etiolated Broad Bean Seedling

The TLC eluates of the extracts in the previous experiments were found to contain other substance or substances which inhibited the growth of the lettuce radicle. These substances could have affected the growth response of the lettuce hypocotyl to gibberellin-like substances in the extracts, and in this case the peaks obtained in the previous experiments might have been distorted to some extent by the inhibitory substances. For this reason, trials were carried out for the further purification of the extracts.

It should be mentioned here too, that the solvent system di-isopropyl ether-acetic acid is not recommended for routine separations of the gibberellins since di-isopropyl ether is a highly dangerous chemical capable of forming explosive peroxides (Dr. J. MacMillan, personal communication quoted by Railton, 1971). So, the fractionation and the separation procedures of endogenous gibberellins used in the previous experiments were subsequently changed, the changes included the following:

a. The first step of tissue extraction was carried out by ice-cold absolute methanol in the ratio of 2 ml of methanol/gm fresh weight of tissue, and stirring the tissue residue for 24 hours with a fresh amount of ice-cold 80% aqueous methanol in a cold room at 4°C using the same ratio of methanol to fresh weight of tissue as before. The reason for this change was to keep down the volume of methanolic extracts as the fresh weight of the sample was increased.

b. Partitioning against ethyl acetate at pH 8.0 was replaced by partitioning against redistilled petroleum ether (B.P. 60-80°C) at the same pH and this ensures that all the free gibberellins are retained in the aqueous phase (Crozier & Reid, 1971).

c. Gibberellin-like substances in the tissue extracts were separated employing silicic acid partition column chromatography (Powell & Tautvydas, 1967; Crozier et al., 1971).

d. The germination period was increased to ten days and the number of seedlings was increased as well, in order to obtain a considerable amount of fresh weight of tissue for analysis.

It should be mentioned here that increasing the size of the sample resulted in a problem concerning the maceration of the cotyledonary tissue with the blender, so for some time, until this problem was solved by using a mincer as described in the Materials and Methods Section, extraction and analysis of the plumule and the radicle tissue only was continued. The next experiment was carried out as follows:

Ten-days old etiolated broad bean seedlings were harvested and the selected ones were treated as described in the Materials and Methods Section. 100 gms fresh weight of the plumule tissue (obtained from 30 seedlings) were frozen immediately with dry ice then extracted twice with ice-cold redistilled methanol.

The methanol was evaporated under vacuum at 35°C, the aqueous phase was freed from pigmented material by partitioning against equal volumes of redistilled petroleum ether (B.P. 60-80°C) at pH 8.0 until no more colour was obtained (Crozier & Reid, 1971), and free gibberellin--like substances were extracted by partitioning against redistilled deacidified ethyl acetate at pH 2.5 as described before. After evaporating the ethyl acetate to dryness in the usual way, the residue obtained was treated and applied to the top of 15 mm inner diameter silicic acid column which was eluted with increasing concentrations of ethyl acetate in n-hexane (see the Materials and Methods Section for all details).

Twenty-five fractions of 25 ml each were collected as shown in Table 7, evaporated separately to dryness under vacuum at 35°C, the residue obtained from each fraction was dissolved in 0.5 ml of re-distilled absolute ethanol of which 0.25 ml (equivalent to 50 gms fresh weight of tissue) was evaporated into individual 4" diameter crystallising dishes and the residue dissolved in 5 ml of distilled water and assayed by the lettuce hypocotyl bioassay.

The results of the bioassay are represented in histogram form in Figure 18. From the Figure, activity was detected in fraction (2), fractions (15-21) with little activity in fraction (17), and a very little activity in fraction (25). It should be mentioned here that some of the column fractions were found to contain phenolic compounds, as indicated by the black discoloration which appeared in the media of these fractions when tested with the lettuce hypocotyl bioassay. For this reason, the step of slurrying the extracts with PVP powder at pH 8.0 was added for the future work, to free the extracts from the phenolic compounds which were found to be abundant in vegetative plant tissue (Loomis & Battaile, 1966).

In the following experiment, extraction of 200.0 gms fresh weight of frozen radicle and/or plumule tissue of 10-days old etiolated broad bean seedlings was carried out separately in exactly the same way as described in the previous experiment, and the residue containing the free gibberellin-like substances from each organ was dissolved in 0.5M phosphate buffer at pH 8.0 and slurried three times with PVP powder. Gibberellins were recovered from the buffer by partitioning four times against half the volume of redistilled di-ethyl ether at pH 2.5 (Glenn et al., 1972). The ether extracts were combined, evaporated to dryness under vacuum at 30°C and the residue obtained was treated and applied to the top of 15 mm inner diameter silicic acid column as described in the

Figure 18

Lettuce hypocotyl bioassay of fractions collected from 15 mm inner diameter silicic acid partition column loaded with the free gibberellins of the plumule tissue extracts.

An amount equivalent to 50 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

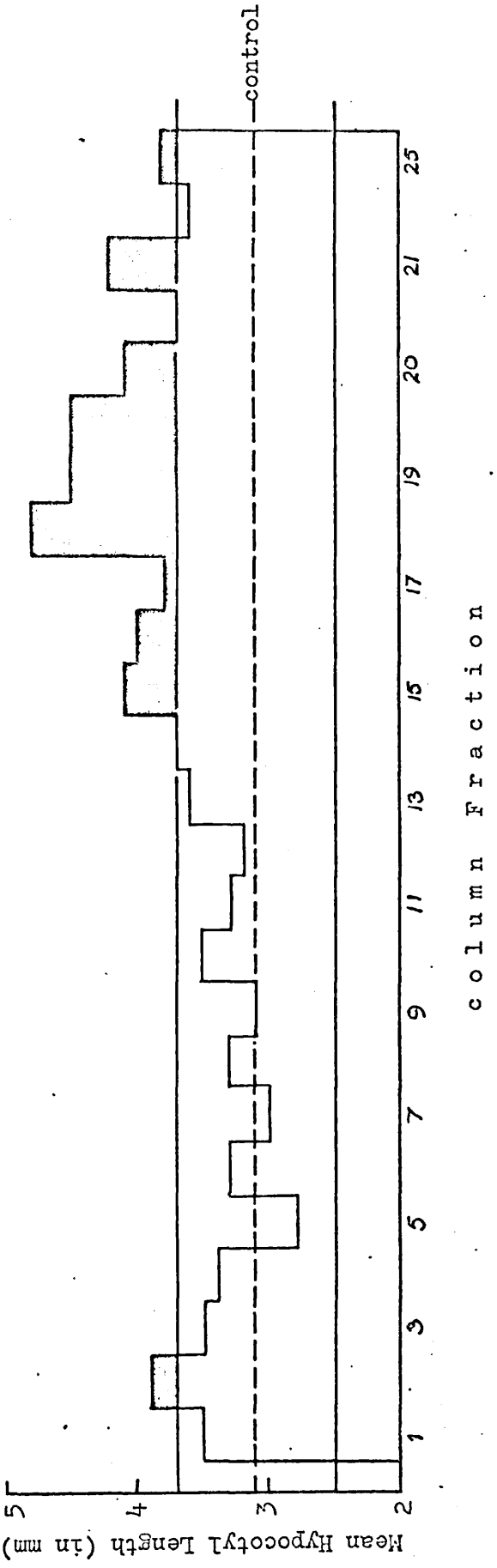

control
Mean Hypocotyl Length (in mm)
5
4
3
2
1
3
5
7
9
11
13
15
17
19
20
21
25
column Fraction

Figure 18

previous experiment. The column was eluted with increasing concentration of ethyl acetate in n-hexane and twenty-five fractions of 25 ml each were collected, as represented in Table 7.

The aqueous phase remaining after the extraction of the free gibberellins was treated and the water-soluble or bound-gibberellins were hydrolysed as described before. The free gibberellins released then were extracted with redistilled deacidified ethyl acetate at pH 2.5 and purification by PVP slurries and silicic acid partition column chromatography proceeded as with the free gibberellins. The columns fractions were evaporated separately as described in the Materials and Methods Section, the residue obtained from each fraction was dissolved in 0.5 ml of redistilled absolute ethanol of which 0.25 ml (equivalent to 100.0 gm fresh weight of tissue) was used for testing the gibberellin--like activity of the columns fractions with the lettuce hypocotyl bioassay test.

The results are represented in histogram form in Figures 19A, 19B, and 20A, 20B, for the radicle free and hydrolysed bound-gibberellins, and for the plumule free and hydrolysed bound-gibberellins respectively.

In the case of radicle extracts, activity was detected in fractions (4-7), (13-15), fraction (22) together with a just significant activity in fractions (17), (20) and fraction (23) of the column loaded with the free gibberellins (Fig. 19A). Fractions collected from the column loaded with the free gibberellins released by acid hydrolysis of the radicle bound-gibberellins showed activity in fractions (6-7), fraction (9), fractions (11-16) with maximum activity in fraction (13), fractions (21-23) and a very small activity in fractions (24-25) when the fractions were tested with the lettuce hypocotyl assay test (Fig. 19B).

In the case of plumule free gibberellins, activity was detected in fractions (2-3), fraction (5), fraction (8), fractions (12-15) with

Figure 19

Lettuce hypocotyl bioassay of fractions collected from 15 mm inner diameter silicic acid partition column loaded with

A - Free gibberellins

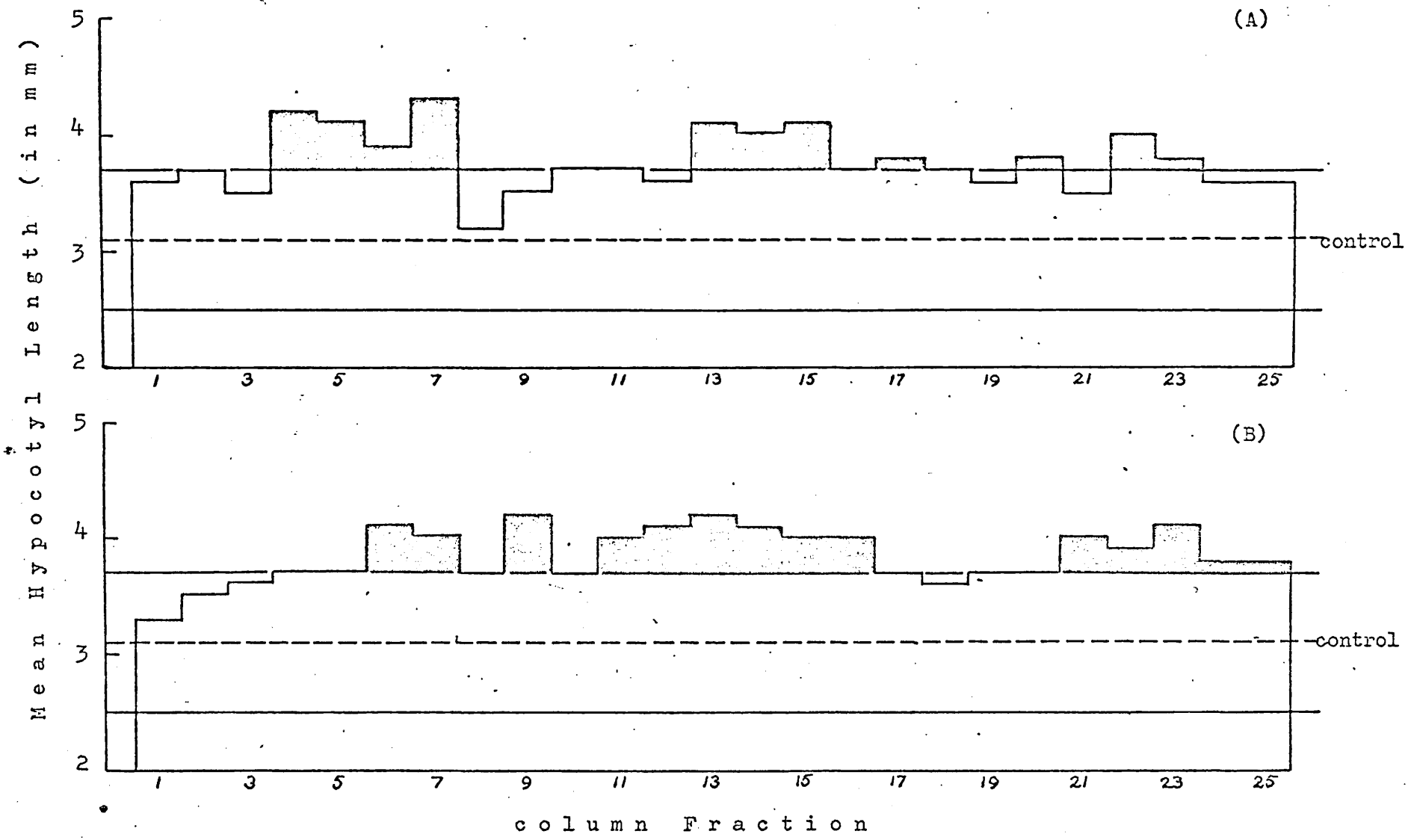
B - Acid hydrolysed bound-gibberellins

from the radicle tissue extracts.

An amount equivalent to 100 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 19


(A)
(B)
control
control
5
4
3
2
1
3
5
7
9
11
13
15
17
19
21
23
25
Mean Hypocotyl Length (in mm)
column Fraction

maximum activity in fraction (12), fractions (18-22) with maximum activity in fraction (19) and fraction (24), together with very small activity in fractions (7), (10-11) and fraction (16) (Fig. 20A). The free gibberellins released by acid hydrolysis of the plumule bound-gibberellins showed activity in fractions (5-6), fraction (9), fraction (12), fractions (16-18), with very small activity in fraction (17) and (19), fractions (21-15) with just significant activity in fraction (23) together with very small activity in fraction (1) and fraction (9), when the silicic acid partition column chromatography fractions were tested for gibberellin-like activity with the lettuce hypocotyl assay test (Fig. 20B).

It should be mentioned here that some of the silicic acid column fractions, especially in the case of columns loaded with free gibberellins, were found to be not very clear and contained some turbidity. This is probably due to the large size of the samples relative to the size of the column, and as the amount of fresh weight to be extracted was to be further increased, a 26 mm inner diameter column was used. The column was packed with 64.0 gm of silicic acid and eluted with increasing concentrations of ethyl acetate in n-hexane as shown in Table 8, and as there was detectable activity in fraction (25) of the previous columns, two more fractions were collected in the coming experiments.

In the following experiment, 300.0 gms fresh weight of tissue from each organ of the etiolated 10-day old broad bean seedling (this amount was obtainable from 200 seedlings in the case of the radicle , 100 seedlings in the case of the plumule, and 150 seedlings in the case of the cotyledons) were extracted separately as described in the previous experiment, the extracts were fractionated into free gibberellins and bound-gibberellins which were hydrolysed as described before, each fraction was purified by means of PVP slurries, and the gibberellins recovered from the slurries

Figure 20

Lettuce hypocotyl bioassay of fractions collected from 15 mm inner diameter silicic acid partition column loaded with

A - Free gibberellins

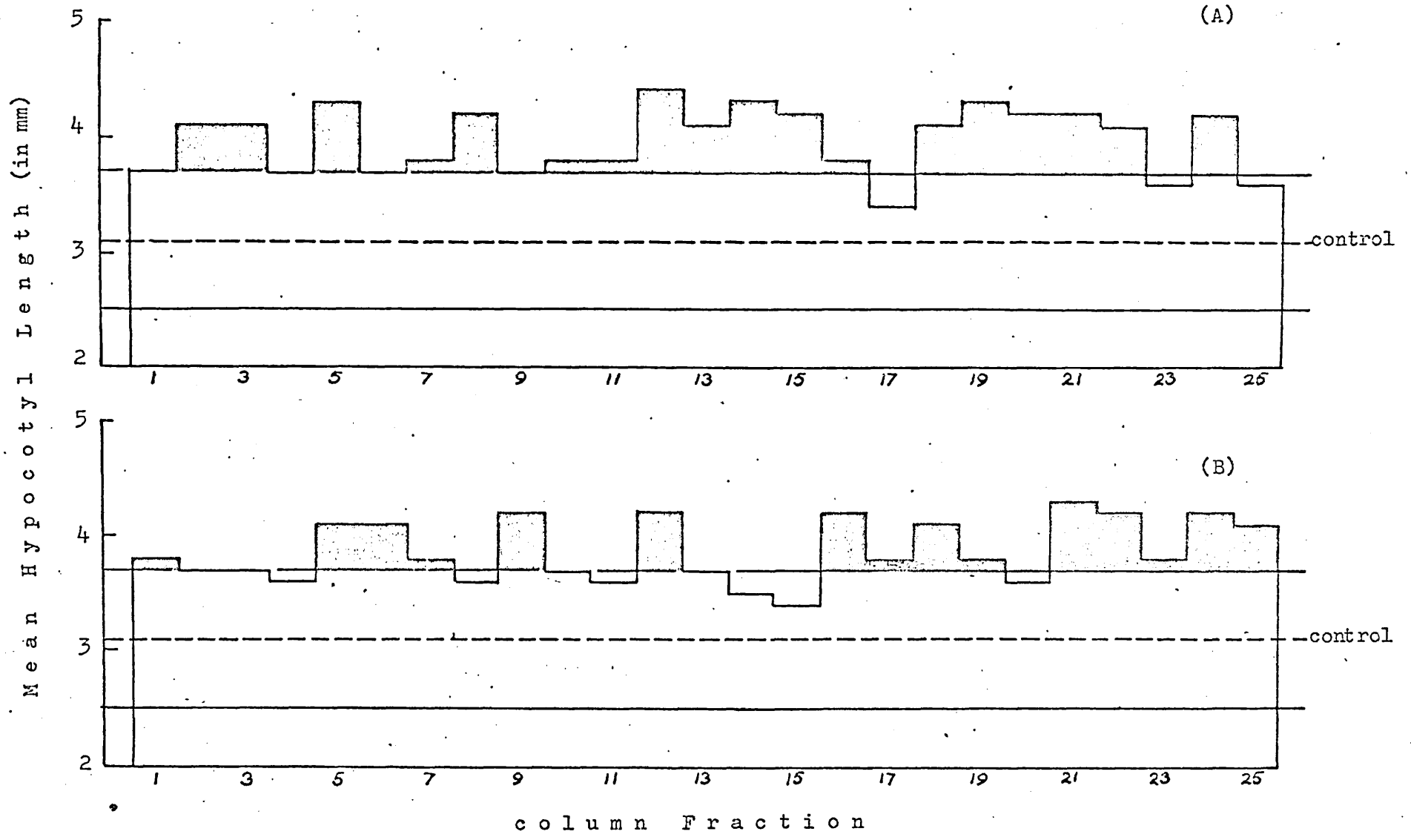
B - Acid hydrolysed bound-gibberellins

from the plumule tissue extracts.

An amount equivalent to 100 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 20


(A)
(B)
5
4
3
2
control
control
1
3
5
7
9
11
13
15
17
19
21
23
25
Mean Hypocotyl Length (in mm)
column Fraction

filtrate were resolved by 26 mm inner diameter silicic acid column chromatography as described in the Materials and Methods Section.

Twenty-seven fractions of 40 ml each were collected as shown in Table 8. The fractions were evaporated separately to dryness using a Büchler portable rotary evapo-mix, the residue of each fraction was dissolved in 1.0 ml of redistilled absolute ethanol of which a volume equivalent to 100.0 gm fresh weight of tissue (0.33 ml), and a volume equivalent to 15.0 gm fresh weight of tissue (0.05 ml) were used for testing the gibberellin-like activity of the column fractions with the lettuce hypocotyl bioassay and the α-amylase bioassay tests respectively. Fractions that showed considerable activity in the bioassay tests were derivatised for GLC analysis.

The results of the bioassay tests for the radicle extracts are represented in Figures 21-24, those for the plumule tissue extracts are represented in Figures 25-28, for the cotyledons tissue extracts, on the other hand, see the Footnote at the end of this Chapter.

The graphs show that, in the case of radicle tissue extracts, the free gibberellin-like substances showed activity in fractions (4), (5), (7), (13-15) and fraction (22) with the lettuce hypocotyl assay test (Fig. 21), while with the α-amylase assay test, activity was detected in fractions (1), (3-8) with considerable activity in fractions (5) and (8), fractions (11-13) with considerable activity in fraction (11), fractions (16-19) with maximum activity in fraction (18) and fractions (22-23) (Fig. 22).

Acid hydrolysis of the radicle "bound" gibberellin-like substances resulted in the release of free gibberellin-like substances which showed activity with the lettuce hypocotyl assay test mainly in fractions (14) and (15) of the silicic acid partition column loaded with these gibberellins (Fig. 23); slight activity was also detected in fractions (5),

Figure 21

Lettuce hypocotyl bioassay fractions collected from 26 mm inner diameter silicic acid partition column loaded with the free gibberellins of the radicle tissue extracts.

An amount equivalent to 100 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

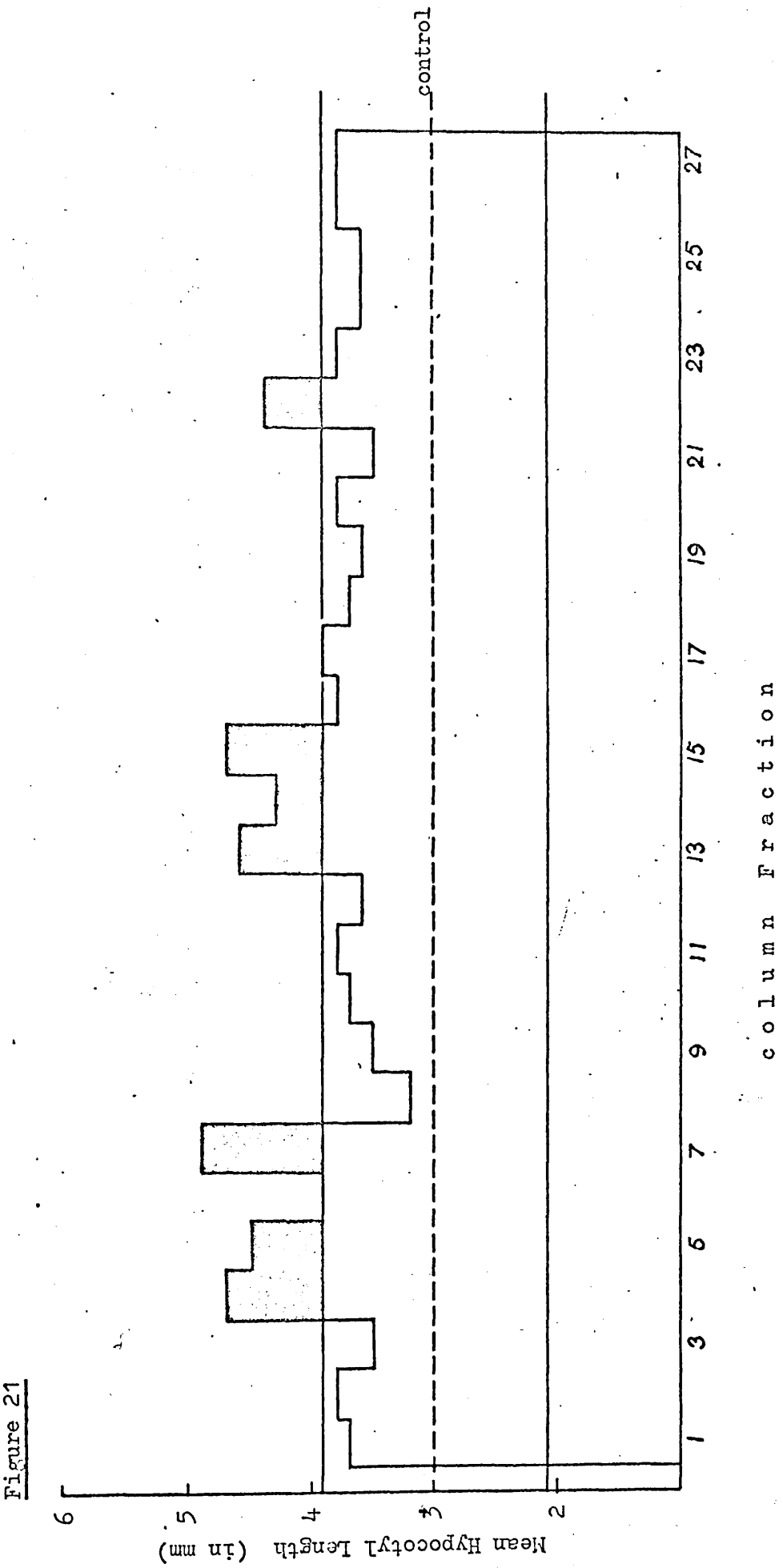

control
Mean Hypocotyl Length (in mm)
6
5
4
3
2
1
3
6
7
9
11
13
15
17
19
21
23
25
27
column Fraction

Figure 21

Figure 22

α-Amylase bioassay of the fractions collected from 26 mm inner diameter silicic acid partition column loaded with the free gibberellins from the radicle tissue extract.

An amount equivalent to 15 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

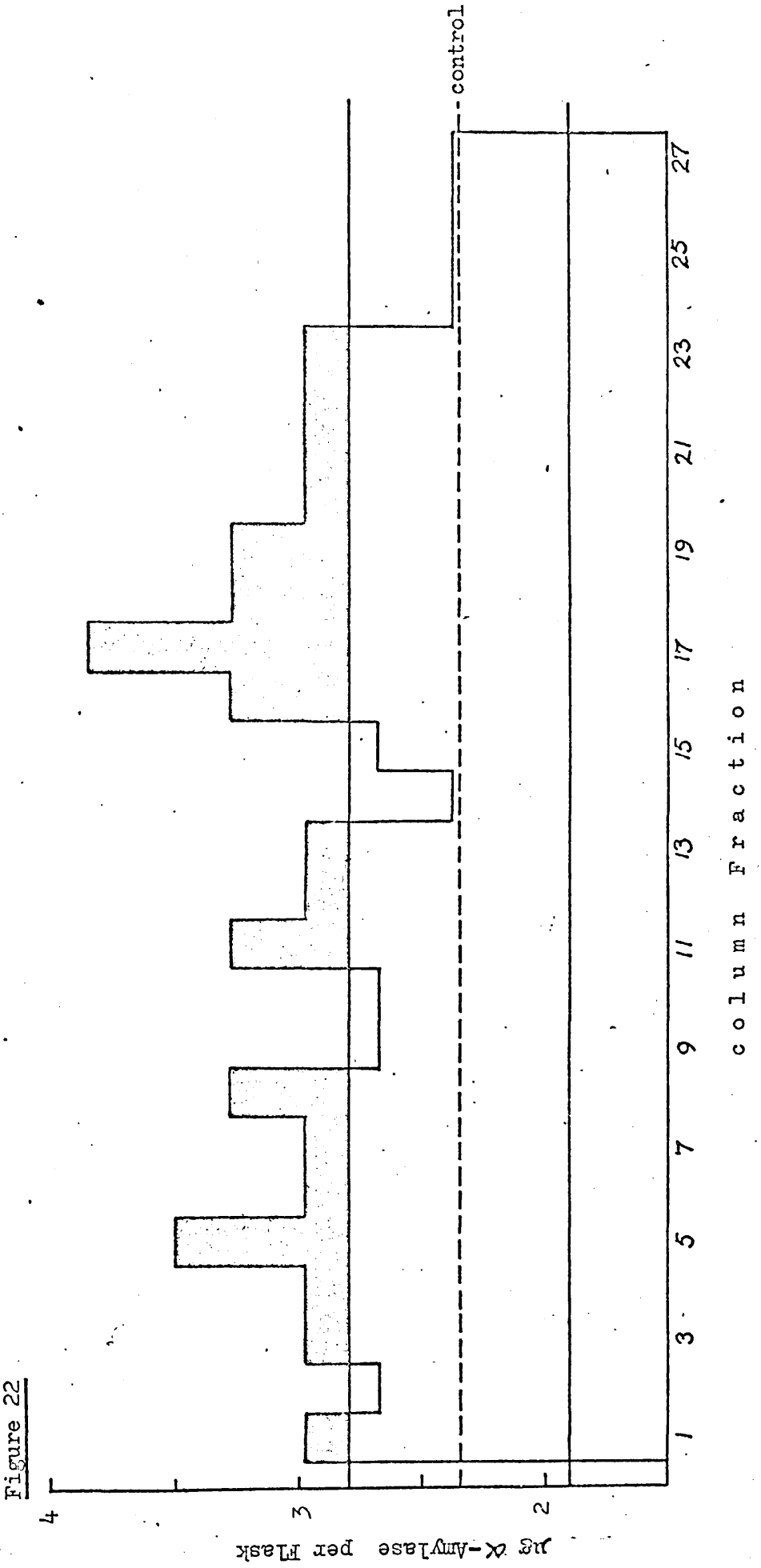

control
μg α-Amylase per Flask
4
3
2
1
3
5
7
9
11
13
15
17
19
21
23
25
27
column Fraction

Figure 22

(11-12) and fraction (17). Just significant activity was detected in fractions (2-4), (6), (8), (13), (16) and fraction (20) of the same sample (Fig. 23). The α-amylase assay test of the same sample showed the presence of active substances in fractions (4), (6-10) with more activity in fractions (6) and (9-10), fractions (12-13) with more activity in fraction (12), fraction (15) and fractions (17-18) (Fig. 24).

The lettuce hypocotyl assay of the fractions collected from a 26 mm inner diameter column packed with silicic acid and loaded with the plumule free gibberellins (see the Materials and Methods Section for all details concerning silicic acid partition column chromatography technique) showed activity in fractions (11), (17-18) and fraction (27) (Fig. 25); while the α-amylase assay test of the same sample showed activity in fractions (4), (8), (11-12), (14) and fraction (17) together with very little activity in fractions (2), (7), (10), (13), (16) and fraction (20) (Fig. 26).

Acid hydrolysis of the plumule bound-gibberellins resulted in the release of free gibberellins which when resolved by silicic acid partition column chromatography and the column fractions were tested biologically with the lettuce hypocotyl bioassay test as described in the Materials and Methods Section, activity was detected in fractions (5), (7-13) with more activity in fractions (7), (8) and (11), fractions (22-26) with more activity in fractions (22) and (25) (Fig. 27). The α-amylase assay test of the same sample showed activity in fractions (2-5), (8), (10), (12), (16) and fraction (26) (Fig. 28).

Ideally, it would be desirable to investigate the behaviour of a wide spectrum of gibberellins on the silicic acid partition column chromatography but the limitation of the available standards (only gibberellic acid at that time) prevented this from being carried out here. In order to find out in what fractions gibberellic acid comes,

Figure 23

Lettuce hypocotyl bioassay fractions collected from 26 mm inner diameter silicic acid partition column loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins of the radicle tissue extracts.

An amount equivalent to 100 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

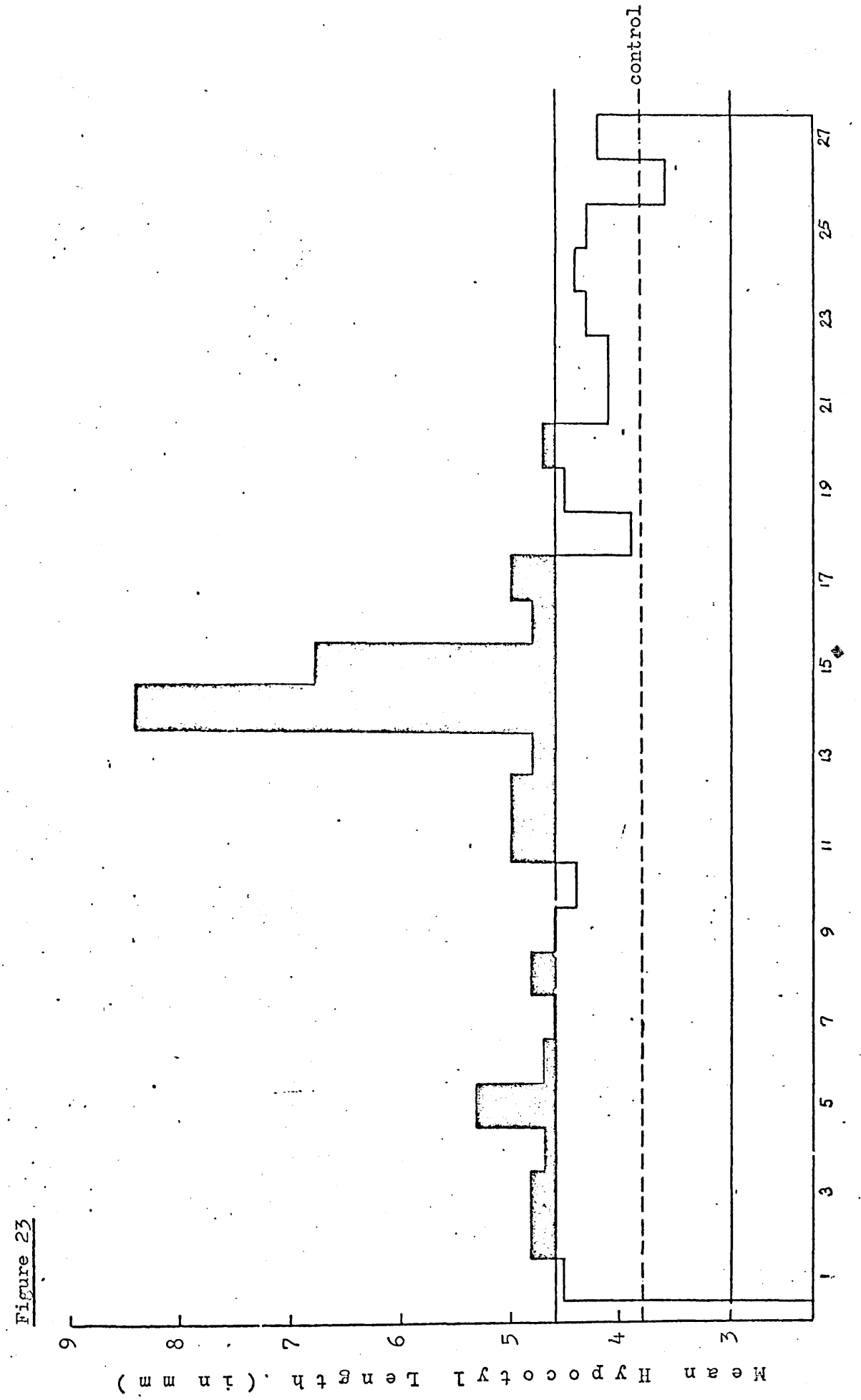

control
Mean Hypocotyl Length (in mm)
9
8
7
6
5
4
3
1
3
5
7
9
11
13
15
17
19
21
23
25
27
Column Fractions

Figure 23

Figure 24

α-Amylase bioassay test of the fractions collected from 26 mm inner diameter silicic acid partition column loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins from the radicle tissue extracts.

An amount equivalent to 15 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 24

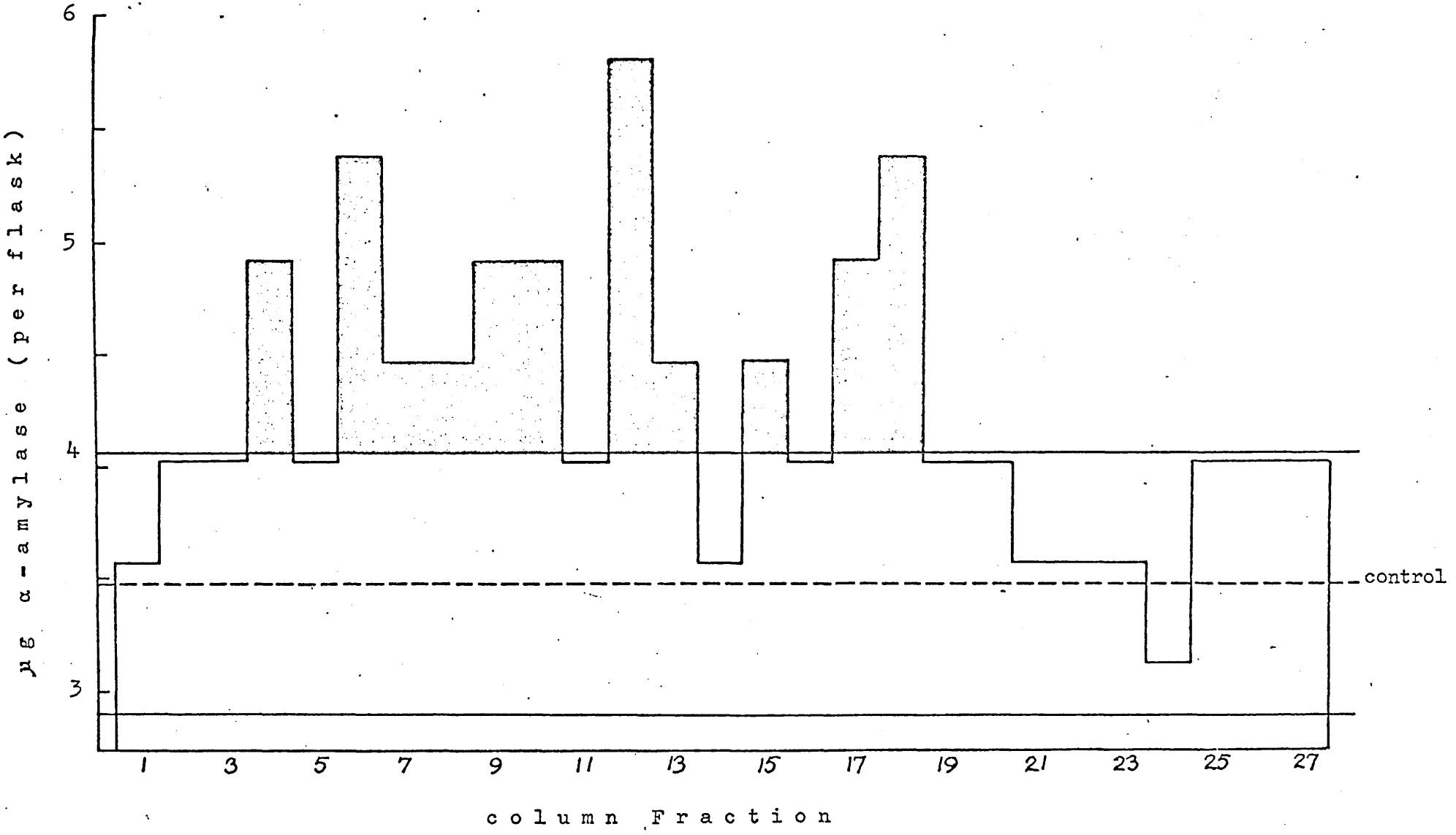

6
5
4
3
μg α-amylase (per flask)
control
1
3
5
7
9
11
13
15
17
19
21
23
25
27
column Fraction

Figure 25

Lettuce hypocotyl bioassay of fractions collected from 26 mm inner diameter silicic acid partition column loaded with the free gibberellins of the plumule tissue extracts.

An amount equivalent to 100 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

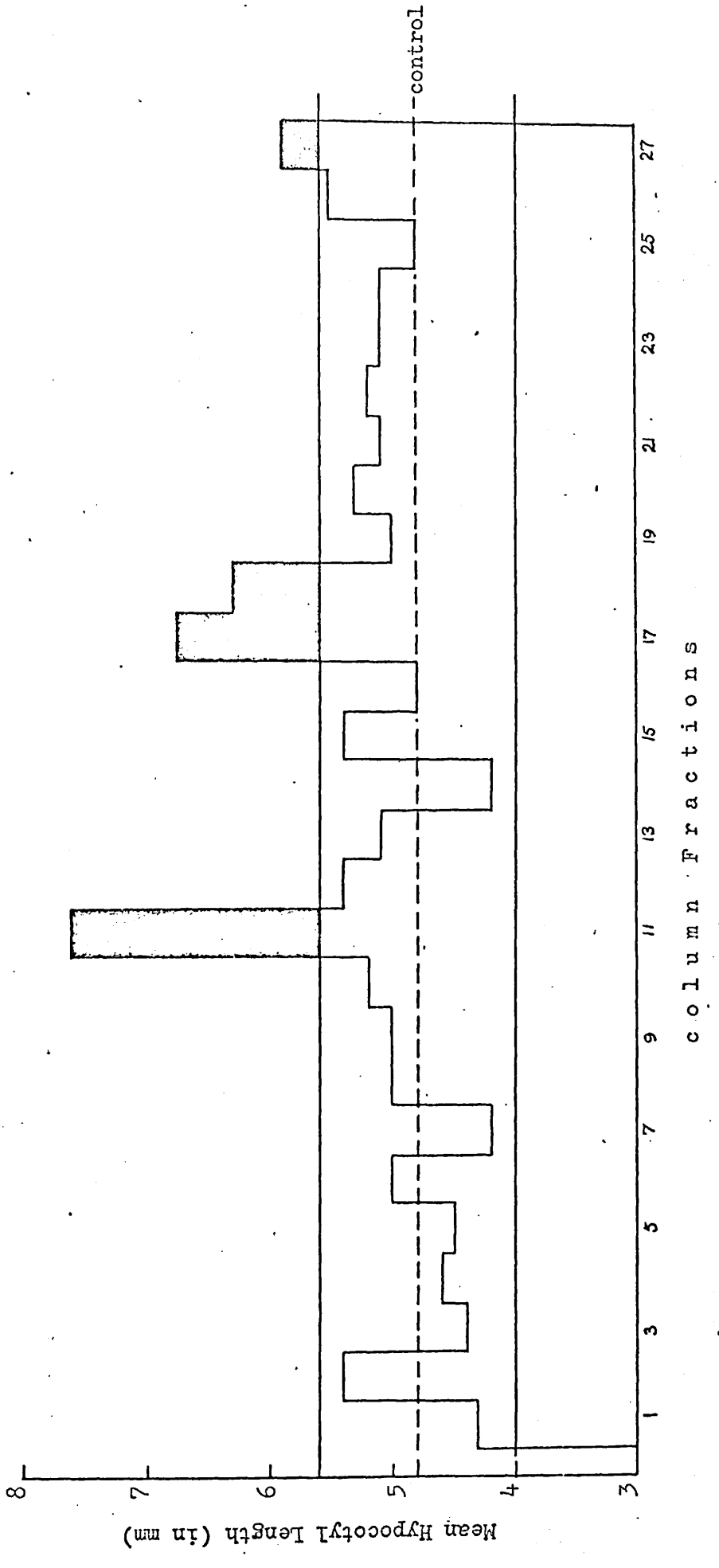

control
8
7
6
5
4
3
1
3
5
7
9
11
13
15
17
19
21
23
25
27
Mean Hypocotyl Length (in mm)
column Fractions

Figure 25

Figure 26

α-Amylase bioassay of the fractions collected from 26 mm inner diameter silicic acid partition column loaded with the free gibberellins from the plumule tissue extracts.

An amount equivalent to 15 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 26

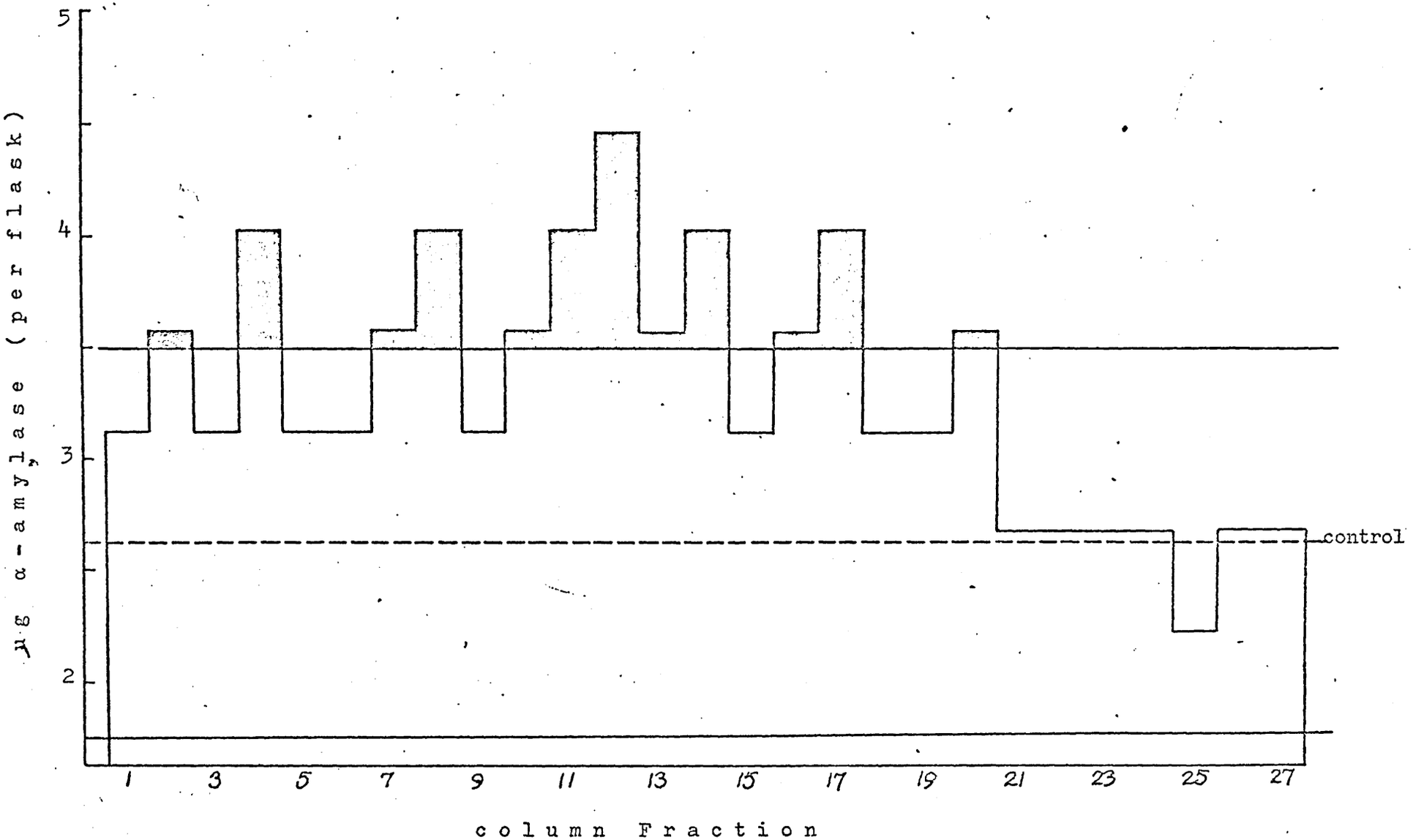

5
4
3
2
µg α-amylase (per flask)
control
1
3
5
7
9
11
13
15
17
19
21
23
25
27
column Fraction

Figure 27

Lettuce hypocotyl bioassay of fractions collected from 26 mm inner diameter silicic acid partition column loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins of the plumule tissue extracts.

An amount equivalent to 100 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 27

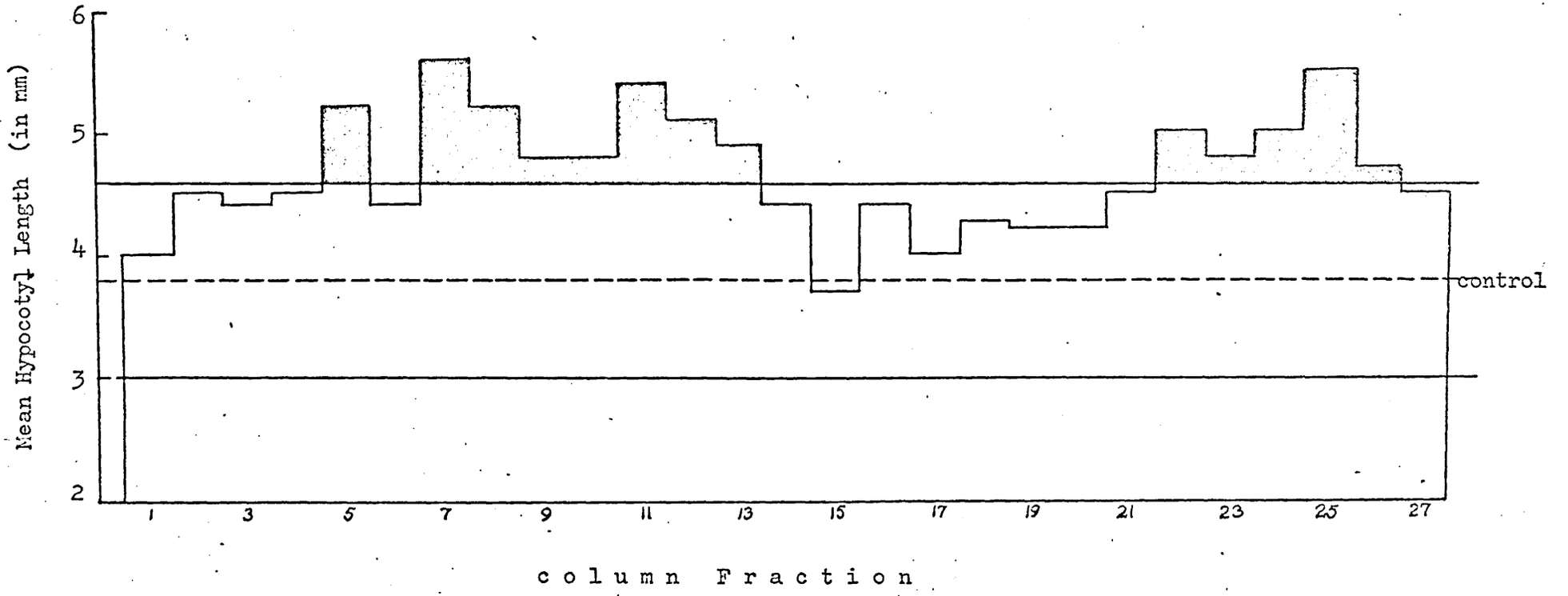

Mean Hypocotyl Length (in mm)
6
5
4
3
2
control
1
3
5
7
9
11
13
15
17
19
21
23
25
27
column Fraction

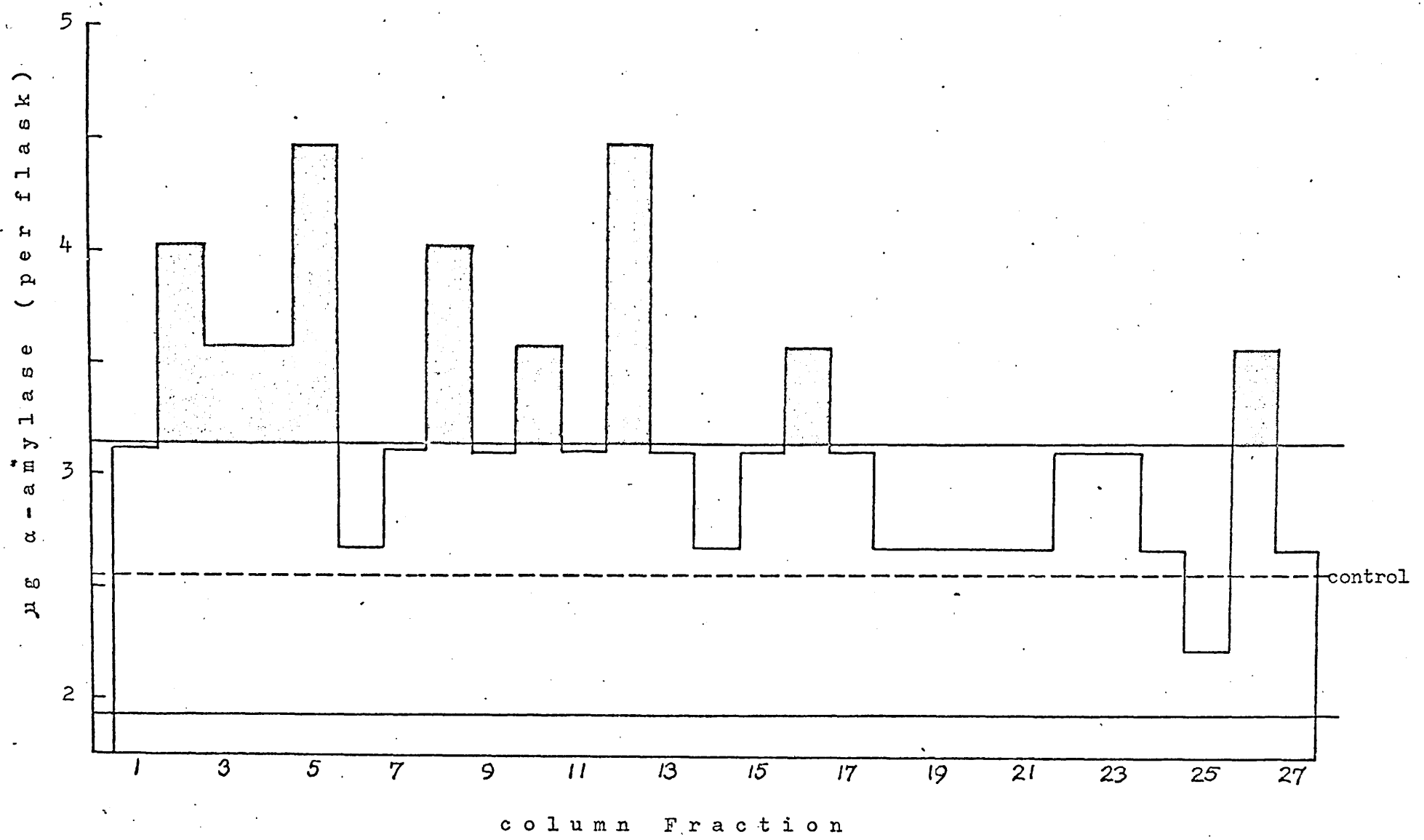
Figure 28

α-Amylase bioassay test of the fractions collected from 26 mm inner diameter silicic acid partition column loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins from the plumule tissue extracts.

An amount equivalent to 15 gm fresh weight of tissue was used in the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 28


5
4
3
2
μg α-amylase (per flask)
control
1
3
5
7
9
11
13
15
17
19
21
23
25
27
column Fraction

100 µg of this standard in ethanolic solution were applied to the top of the 15 mm inner diameter column by the technique described before and twenty-five fractions of 25 ml each were collected as shown in Table 7. The fractions were evaporated to dryness separately as described in the Materials and Methods Section, and each residue was dissolved in 0.5 ml of redistilled absolute ethanol. When the column fractions were tested biologically with the lettuce hypocotyl bioassay test (an amount equivalent to 40 µg GA_3 was used), activity was detected in fractions (22-24).

Although the data obtained earlier had indicated biological activity of the whole broad bean seedling extracts, separation of the seedlings into radicle, plumule, and cotyledons and the purification of the extracts with PVP slurries and silicic acid partition column chromatography revealed an entirely different picture when the column fractions were tested for gibberellin activity with the lettuce hypocotyl bioassay and the α-amylase bioassay tests. By reference to the elution pattern of gibberellins on the 26 mm inner diameter silicic acid partition column published by Crozier *et al.* (1971), it is possible to suggest that fraction (2) could contain GA_9, fraction (4) could contain GA_4 and/or GA_7, fraction (8) could contain GA_6, fraction (11) could contain GA_{13}, fractions (12-14) could contain GA_1 and GA_3, and fraction (17) could contain GA_8.

Attempts to analyse the fractions that showed considerable activity in the bioassay tests used with the GLC were unsuccessful for two main reasons, as reported by Dr. Crozier (personal communication):

1. The fractions were not pure enough and, in his opinion, additional purification steps prior to separation of the gibberellins by silicic acid partition column chromatography was very important.

2. Seedling tissues contain small quantities of gibberellin, certainly several orders of magnitude less than that found in immature seed, which makes identification of seedling gibberellin very difficult because, even after employing elaborate purification procedures, the ratio of gibberellins to extraneous material is at best about one part in 10,000.

For these reasons, it was necessary to increase the size of the samples as much as the provided growing space allows, and to add one or more steps for further purification of the extracts.

Footnote

i. For the cotyledons extract, three experiments were grown and the cotyledons tissue was taken for extraction, but unfortunately accidents happened (drop in the water pressure during the evaporation of the samples in the rotary evaporator), which resulted in the loss of the samples.

ii. The variety Green Sleeves became unobtainable for quite a long time, and the only solution for this problem was the replacement of this variety with variety Green Windsor, which is recommended to be more available at any time.

CHAPTER IV

1. PVP Column Chromatography of Gibberellin-Like Substances in the Different Organs of Etiolated Broad Bean (Vicia faba) Seedlings

As a result of the experiments carried out in the previous Chapter (Chapter III), it appeared necessary for the size of the sample to be increased as much as possible, and for further purification of the extracts to be carried out before silicic acid partition column chromatography, as recommended by Dr. Crozier. In the meantime, the broad bean variety Green Sleeves became unobtainable for a considerable period of time and eventually had to be replaced by variety Green Windsor. This meant that some preliminary experiments had to be done with this new variety to qualify the endogenous gibberellins of the different organs of its etiolated seedling.

Since silicic acid partition column chromatography was a time-consuming technique, due to the length of time necessary for the collection of twenty-seven fractions (6-7 hours), the evaporation and bioassay of these fractions, I looked round for another technique to purify the extracts, I therefore tried the PVP column chromatography, followed by TLC of the gibberellins recovered from the column effluents on 20 x 20 cm thin-layer plates coated with 0.25 mm thick layer of Keiselgel 60 F_{254} (Merck) and developed with solvent system (1). Each plate was divided into 10 equal zones, each of which was eluted with a known volume of wet ethyl acetate as described in the Materials and Methods Section, and the eluates were tested for gibberellin-like activity with the lettuce hypocotyl bioassay and the α-amylase bioassay tests.

For further purification and separation of plant extracts that had been partially purified by PVP slurries, a column chromatographic procedure using PVP was used, the main details of which have already been

described in the Materials and Methods Section. In PVP column chromatography, some workers have collected ten or more fractions of the column effluent (100 ml each), and tested the activity of each fraction for gibberellin-like substances by means of bioassay tests (e.g. Bowen et al., 1973); other workers have discarded the first 50 ml of the column effluent for reasons not specified, and collected the next 200 ml only for separation of gibberellins by TLC and subsequent bioassay of the TLC eluates (e.g. Williams et al., 1974). Because of these differences in the use of the same technique, and in order that none of the gibberellin-like substances of the tissue extracts should be missed, the first 50 ml of the PVP columns effluents were collected separately, and five consecutive fractions of 200 ml each were collected in turn.

Gibberellin-like substances were recovered from each of the five 200 ml fractions and resolved separately by TLC/bioassay as described in the Materials and Methods Section, while gibberellin-like substances recovered from the first 50 ml of the columns effluents were tested biologically without further purification with TLC in order to obtain early information about the gibberellin content of this fraction and to see whether it could be discarded in the future work or not. Ideally, one should investigate the behaviour of a wide range of known gibberellins on the PVP column, but limitation in the amount of the available standards prevented this being done here.

The following experiments were carried out as follows:
Ten-days old etiolated broad bean seedlings (cv Green Windsor) were harvested, selected for uniformity, and treated as described in the Materials and Methods Section. The testas were discarded, and the fresh weight of each organ was determined (from 450 seedlings radicle ca 1000 gms, plumule ca 1500 gms, and cotyledons ca 1100 gms). The

tissues were frozen immediately with dry ice and stored in a deep freezer at -20°C until required for extraction.

Endogenous gibberellin-like substances of the different organs were extracted separately by ice-cold redistilled methanol and the methanolic extracts were processed as described before to give an acidic ethyl acetate-soluble fraction which contains the free gibberellins, and a water-soluble fraction which contains bound-gibberellins and these were hydrolysed with 0.4N HCl on a water bath at 60°C for 60 minutes (Barendse et al., 1968). Free gibberellins and the free gibberellins released by acid hydrolysis of bound-gibberellins were purified separately by means of PVP slurries, PVP column chromatography, and TLC of the column effluents (for full details see the Materials and Methods Section). The thin-layer plates were air dried and divided as mentioned before, and each zone was eluted with 5 ml of wet ethyl acetate overnight at 2°C (Aung & DeHertogh, 1968). A volume of 0.8 ml of the eluate of each thin-layer zone (equivalent to 160 gm, 240 gm, and 176 gm fresh weight of the radicle, plumule, and cotyledons tissue respectively) was used for testing the gibberellin-like activity of the eluates with the lettuce hypocotyl bioassay test, and a volume of 0.20 ml (equivalent to 40 gm, 60 gm, and 44 gm fresh weight of the radicle, plumule, and cotyledons tissue respectively) were used for the detection of gibberellin-like activity of the extracts with the α-amylase bioassay test. Eluates of the TLC zones that showed considerable activity in the bioassay tests were derivatised for GLC analysis.

It should be mentioned here that in the case of radicle tissue extracts, the thin-layer eluates, of the first 200 ml fraction from the PVP column, corresponding to R_f 0.0-0.5 were found to contain other substance or substances which inhibited the growth of the lettuce radicle, and perhaps might have affected the growth of the lettuce hypocotyl.

For this reason, the eluates of these zones were mixed together (2.5 ml from each zone equivalent to 500 gm fresh weight of tissue), evaporated to dryness under vacuum at 35°C, the residue obtained was dissolved in the least possible amount of redistilled absolute ethanol and streaked quantitatively on the starting line of 20 x 20 cm thin-layer plates. The plates were developed as usual with solvent system (2), dried, divided and the gibberellins eluted as mentioned before.

The TLC eluates were stored in 1.5 x 5 cm glass specimen tubes with screw tops lined with aluminium foil, and the tubes were kept in a deep freezer at -20°C until required for bioassay tests and GLC analysis.

The results of the bioassay are represented in histogram form in Figures 29-33 for the radicle tissue extracts, Figures 34-37 for the plumule tissue extracts, and Figures 38-39 for the cotyledon tissue extracts.

As a general observation from the Figures, no activity was detected in the first 50 ml of the PVP column effluent in the case of radicle free gibberellins (Figs. 29A, 31A), while it showed significant activity in the case of the free gibberellins released by acid hydrolysis of bound-gibberellins (Figs. 30A, 32A). In the case of the plumule extracts the first 50 ml of the PVP column effluents did not show any activity as free or acid hydrolysed "bound" gibberellin-like substances (Figs. 34A, 35A, 36A, and 37A), while in the case of the cotyledons extracts the first 50 ml of the PVP column effluents showed activity with the bioassay tests used in the case of free and acid hydrolysed "bound" gibberellin-like substances (Figs. 38A, 39A). No significant activity corresponding to gibberellin-like substance or substances was detected in the fourth and the fifth 200 ml fractions of the PVP column effluents in all the extracts (part D, E of Figs. 29-32, and 34-39); in some cases, inhibitory substance or substances were detected in either or both these fractions (Figs. 31D,E; 34D,E; and 39E).

1.1. Gibberellin-Like Substances in the Radicle's Tissue Extracts

Gibberellins recovered from the effluents of a PVP column loaded with the acidic ethyl acetate-soluble fraction from the radicle's tissue extract were resolved by TLC; the TLC eluates were tested biologically with the lettuce hypocotyl bioassay, the results of which are represented in histogram form in Figure 29 (A-E); and with the α-amylase bioassay test which is represented in Figure 30 (A-E), (see the Materials and Methods Section for full details of techniques).

With the lettuce hypocotyl assay, the first 50 ml of the PVP column effluent showed no activity; gibberellin-like substances of the first 200 ml column fraction were resolved by TLC into at least two peaks of activity (Fig. 29A); and those of the second 200 ml column fraction were resolved into two separate peaks of activity, one at R_f 0.4-0.5, and the second at R_f 0.6-0.8 with maximum activity at R_f 0.7-0.8 (Fig. 29B). The next consecutive three column fractions of 200 ml each (i.e. the third, the fourth, and the fifth fractions) did not show any sort of peaks either positive or negative ones (Fig. 29C-E) when their TLC eluates were tested with the lettuce hypocotyl bioassay.

With the α-amylase bioassay test on the other hand, no activity was detected in the first 50 ml of the PVP column effluent; in the first 200 ml fraction, two separate peaks were detected, one at R_f 0.2-0.3 and the second at R_f 0.7-0.9 with maximum activity at R_f 0.7-0.8 (Fig. 30A). No activity was detected in any of the next four consecutive fractions (200 ml each) when their TLC eluates were tested with the α-amylase bioassay test (Fig. 30B-E).

Acid hydrolysis of the water-soluble gibberellins in the radicle tissue extracts released free gibberellins which were purified in exactly the same way as described for the acidic ethyl acetate-soluble gibberellins, resolved by TLC and the TLC eluates were tested biologically

with the lettuce hypocotyl bioassay (Fig. 31A-E), and the α-amylase bioassay tests (Fig. 32A-E).

With the lettuce hypocotyl bioassay test, activity was detected in the first 50 ml of the PVP column effluent (Fig. 31A); in the TLC eluates of the first 200 ml column fraction at R_f 0.4-0.6 (Fig. 31A); and in the TLC eluates of the second 200 ml column fraction at R_f 0.2--0.3 and R_f 0.7-0.8 (Fig. 31B). The fourth and fifth column fractions (200 ml each) showed negative peaks at R_f 0.4-0.5 and R_f 0.9-1.0 in fraction four (Fig. 31D), and at R_f 0.0-0.1 and R_f 0.4-0.8 in fraction five (Fig. 31E); while the third 200 ml fraction did not show any positive or negative peaks when its TLC eluates were tested with the lettuce hypocotyl bioassay (Fig. 31C).

With the α-amylase bioassay test on the other hand, activity was detected in the first 50 ml of the PVP column effluents (Fig. 32A); in the first 200 ml fraction activity was detected at R_f 0.0-0.3, 0.4-0.7, and 0.8-1.0 (Fig. 32A); and in the second 200 ml column fraction activity was detected in at least three peaks as follows (Fig. 32B): one peak at R_f 0.0-0.2 with maximum activity at R_f 0.1-0.2, a second peak at R_f 0.3-0.6 with maximum activity at R_f 0.4-0.6, and a third peak at R_f 0.8-1.0 with maximum activity at R_f 0.8-0.9. No activity was detected in the next three consecutive fractions of the PVP column effluents (i.e. fraction number three, fraction number four, and fraction number five) when their TLC eluates were tested with the α-amylase bioassay test (Fig. 32C-E).

Figure 29

Lettuce hypocotyl bioassay of eluates from TLC loaded with the free gibberellins in extracts from the radicle tissue of etiolated broad bean seedling (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of:

Ethyl acetate-chloroform-acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 160 gm fresh weight of tissue was used in the assay.

A - The first 50 ml and the first 200 ml fraction of the column effluents.

B - The second 200 ml fraction of the column effluents

C - The third " " " " " " "

D - The fourth " " " " " " "

E - The fifth " " " " " " "

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 29

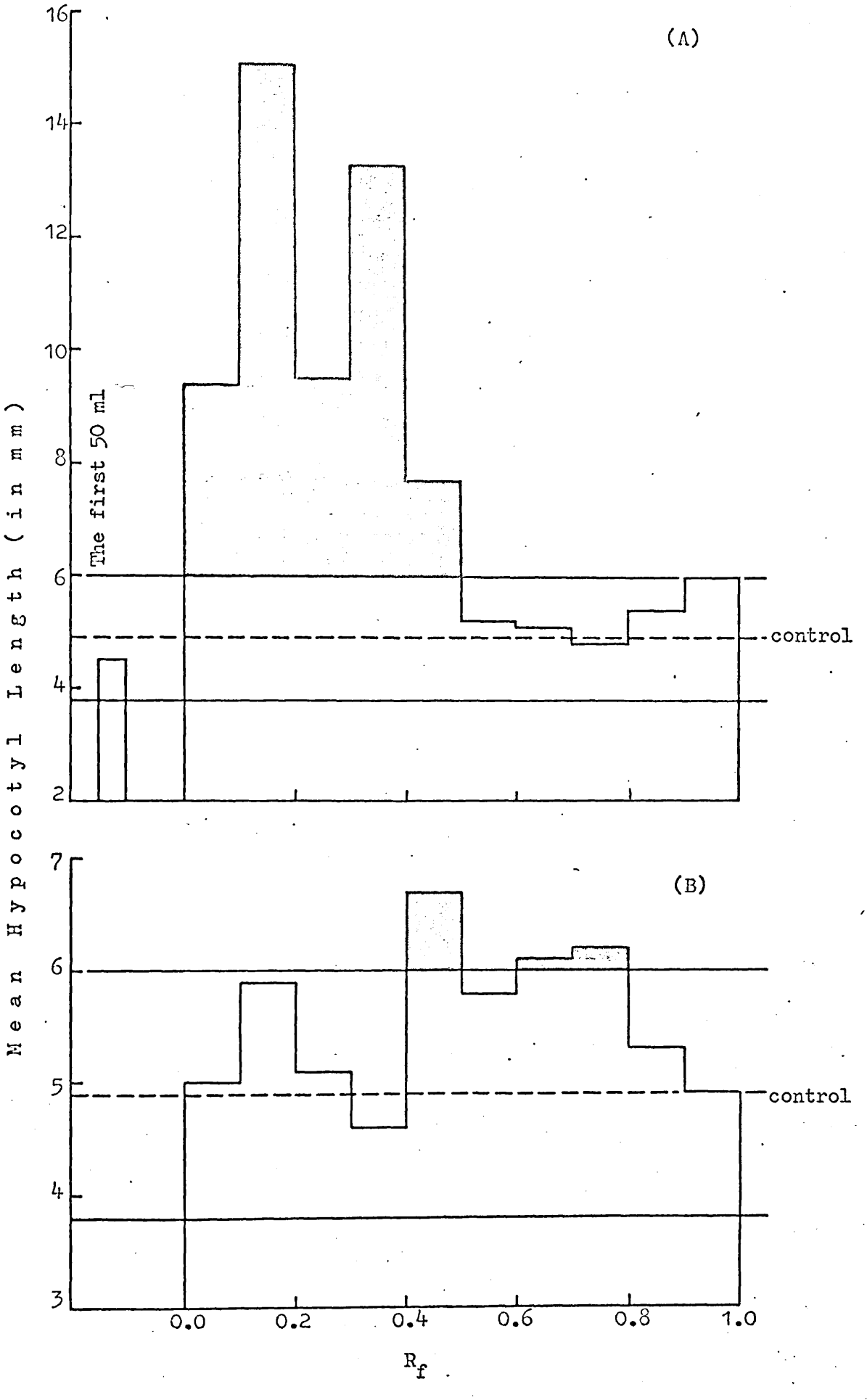

(A)
(B)
The first 50 ml
control
control
Mean Hypocotyl Length (in mm)
R_f
0.0
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
12
14
16
3
5
7

Figure 29 (continued)

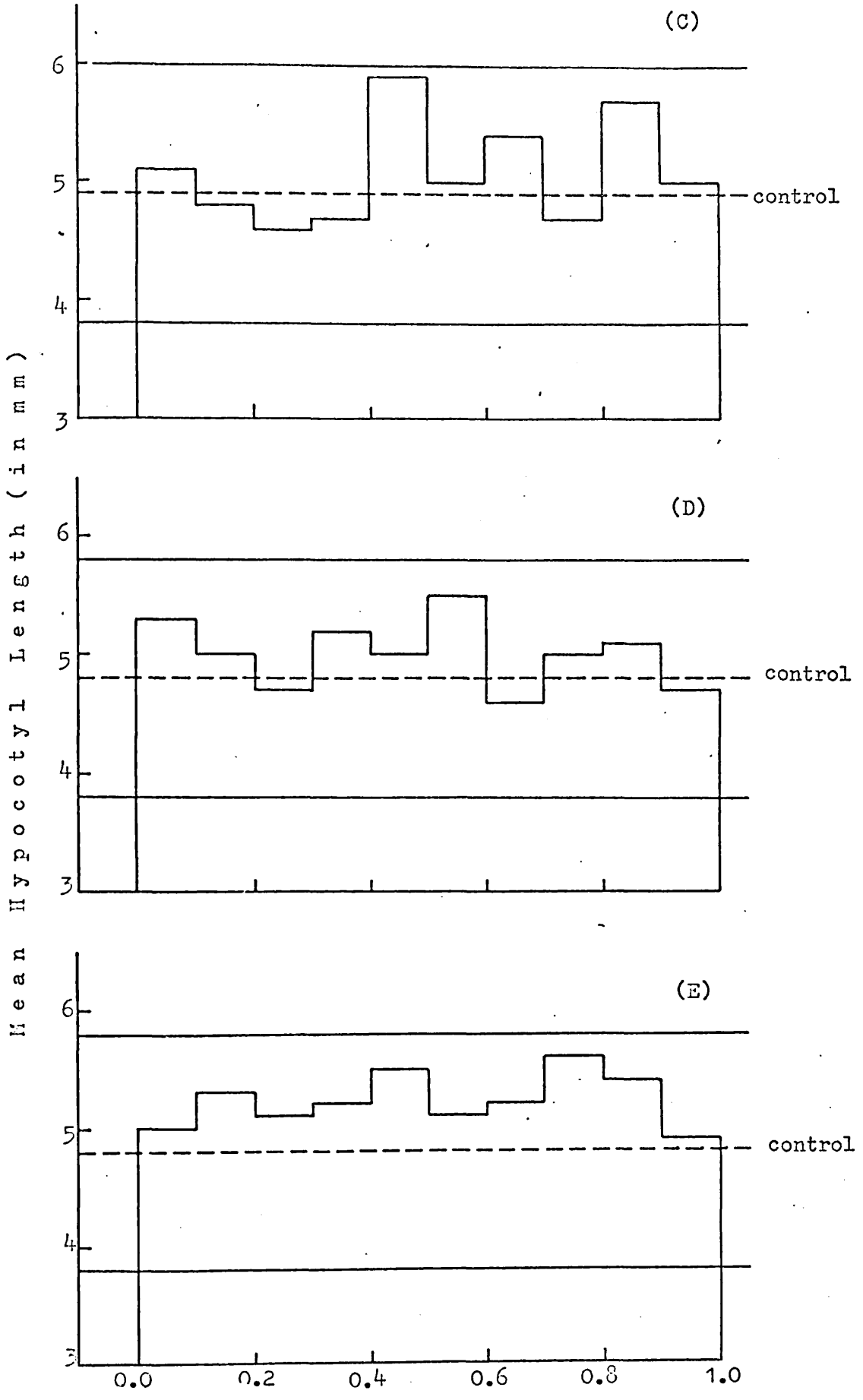


Figure 30

α-Amylase bioassay of eluates from TLC loaded with the free gibberellins of extracts from the radicle tissue of etiolated broad bean seedling (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 4.0 gm fresh weight of tissue was used in the assay.
The letters (A-E) represent the same fractions as in Figure 29.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 30

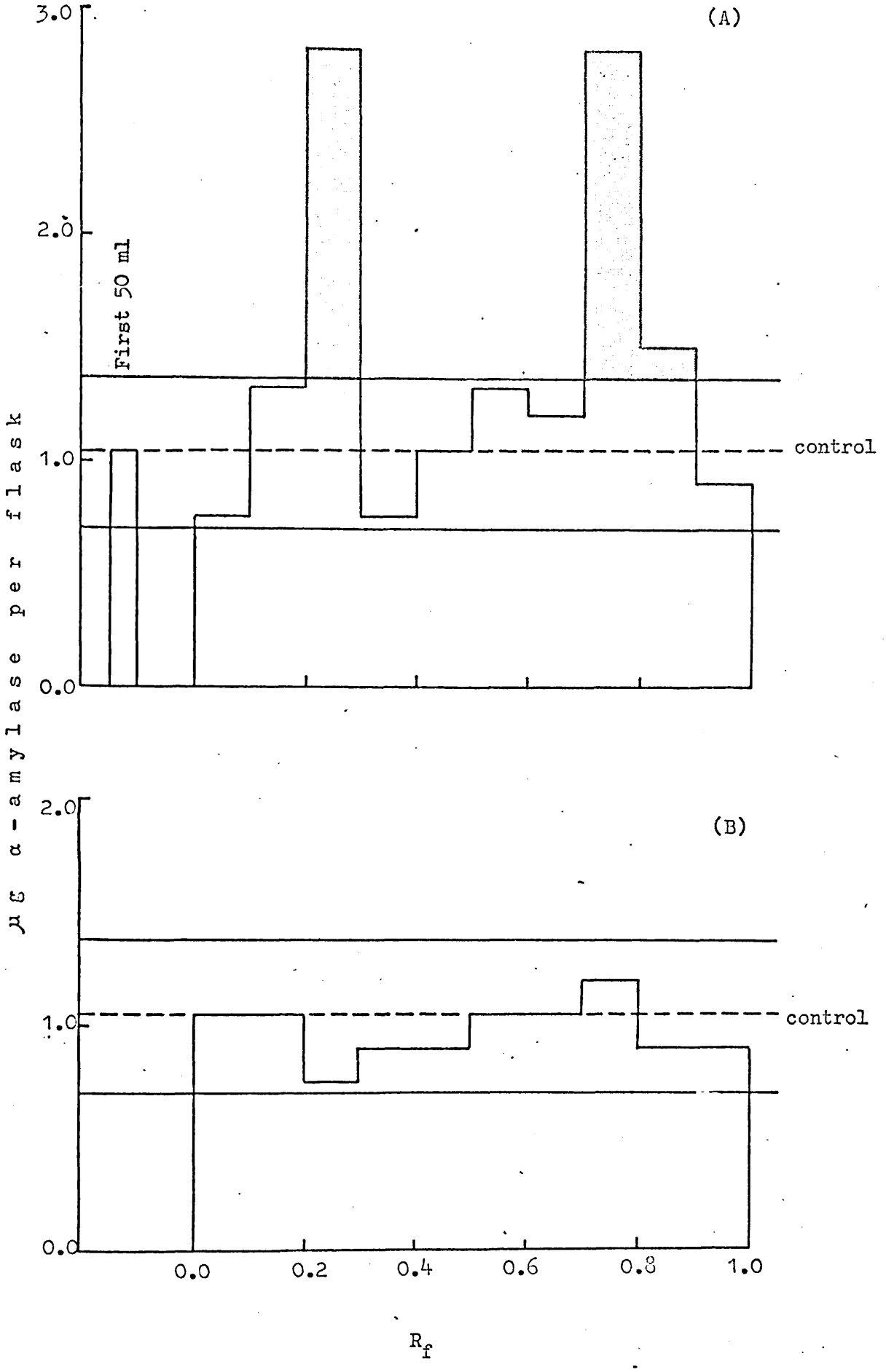

(A)
3.0
2.0
1.0
0.0
First 50 ml
control
μg α-amylase per flask
(B)
2.0
1.0
0.0
control
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 30 (continued)

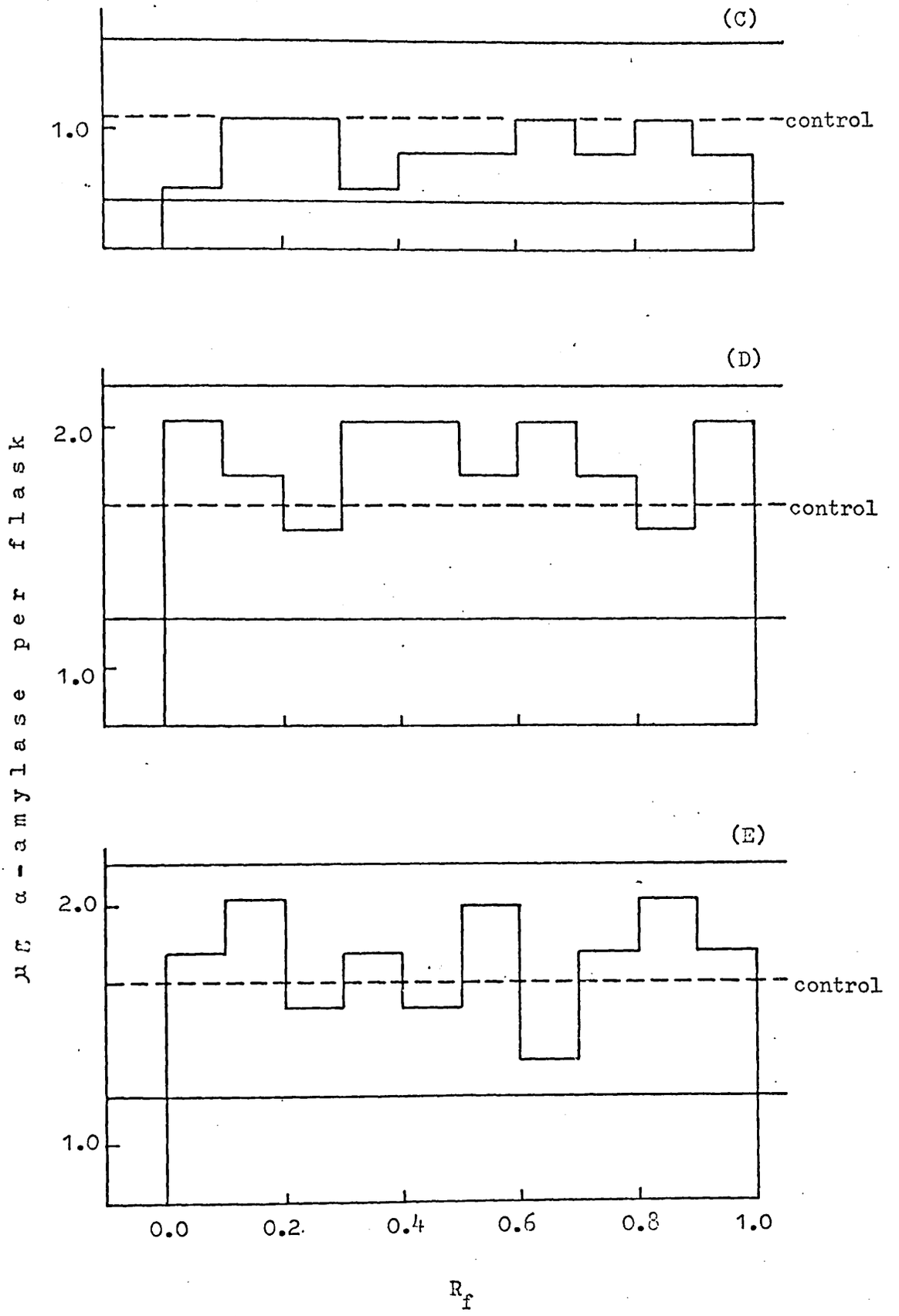


Figure 31

Lettuce hypocotyl bioassay of eluates from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins in extracts of the radicle tissue of etiolated broad bean seedlings (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of

Ethyl acetate-chloroform-acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 160 gm fresh weight of tissue was used in the assay. The letters (A-E) represent the same fractions as in Figure 29.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 31

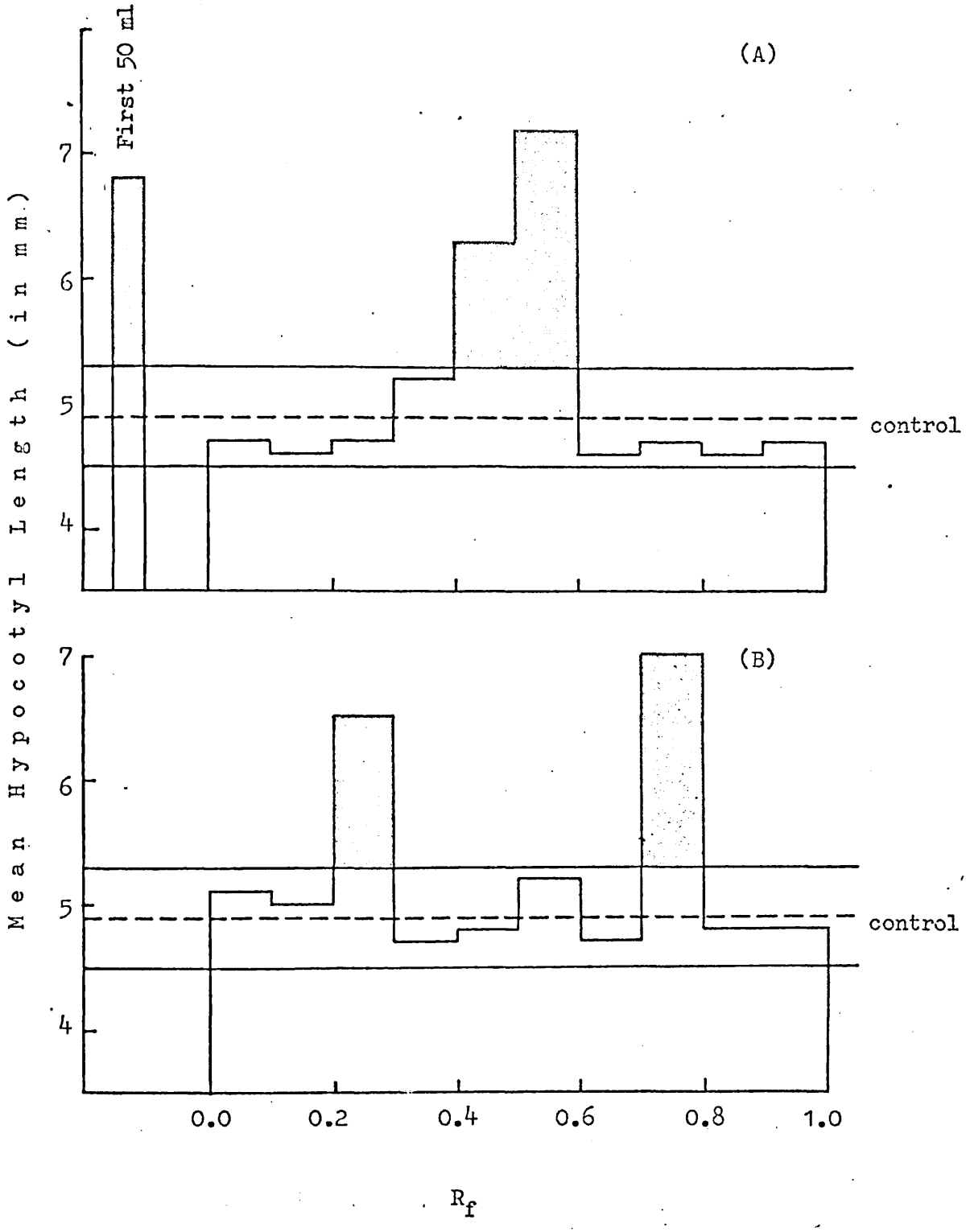

First 50 ml
(A)
(B)
control
control
Mean Hypocotyl Length (in mm)
7
6
5
4
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 31 (continued)

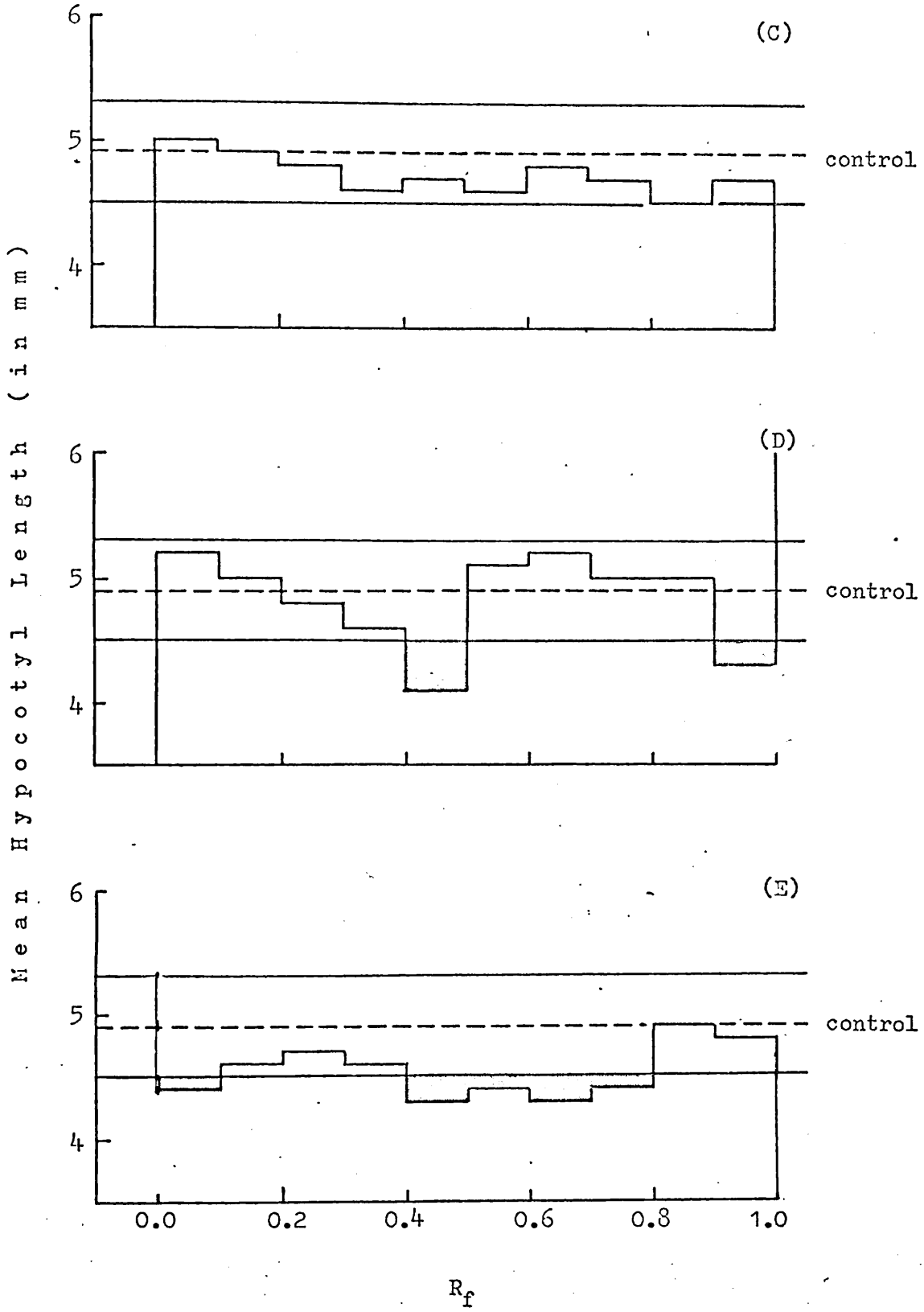


Figure 32

α-Amylase bioassay of eluates from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins from extracts of the radicle tissue of etiolated broad bean seedling (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 4.0 gm fresh weight of tissue was used in the assay.
The letters (A-E) represent the same fractions as in Figure 29.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 32

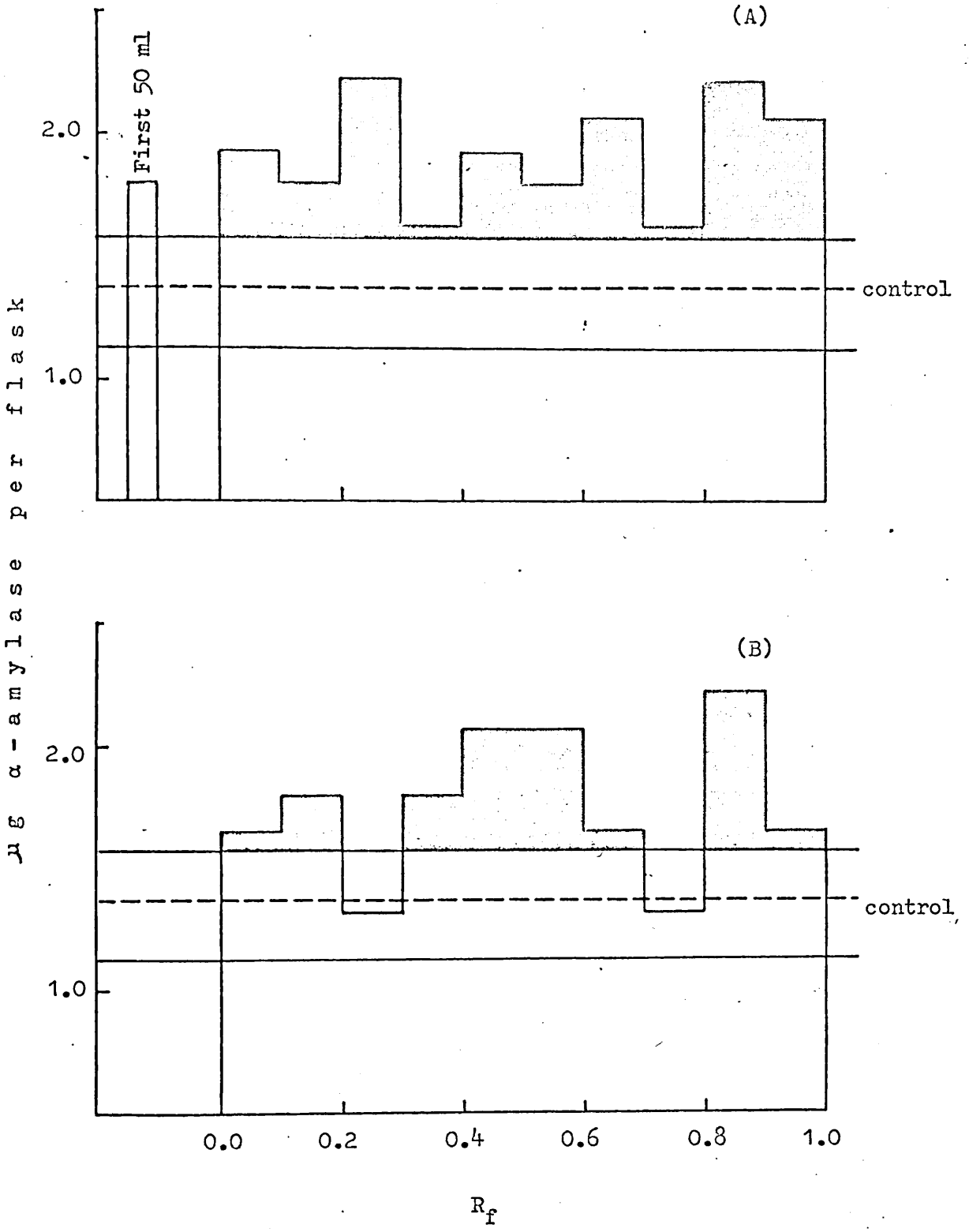
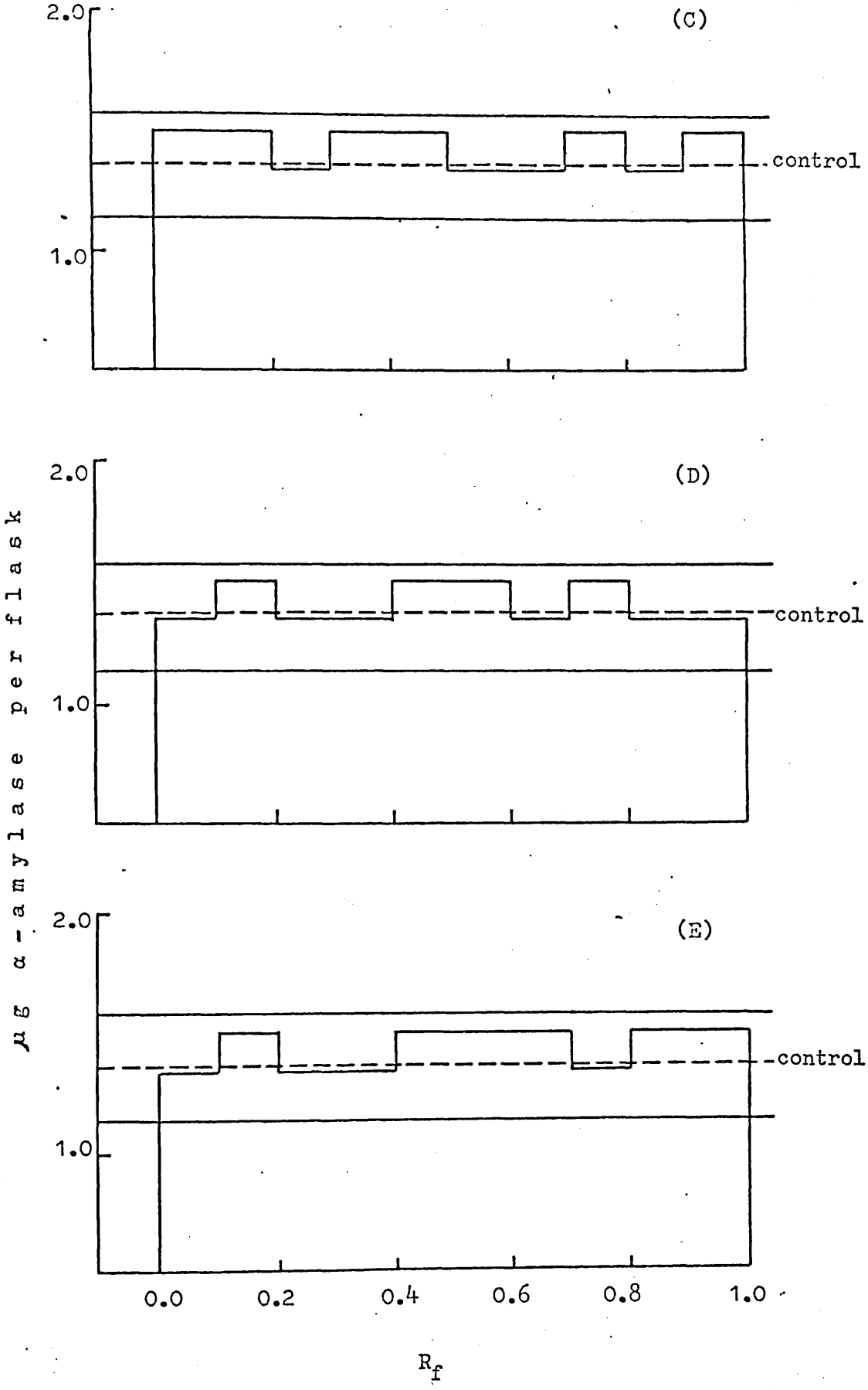

(A)
First 50 ml
2.0
1.0
control
(B)
2.0
1.0
control
μg α-amylase per flask
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 32 (continued)



Re-Chromatography of Free Gibberellins in Extracts from the Radicle Tissue

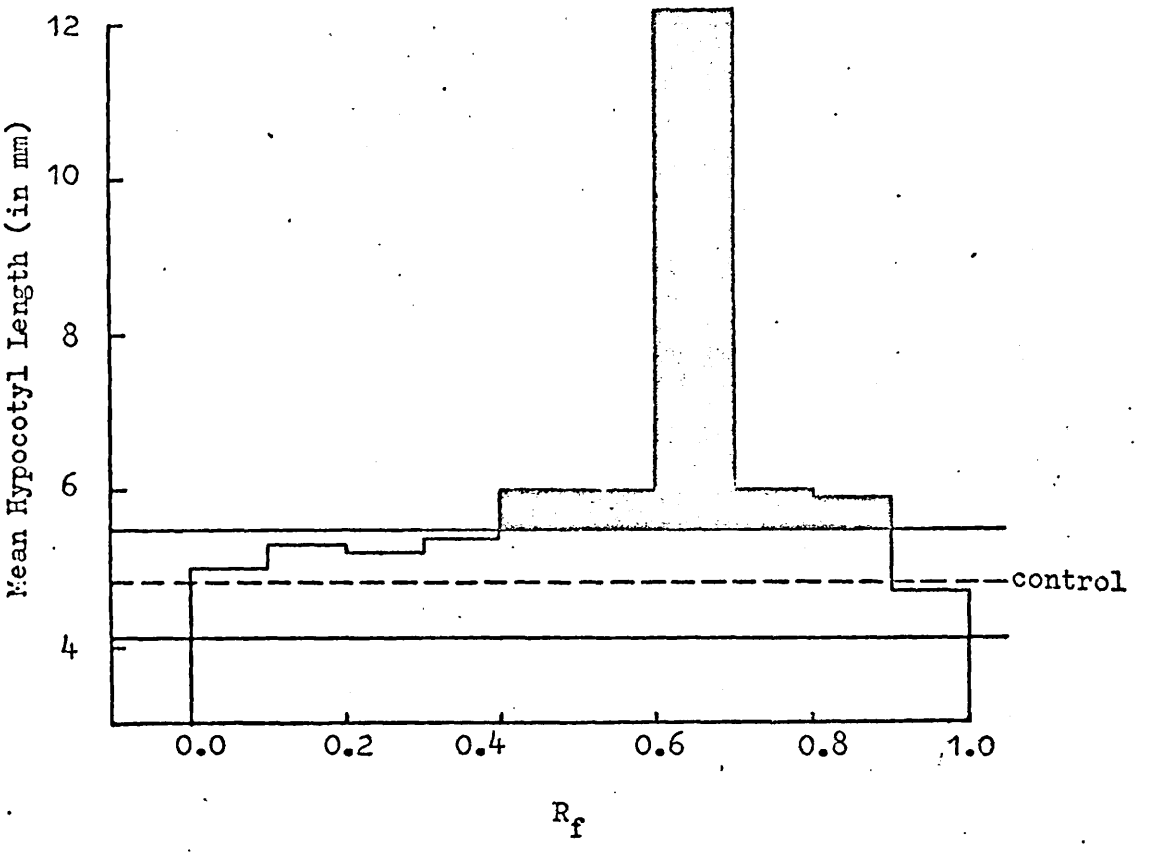
Due to the presence of substance or substances in the TLC eluates of the first 200 ml fraction of the PVP column effluents at R_f 0.0-0.5 which inhibited the growth of the lettuce radicle, the eluates of these zones were re-chromatographed as described earlier in this Chapter. The eluates of the TLC were tested biologically with the lettuce hypocotyl bioassay as described in the Materials and Methods Section, and the results are represented in histogram form in Figure 33.

The Figure shows activity at R_f 0.4-0.9 with maximum activity at R_f 0.6-0.7. Significant amount of inhibitory substances were no longer present to affect the growth of the lettuce radicle, and there was a decrease in the amount of gibberellin-like substances detected (in comparison with those detected in Fig. 29A) because elution of the TLC plate resulted in recovery of only about 30% of the applied gibberellin (see the Materials and Methods Section).

Chromatography in solvent system (2) did not reveal any separate inhibitory substances as it was expected (as no inhibition of the lettuce radicle was noticed in any of the TLC zones eluates), and it is not apparent in the assay, probably due to loss during elution of the TLC or to dilution below the concentration where it is active in the bioassay. So, the re-chromatography technique was not subsequently used, and more dilutions of the extracts were carried out in the following experiments.

For the α-amylase bioassay test of this sample, see the Footnote at the end of this Chapter.

Figure 33



Lettuce hypocotyl bioassay of eluates from TLC loaded with free gibberellins of radicle extracts after their separation on TLC with solvent system (1). A volume equivalent to 500 gm fresh weight of tissue from the zones at R_f 0.0-0.5 was rechromatographed on TLC and developed with solvent system (2) which consisted of:

Benzene - n-butanol - acetic acid

(70:25:5 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 100 gm fresh weight of tissue was used for the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

1.2. Gibberellin-Like Substances in the Plumule Tissue Extracts

Using PVP slurries, PVP column chromatography followed by TLC of the gibberellins recovered from the column effluents, free gibberellins of the plumule tissue extracts were resolved into separate zones showing activity when the TLC eluates were tested biologically with the lettuce hypocotyl bioassay and the α-amylase bioassay tests, the results of which are represented in histogram form in Figures 34 (A-E), and 35 (A-E) for the lettuce bioassay and the α-amylase bioassay test respectively.

With the lettuce hypocotyl bioassay, no activity was detected in the first 50 ml of the column effluent (Fig. 34A); gibberellins of the first 200 ml column fraction were resolved by TLC into a major peak at R_f 0.4-0.6 with maximum activity at R_f 0.4-0.5 (Fig. 34A); those of the second 200 ml column fraction were separated into two small peaks of activity, one at R_f 0.3-0.4, and the second at R_f 0.5-0.6 (Fig. 34B); and the gibberellins of the third 200 ml column fraction showed activity in one peak only at R_f 0.0-0.1 (Fig. 34C). The next two consecutive column fractions of 200 ml each (i.e. the fourth and the fifth fraction) contained inhibitory substance or substances at R_f 0.1-0.3, 0.8-1.0 in the case of the fourth fraction (Fig. 34D), and at R_f 0.8-0.9 in the case of the fifth fraction (Fig. 34E) when the eluates of their TLC were tested biologically with the lettuce hypocotyl bioassay test.

With the α-amylase bioassay test on the other hand, the TLC eluates of gibberellins recovered from the PVP column effluents showed activities which are nearly identical with those detected with the lettuce hypocotyl bioassay test (Figs. 35A, 35B, 35C, 35D, and 35E).

The free gibberellins released by acid hydrolysis of the plumule's bound-gibberellins were purified as described before, separated by TLC, the the TLC eluates were tested biologically with the lettuce hypocotyl bioassay test, Figure 36 (A-E) and the α-amylase bioassay test, Figure 37 (A-E).

Figure 34

Lettuce hypocotyl bioassay of eluates from TLC loaded with the free gibberellins in the plumule tissue extracts of etiolated broad bean seedling (after PVP column chromatography). The plates were developed in solvent system (1) which consisted of

Ethyl acetate-chloroform-acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 240 gm fresh weight of tissue was used in the assay. The letters (A-E) represent the same fractions as in Figure 29.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 34

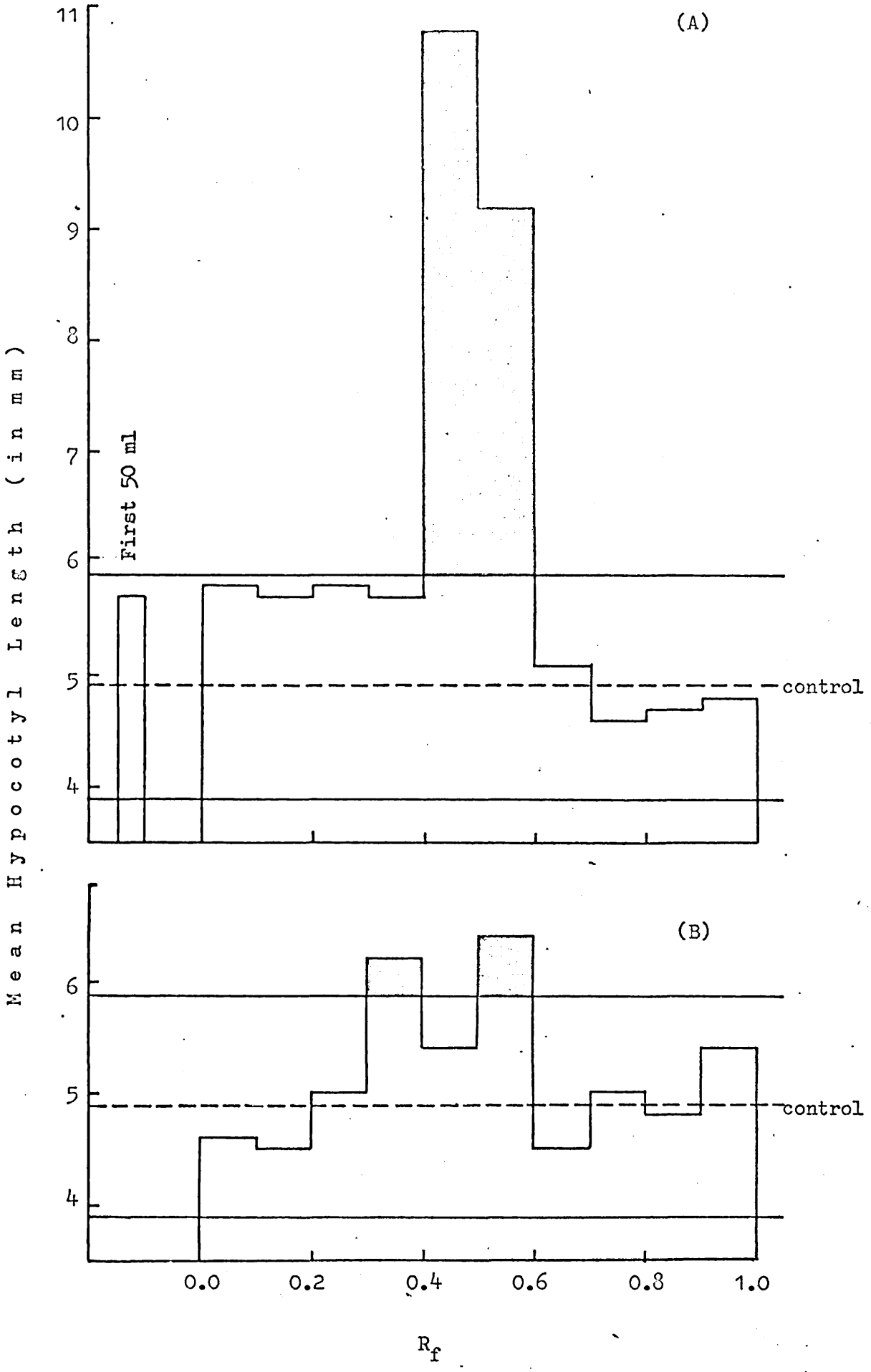

(A)
(B)
First 50 ml
control
control
Mean Hypocotyl Length (in mm)
11
10
9
8
7
6
5
4
6
5
4
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 34 (continued)

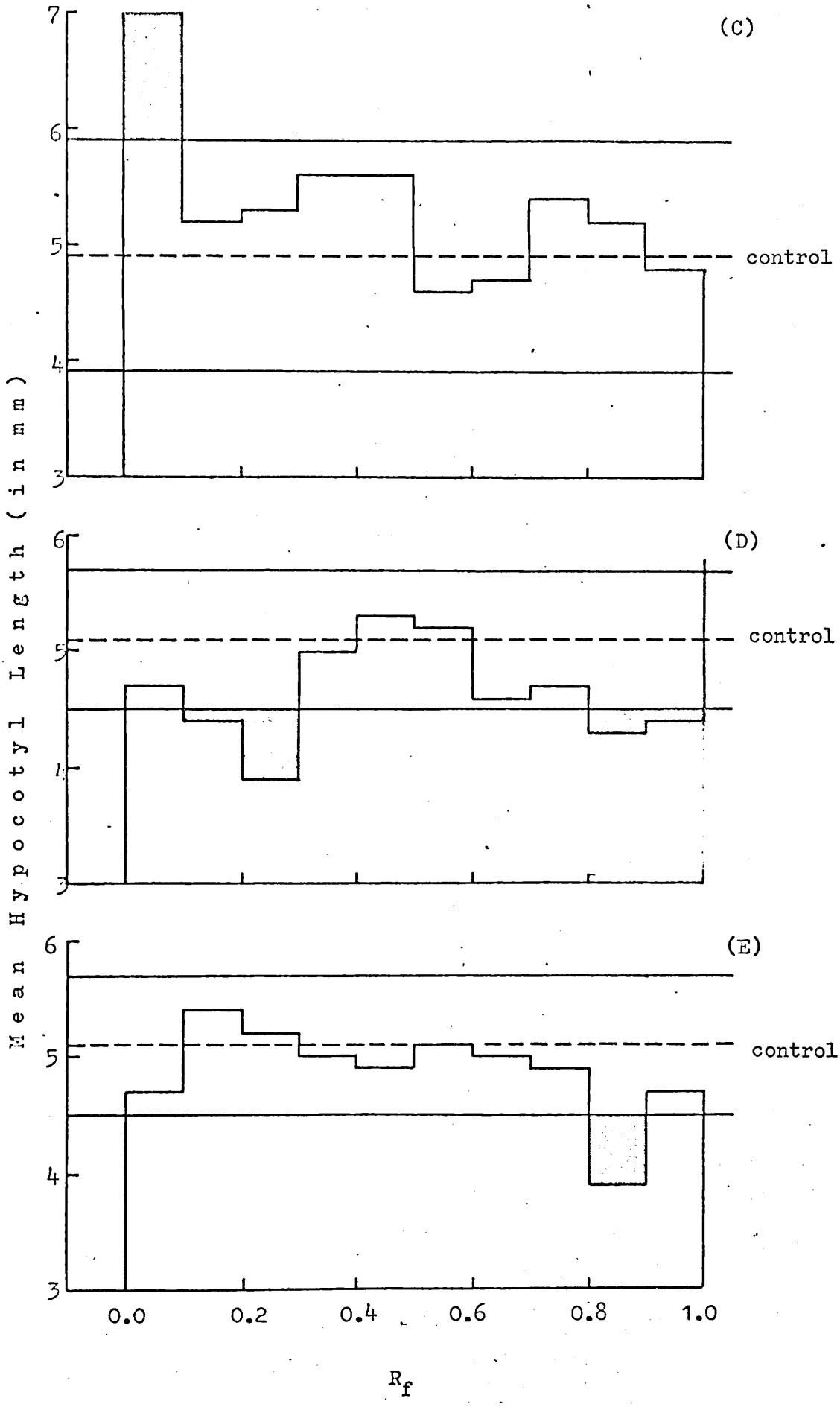


Figure 35

α-Amylase bioassay of the eluates from TLC loaded with the free gibberellins from the plumule tissue extracts of etiolated broad bean seedling (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 6.0 gm fresh weight of tissue was used in the assay.

The letters (A-E) represent the same fractions as in Figure 29.

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 35

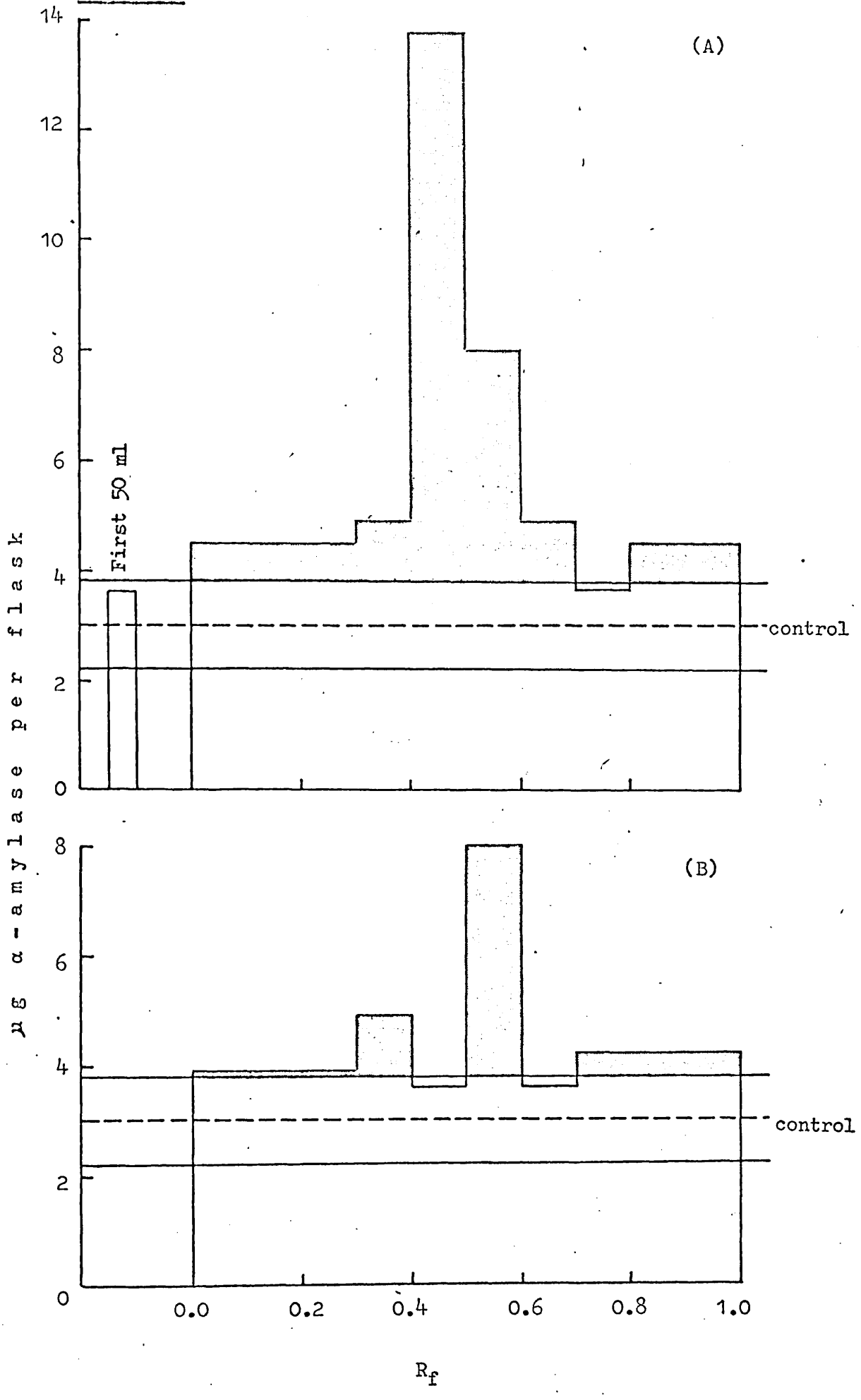

(A)
(B)
First 50 ml
control
control
μg α-amylase per flask
0
2
4
6
8
10
12
14
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 35 (continued)

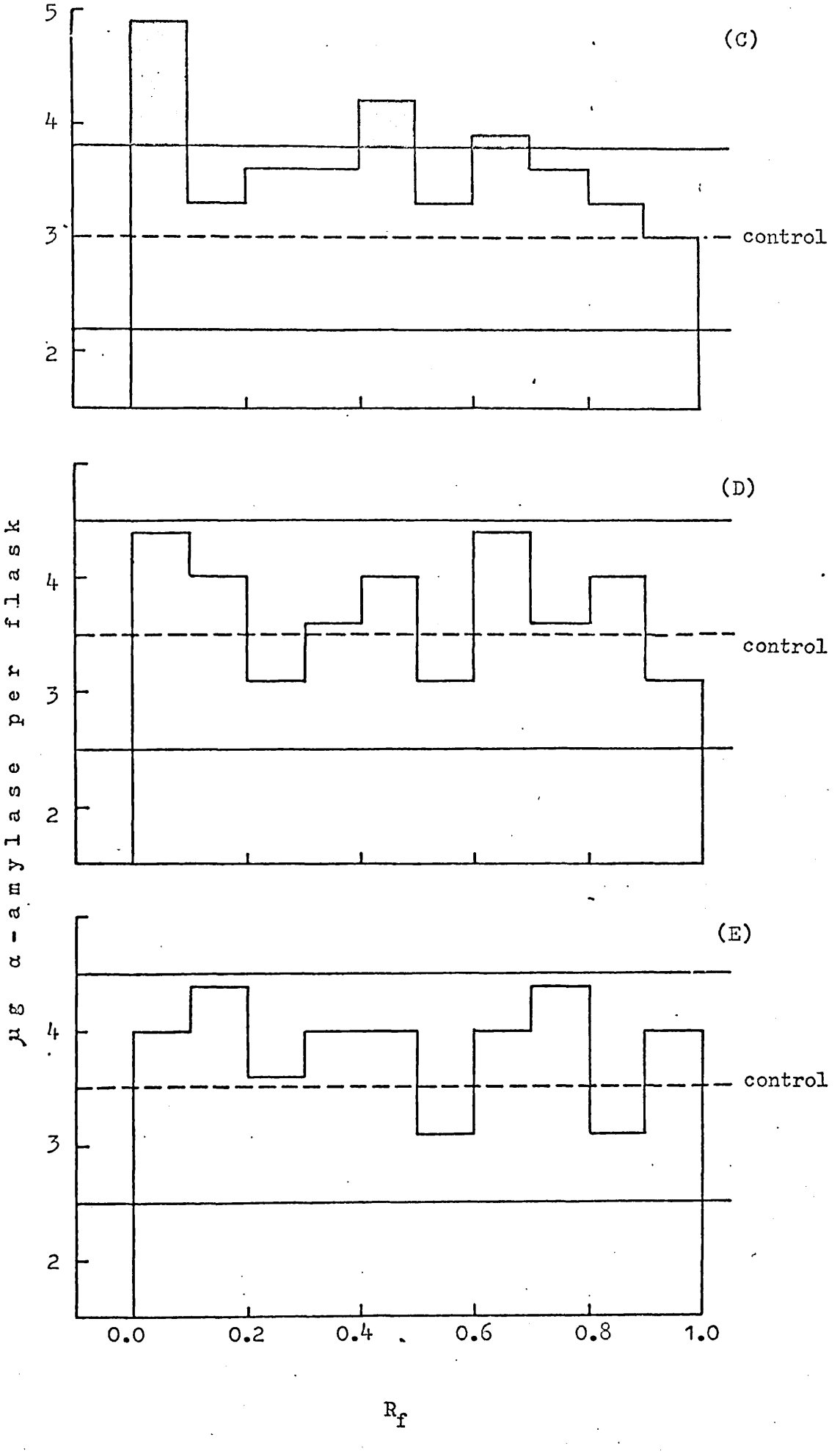


Figure 36

Lettuce hypocotyl bioassay of eluates from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins in the plumule tissue extracts of etiolated broad bean seedling (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of

Ethyl acetate-chloroform-acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 240 gm fresh weight of tissue was used in the assay. The letters (A-E) represent the same fractions as in Figure 29.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 36

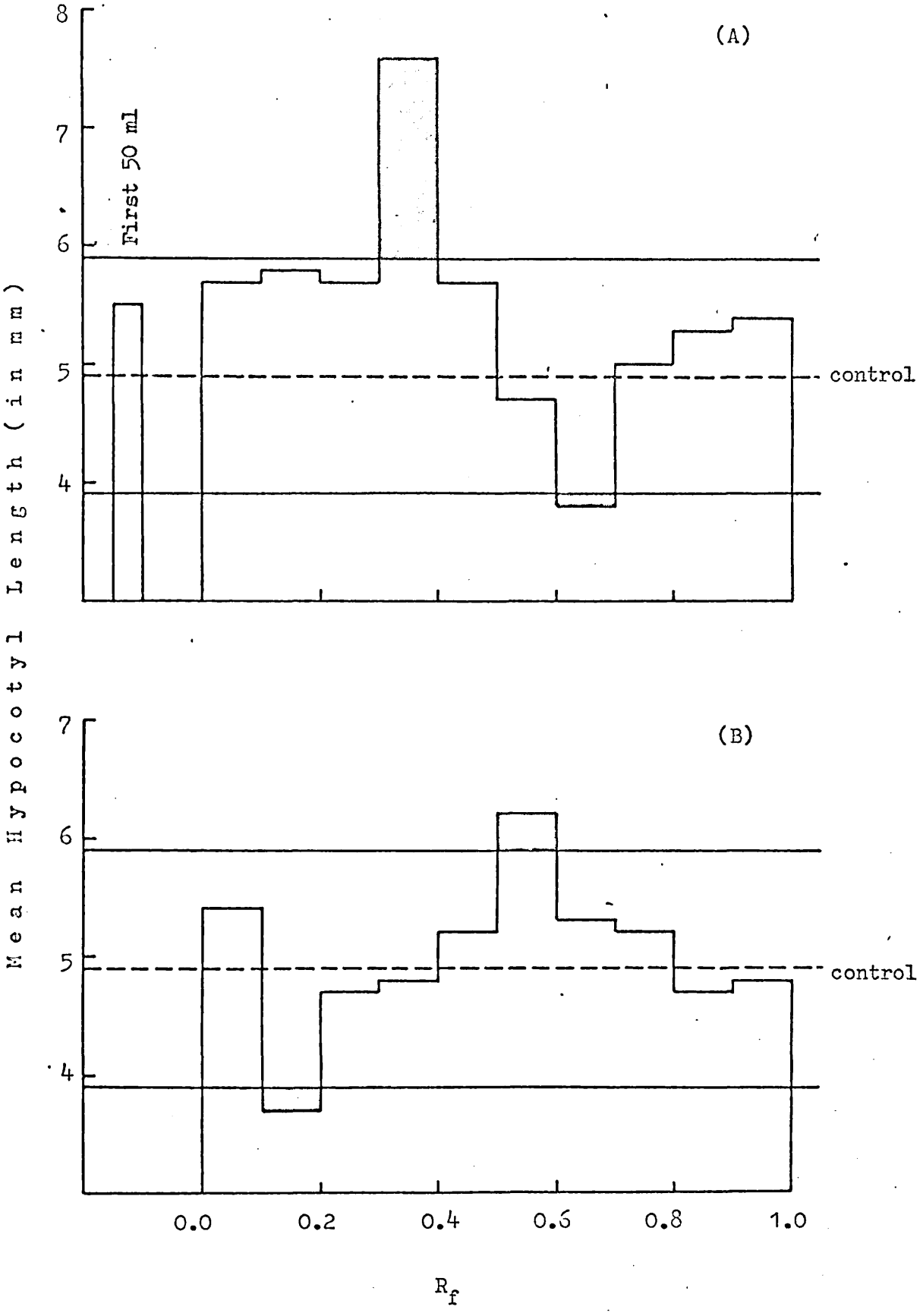

(A)
First 50 ml
control
(B)
control
Mean Hypocotyl Length (in mm)
8
7
6
5
4
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 36 (continued)

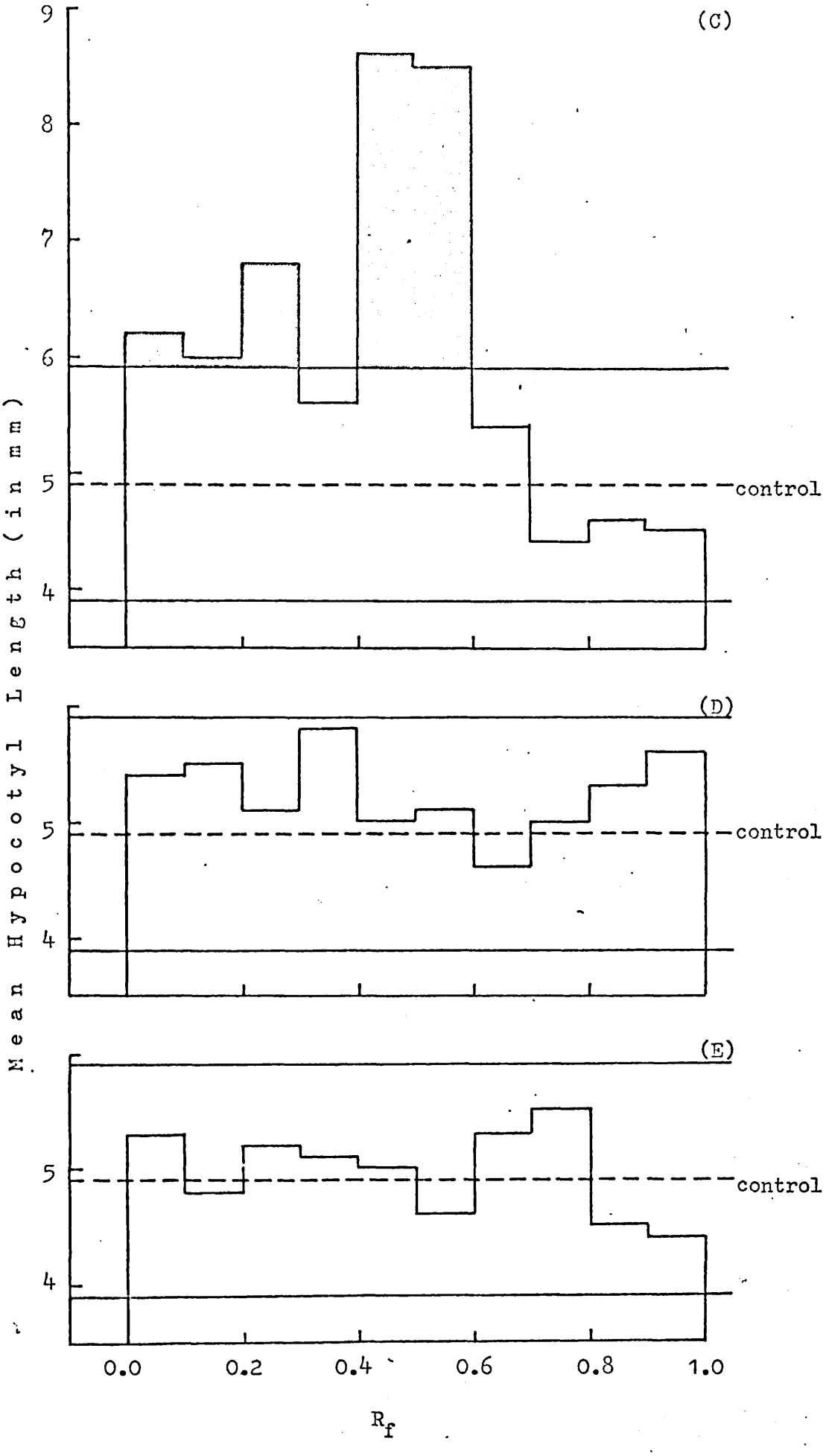


Figure 37

α-Amylase bioassay of the eluates from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins from the plumule tissue extracts of etiolated broad bean seedling (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 6.0 gm fresh weight of tissue was used in the assay.
The letters (A-E) represent the same fractions as in Figure 29.

Shaded parts of the histograms represent promotion significant at 5% level of probability.

Figure 37

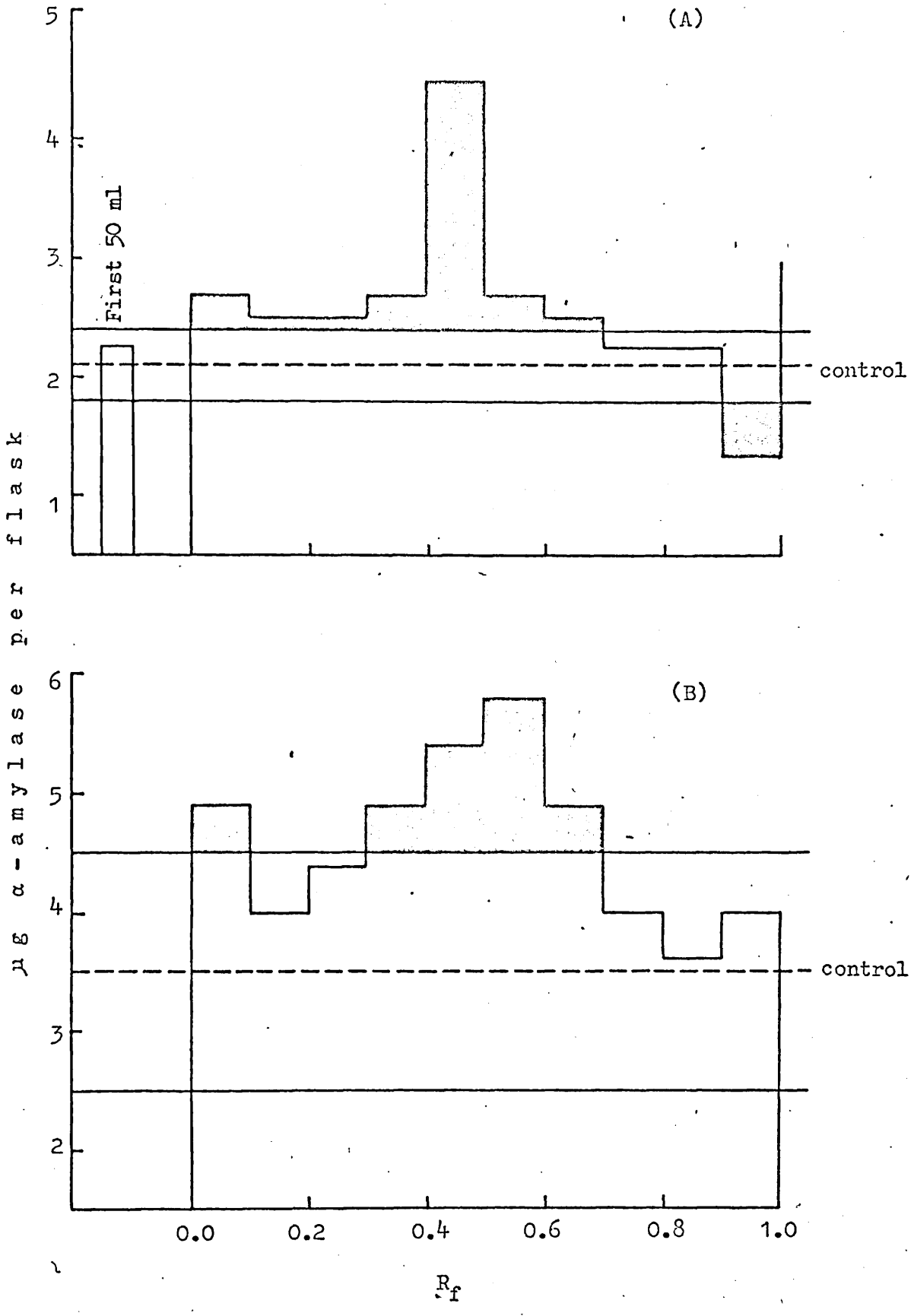
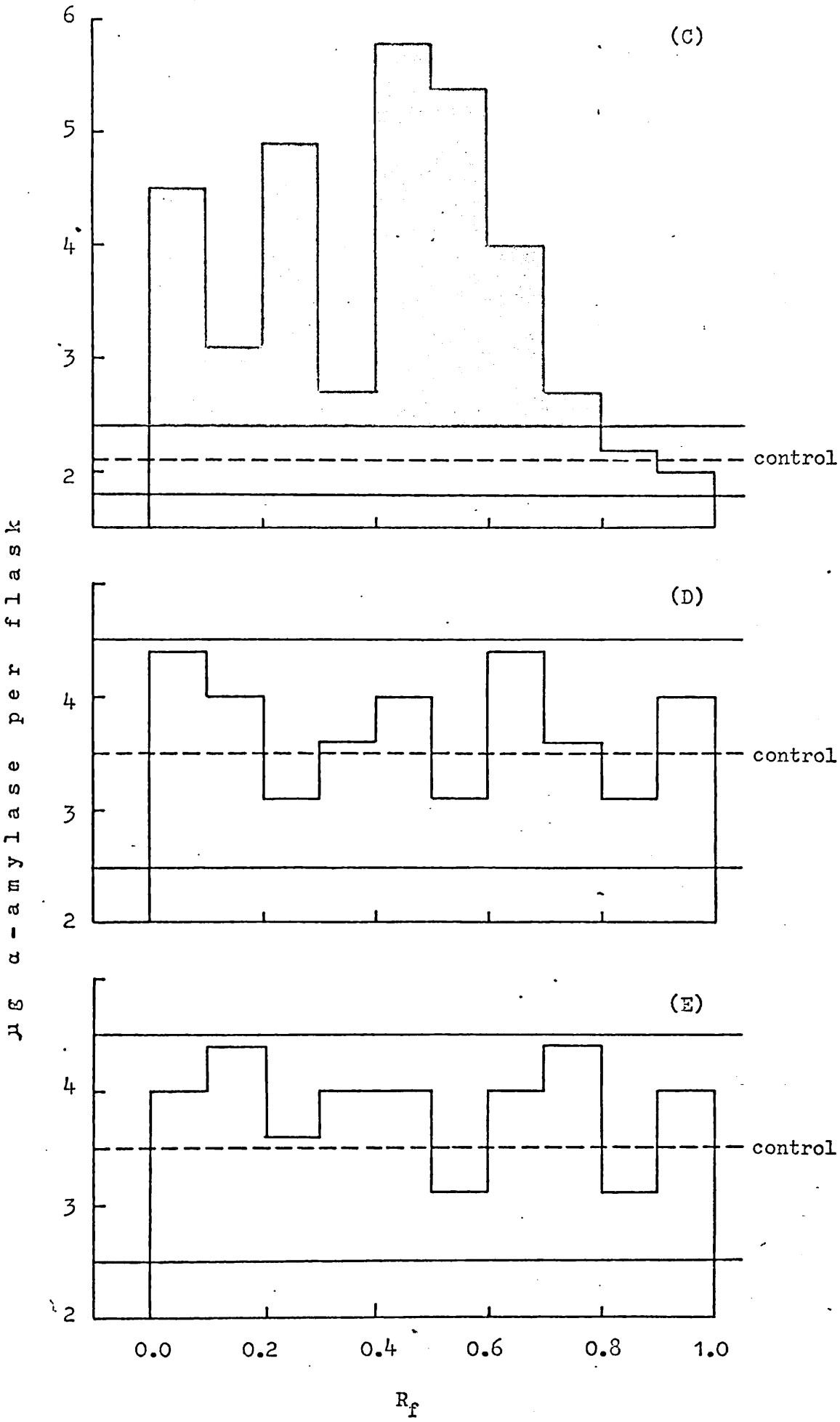

(A)
First 50 ml
control
(B)
control
μg α-amylase per flask
5
4
3
2
1
6
5
4
3
2
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 37 (continued)



With the lettuce hypocotyl bioassay, no activity was detected in the first 50 ml of the column effluent (Fig. 36A); gibberellins of the first 200 ml fraction showed activity at R_f 0.3-0.4, this column fraction contained also inhibitory substance or substances at R_f 0.6-0.7 (Fig. 36A); the second 200 ml column fraction contained active substances together with inhibitory substances, these were separated by TLC at R_f 0.5-0.6 and R_f 0.1-0.2 for the active and the inhibitory substances respectively (Fig. 36B); the third 200 ml column fraction, on the other hand, contained the majority of free gibberellins released by acid hydrolysis of the plumule's bound-gibberellins, these were separated by TLC into at least three peaks of activity with the following R_f values (Fig. 36C): 0.0-0.2, with maximum activity at R_f 0.0-0.1; 0.2-0.3; and 0.4-0.6 which constitute the major peak present. Neither positive nor negative peaks were detected in the fourth and the fifth 200 ml column fractions (Fig. 36D, 36E).

With the α-amylase bioassay test, the results are nearly identical with those of the lettuce hypocotyl bioassay test, Figures 36A, 36B, 36C, 36D, and 36E.

It was not possible to state whether each of the previous mentioned peaks was composed of a single biologically active substance or several such substances.

1.3. Gibberellin-Like Substances in the Cotyledon Tissue Extracts

The cotyledonary tissue extracts were purified by means of PVP slurries, PVP column chromatography followed by TLC of the column effluents, then testing the TLC eluates for gibberellin-like activity with bioassay tests.

The free gibberellins were resolved into separate zones of activity, as detected by the lettuce hypocotyl bioassay test, as follows (Fig. 38 A-E):

Figure 38

Lettuce hypocotyl bioassay of the eluates from TLC loaded with the free gibberellins in the cotyledon tissue extracts of the etiolated broad bean seedling (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of

Ethyl acetate-chloroform-acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 176 gm fresh weight of tissue was used in the assay.
The letters (A-E) represent the same fractions as in Figure 29.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 38

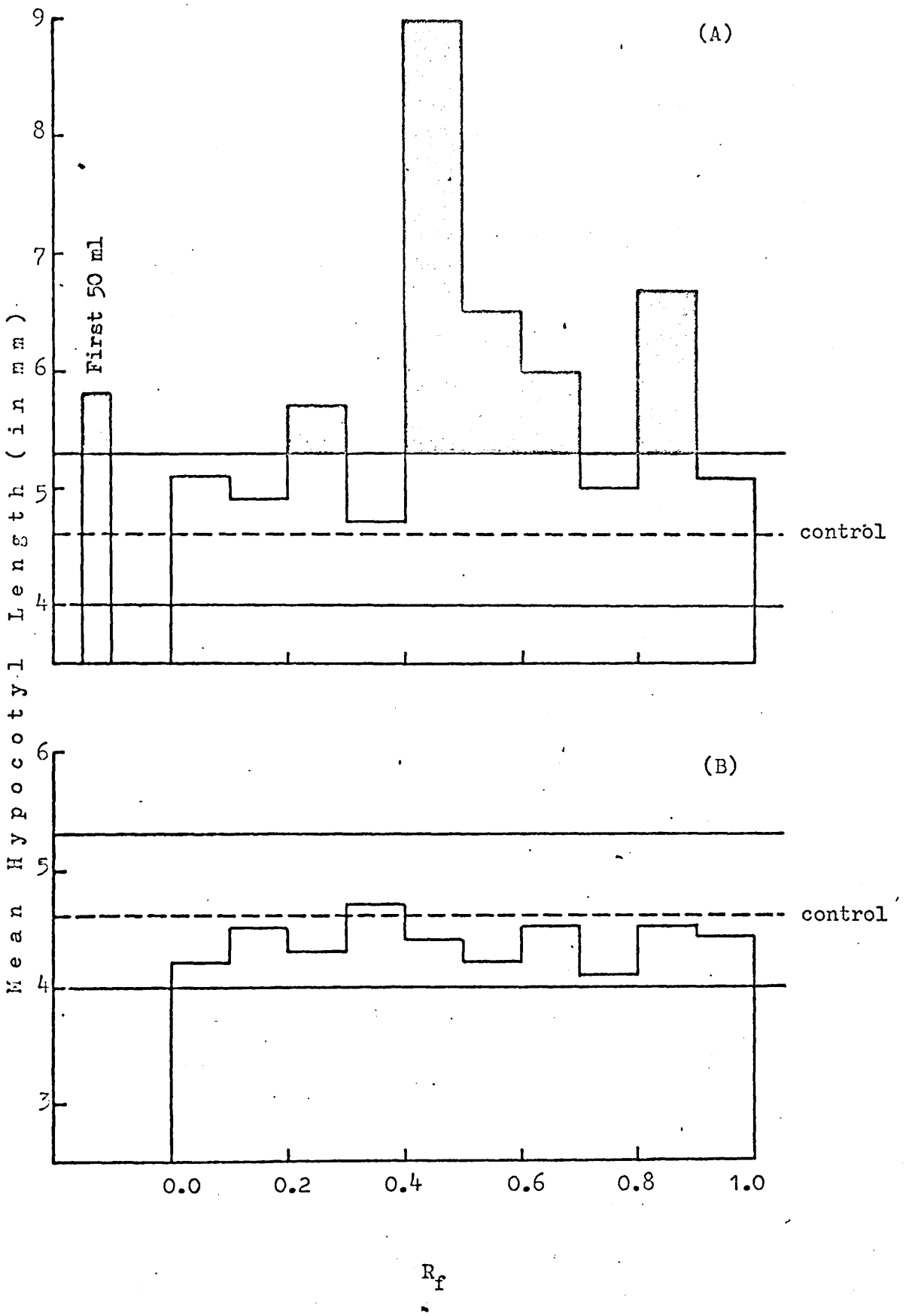

(A)
(B)
First 50 ml
control
control
Mean Hypocotyl Length (in mm)
9
8
7
6
5
4
3
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 38 (continued)

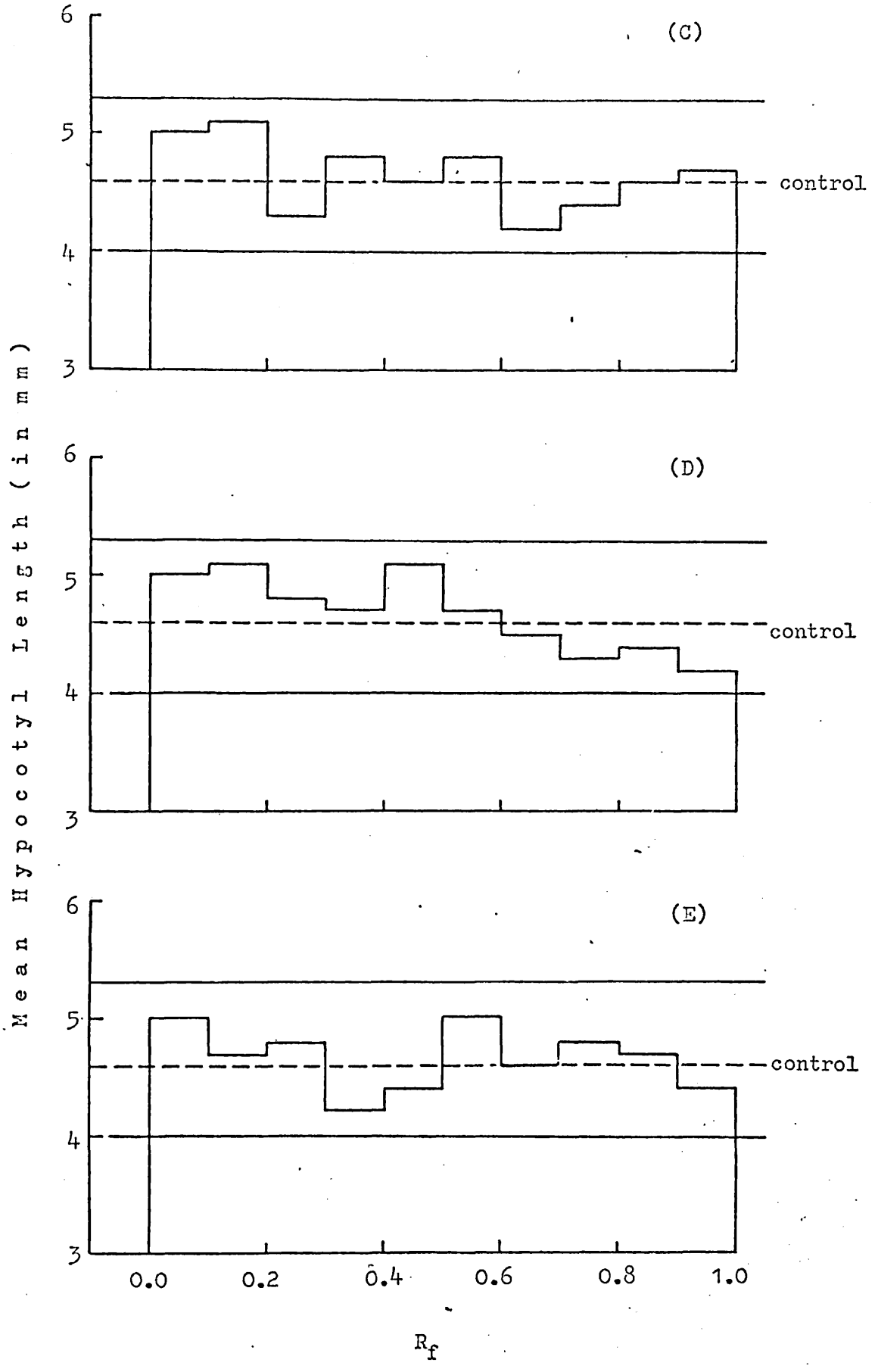


Figure 39

Lettuce hypocotyl bioassay of the eluates from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins from cotyledons tissue extracts of etiolated broad bean seedlings (after PVP column chromatography). The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

An amount of the eluate equivalent to 176 gm fresh weight of tissue was used in the assay.

The letters (A-E) represent the same fractions as in Figure 29.

The shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 39

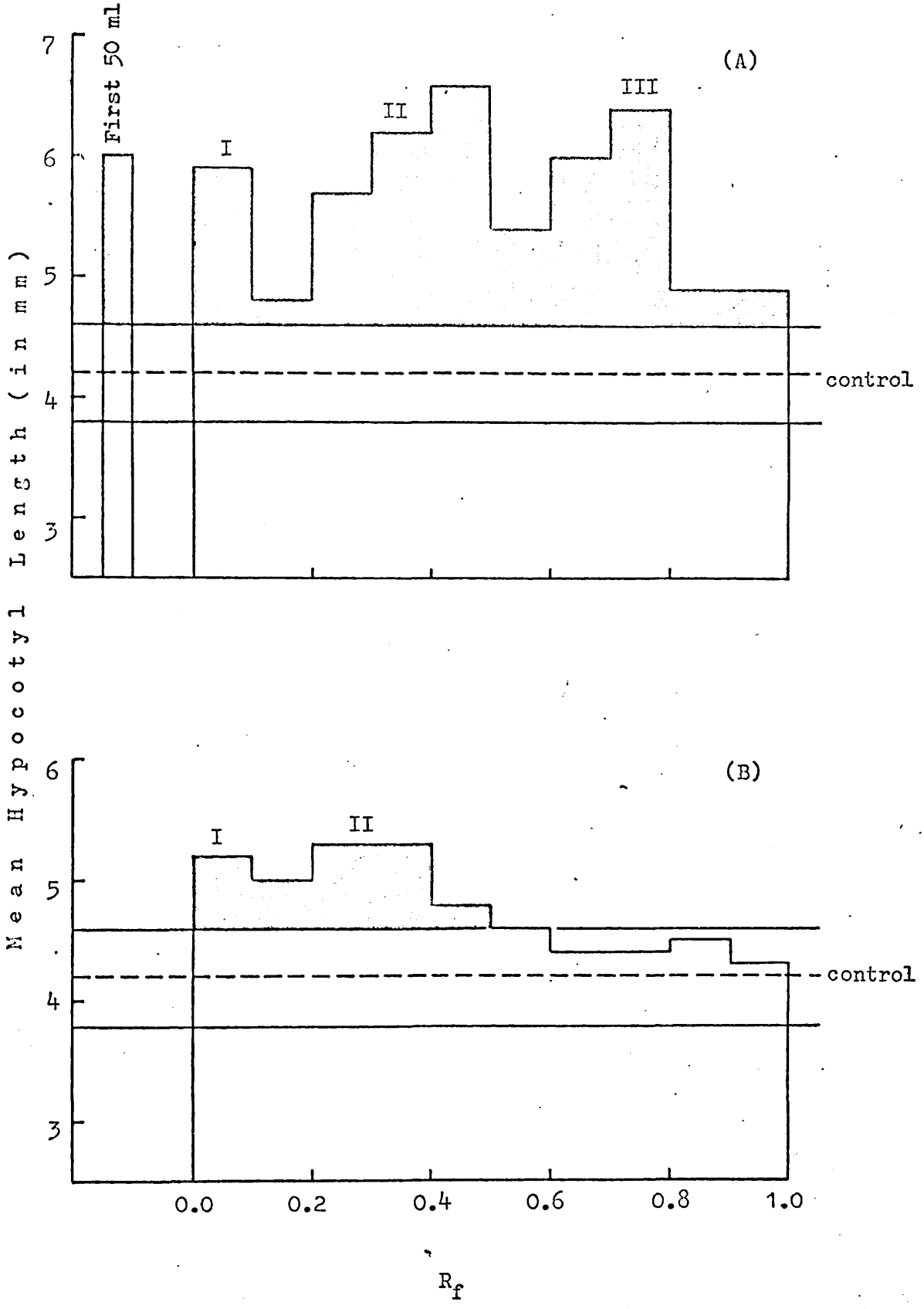
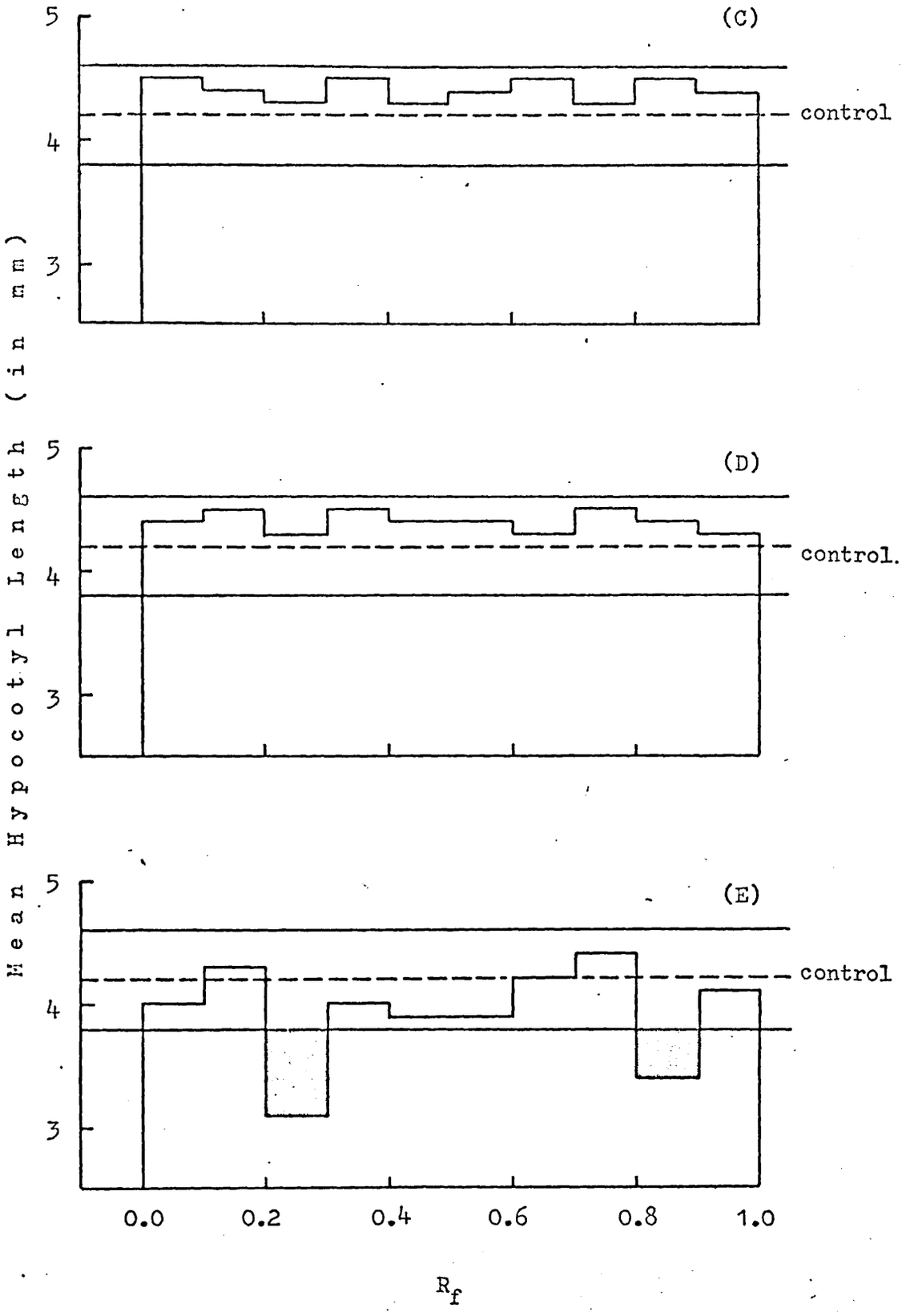

Mean Hypocotyl Length (in mm)
7
6
5
4
3
First 50 ml
I
II
III
(A)
control
(B)
I
II
control
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 39 (continued)



The first 50 ml of the column effluents contained stimulatory substance or substances (Fig. 38A); of the five consecutive column fractions of 200 ml each, only the first one showed peaks of activity at R_f 0.2-0.3, 0.4-0.7 (with maximum activity at R_f 0.4-0.5), and 0.8-0.9 (Fig. 38A). No peaks, either inhibitory or stimulatory, were detected in the second, the third, the fourth, or the fifth fractions (200 ml each) of the PVP column (Fig. 38 B-E).

In the case of the free gibberellins released by acid hydrolysis of the cotyledon's bound-gibberellins on the other hand, activity was detected in the first 50 ml of the PVP column effluent and also in the first two fractions (200 ml each). The gibberellin content of the first 200 ml fraction was resolved by TLC into at least three peaks of activity (Fig. 39A), peak marked (I) which behaved as a fairly polar component and was barely mobile in the solvent system used and under the conditions employed, peak marked (II), and peak marked (III) which are less polar in comparison with peak (I). The gibberellin content of the second 200 ml column fraction was resolved by TLC into at least two peaks, peak marked (I), and peak marked (II) (Fig. 39B). No activity was detected in either the third or the fourth column fractions (Fig. 39 C,D); while in the fifth fraction two peaks which contained inhibitory substance or substances were detected at R_f 0.2-0.3, and 0.8-0.9 (Fig. 39E).

Footnote

1. Unfortunately, at the time when re-chromatography of the free gibberellins from the radicle tissue extracts and extraction of the cotyledon's tissue were carried out, the barley seeds var Sultan (which has been used in all the α-amylase bioassay tests mentioned in the previous experiments) became unobtainable from the same or other sources.

For this reason, the samples mentioned above were tested biologically with the lettuce hypocotyl assay test only.

2. GLC analysis of the TLC eluates of the zones that showed considerable activity with the bioassay tests used were successful, but on some occasions (e.g. the zone with R_f 0.7-0.8 in the case of the radicle's free gibberellins, Fig. 30A) an enormous peak was detected. The retention time of this peak corresponds to the retention time of the methyl ester of gibberellin A_{17} which is inactive in the bioassay tests used (Table 1). An attempt was made in collaboration with Dr. J. MacMillan, of the Department of Chemistry, University of Bristol, to determine the identity of this peak by combined gas chromatography--mass spectrometry (GC-MS), the mass spectrum showed the presence of acetyl tri-n-butyl citrate. This is a common laboratory contaminant that occurs in most organic solvents, in PVC tubing and G.C. septa. It has been found also in solvents that had not been exposed to plastic containers or tubing from the time of delivery in metal drums (Binks et al., 1970). Dr. MacMillan's advice was to partition the extracts against a diluted solution of sodium bicarbonate to separate this contaminant from the gibberellins and these can be back extracted from the bicarbonate phase by partitioning against redistilled deacidified ethyl acetate at pH 2.5.

Consequently, the step of partitioning the extracts against sodium bicarbonate was added to the extraction steps, and the extracts in the coming experiments were purified in exactly the same way as represented in the Flow Diagram (Fig. 5). The following experiments also included the separation of acidic butanol-soluble gibberellins and the hydrolysis of gibberellin-glucosides.

CHAPTER V

Distribution of Endogenous Gibberellins in Etiolated Broad Bean (Vicia faba) Seedling

1. Gibberellin Activity Determined by Bioassay Tests

Broad bean seeds (cv Green Windsor) were soaked for 40 hours, grown for ten days in the dark at 25°C (± 1°C), and the seedlings harvested, selected and treated as described in the Materials and Methods Section; the gibberellin content of the different organs were extracted separately; fractionated into an acidic ethyl acetate-soluble fraction, which contains most of the free gibberellins; an acidic butanol-soluble fraction, which contains gibberellin glucosides [together with the most polar free gibberellin (GA_{32}) and a part of the less polar one (GA_{28}) if present (Takahashi, 1974)]; and a water--soluble fraction which contains other bound-gibberellins (see Flow Diagram of Extraction and Purification Technique, Figure 5).

1.1. Acidic Ethyl Acetate-Soluble Fraction (Free Gibberellins)

Extracts containing the free gibberellins were purified by means of PVP slurries and PVP column chromatography. Gibberellins recovered from the column effluents were separated by thin-layer chromatography on activated Keiselgel 60 F_{254} plates (20 x 20 cm) using solvent system (1). The plates were divided into 10 equal zones which were eluted separately with wet ethyl acetate, and the eluates were tested biologically for gibberellin-like activity with the lettuce hypocotyl and the α-amylase bioassay tests.

Due to the results obtained in the previous Chapter (Chapter IV) with the PVP column chromatography of the different tissue extracts,

the column effluents in the following experiments were collected for gibberellin separation as follows:

A. In the case of the radicle extracts, the first 50 ml were discarded and the next 400 ml were collected together.

B. In the case of the plumule extracts, the first 50 ml were discarded and the next 600 ml were collected together.

C. In the case of the cotyledons extracts, the first 50 ml were collected together with the next 200 ml of the PVP column effluents.

The results of the bioassay of the TLC eluates are illustrated in histogram form, each histogram represents a replicate extract from a separate seedling sample.

A. Free Gibberellin Content of the Radicle Extracts

Three replicates were extracted separately, the extracts were purified, and gibberellins separated by TLC as described earlier. The thin-layer plates were divided into 10 equal zones, each of which was eluted with a known volume of wet ethyl acetate as described in the Materials and Methods Section. The TLC eluates of each of the three replicates were tested for gibberellin-like activity with the lettuce hypocotyl bioassay test, and the eluates of one of these replicates was tested with the α-amylase bioassay test. The fresh weight of each replicate, the number of seedlings used every time, the volume of wet ethyl acetate used for elution of each TLC zone in each case, and the volume of eluates used for the bioassay tests are shown in the following Table:

Rep. No.	No. seedlings extracted	F.W.* (in gms.)	Vol. of EtoAC for elution (ml)	Vol. for lettuce assay	Vol. for α-amylase assay
1	400	910	6	0.6 (eq. to 91.0 gm F.W.)	-
2	475	925	6	1.0 (eq. to 154 gm F.W.)	-
3	350	725	5	1.0 (eq. to 145 gm F.W.)	0.05 (eq. to 7.2 gm F.W.)

*F.W. = Fresh Weight

The results of the lettuce assay for the first two replicates are represented in Figure 40A and 40B for replicate (1) and replicate (2) respectively, while Figure 41A and 41B shows the lettuce bioassay and the α-amylase bioassay tests for replicate (3) respectively.

With the lettuce hypocotyl bioassay, each extract contains substance or substances with gibberellin-like activity in at least three peaks; two of these peaks (peak marked II, and peak marked III) were found to be reproducible in all cases with the lettuce hypocotyl bioassay and the α-amylase bioassay (Figs. 40A,B and 41A,B), and a third peak which was found to be reproducible in two extracts (peak marked (IV), Fig. 40A,B), and another peak (peak marked I) which appeared on one occasion only (Fig. 41A).

It should be mentioned here that with replicate number (1), the eluate of the TLC zones at R_f 0.5-0.7 was found to contain substance or substances toxic to the lettuce seedling when an amount of the eluate equivalent to 150.0 gm fresh weight of tissue was used for bioassay. Using a smaller amount of the TLC eluate equivalent to only 91.0 gm fresh weight of tissue was sufficient to remove the effect of the toxic substance but it was not sufficient to show the full activity of some peaks (Fig. 40A).

Figure 40

Lettuce hypocotyl bioassay of eluates from TLC loaded with the free gibberellins of the radicle extracts, after their recovery from PVP column effluents. The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

A. Replicate No. 1 : the plate was loaded with an extract from 910 gm fresh weight of tissue, and an amount of the eluates equivalent to 91 gm fresh weight of tissue was used for the assay.

B. Replicate No. 2 : the plate was loaded with an extract from 925 gm fresh weight of tissue, and an amount of the eluates equivalent to 154 gm fresh weight of tissue was used for the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 40

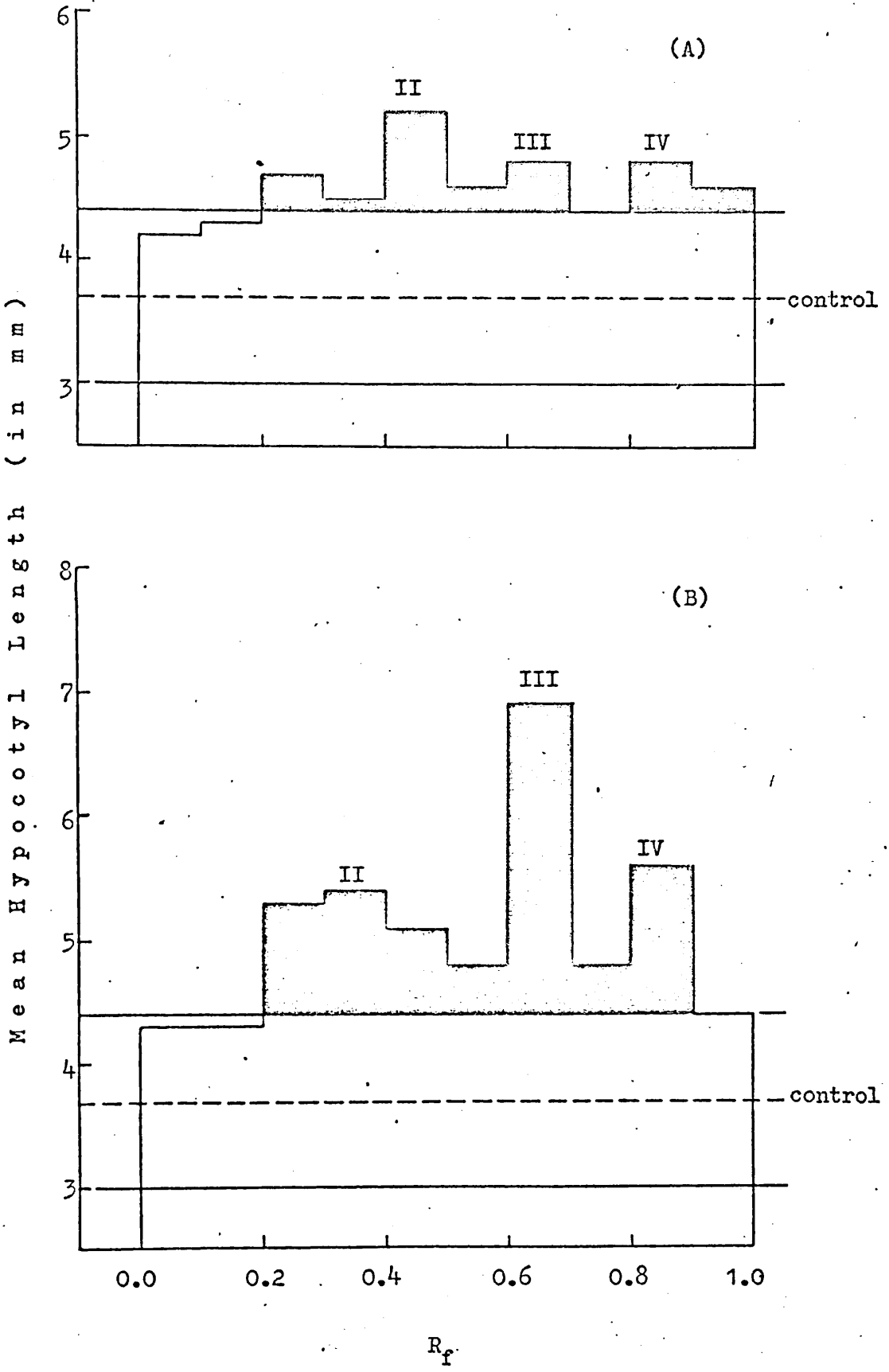

(A)
II
III
IV
control
(B)
III
II
IV
control
Mean Hypocotyl Length (in mm)
6
5
4
3
8
7
6
5
4
3
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 41

Lettuce hypocotyl bioassay (41A) and α-amylase bioassay (41B) of eluates from TLC loaded with the free gibberellins of the radicle tissue extracts (replicate No. 3) after their recovery from the PVP column effluents. The plate was developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

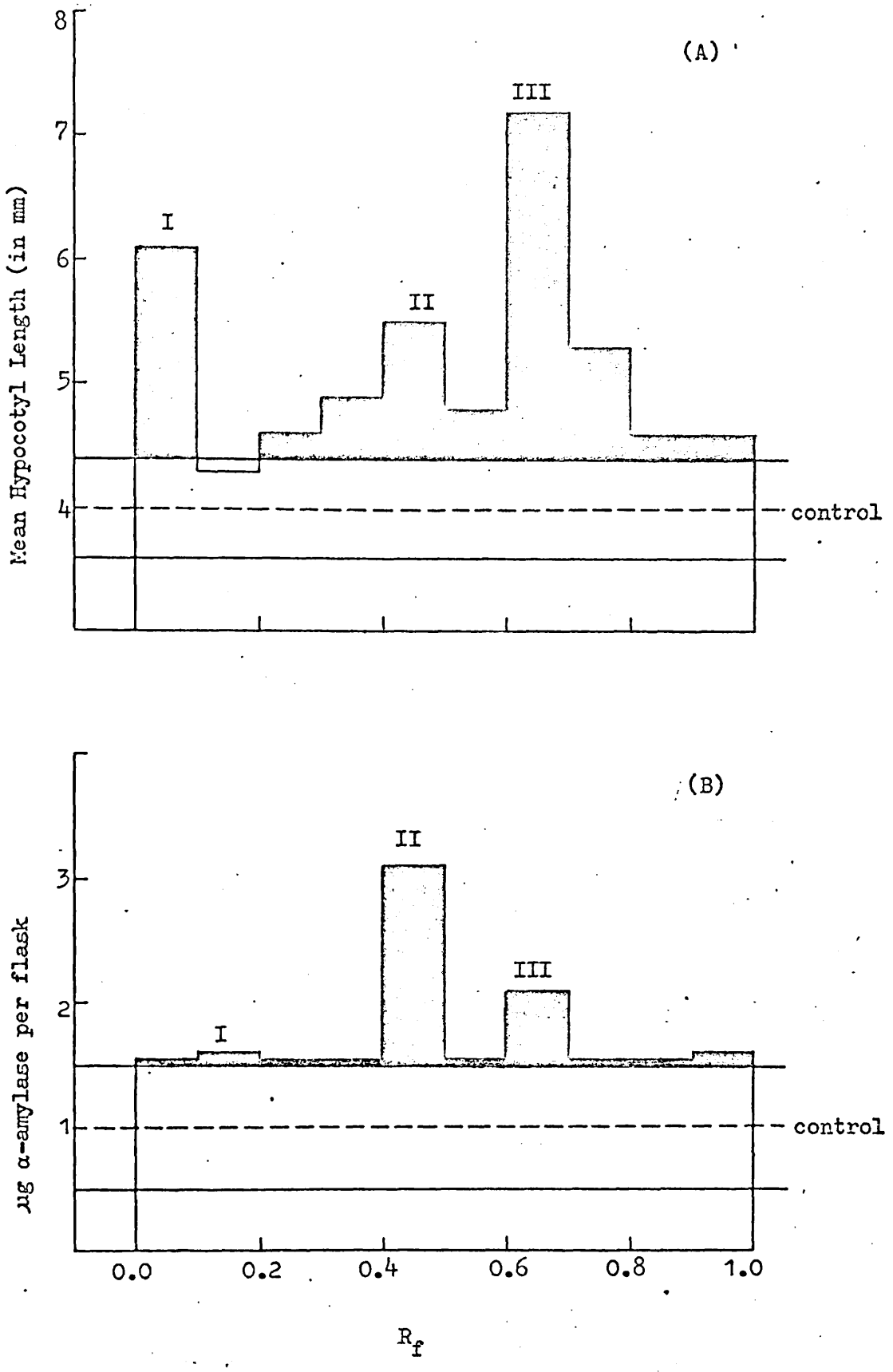
(15:5:1 v/v)

and eluted with wet ethyl acetate.

The plate was loaded with an extract from 725 gm fresh weight of tissue and an amount of the thin-layer eluates equivalent to 145 gms and 7.2 gms fresh weight of tissue were used for the lettuce assay and the α-amylase assay tests respectively.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 41


(A)
Mean Hypocotyl Length (in mm)
8
7
6
5
4
I
II
III
control
(B)
µg α-amylase per flask
3
2
1
I
II
III
control
0.0
0.2
0.4
0.6
0.8
1.0
R_f

B. Free Gibberellin Content of the Plumule Extracts

Three replicates were extracted separately for free gibberellin content of the plumule tissue. The fresh weight of each replicate, the number of seedlings used in each case, the volume of wet ethyl acetate used for elution of each zone of the TLC, and the volume of eluates used for bioassay tests are shown in the following Table:

Rep. No.	No. seedlings extracted	F.W. (in gms.)	Vol. of EtoAC for elution (ml)	Vol. for lettuce assay	Vol. for α-amylase assay
1	400	1400	6	0.7 (eq. to 163.3 gm F.W.)	-
2	350	1250	5	1.0 (eq. to 250.0 gm F.W.)	-
3	415	1470	6	0.8 (eq. to 176.0 gm F.W.)	0.08 (eq. to 17.6 gm F.W.)

The extracts were purified as described earlier, the gibberellins were resolved by TLC/bioassay (for full details, see Flow Diagram of Extraction and Purification Technique, Fig. 5). The TLC eluates of the three replicates were tested for gibberellin-like activity with the lettuce hypocotyl bioassay test, and one of the replicates was tested with the α-amylase bioassay test. The results are represented in Figure 42 A,B for the lettuce hypocotyl assay of replicates (1) and (2), and Figure 43 A,B for the lettuce hypocotyl and α-amylase bioassay tests for replicate (3) respectively.

From the figures, at least three peaks that showed activity with the bioassay tests used were present in all the replicates (peaks marked I, II, and III, Figs. 42 A,B, and 43 A,B); on two occasions a small peak appeared in the extracts (peak marked IV, Fig. 42 A,B).

Figure 42

Lettuce hypocotyl bioassay of eluates from TLC loaded with the free gibberellins of the plumule tissue extracts, after their recovery from the PVP column effluents. The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

A. Replicate No. 1 : the plate was loaded with an extract of 1400 gm fresh weight of tissue, and an amount of the thin-layer eluates equivalent to 163.3 gm fresh weight of tissue was used for the assay.

B. Replicate No. 2 : the plate was loaded with an extract of 1250 gm fresh weight of tissue and an amount of the thin-layer eluates equivalent to 250 gm fresh weight of tissue was used for the assay.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 42

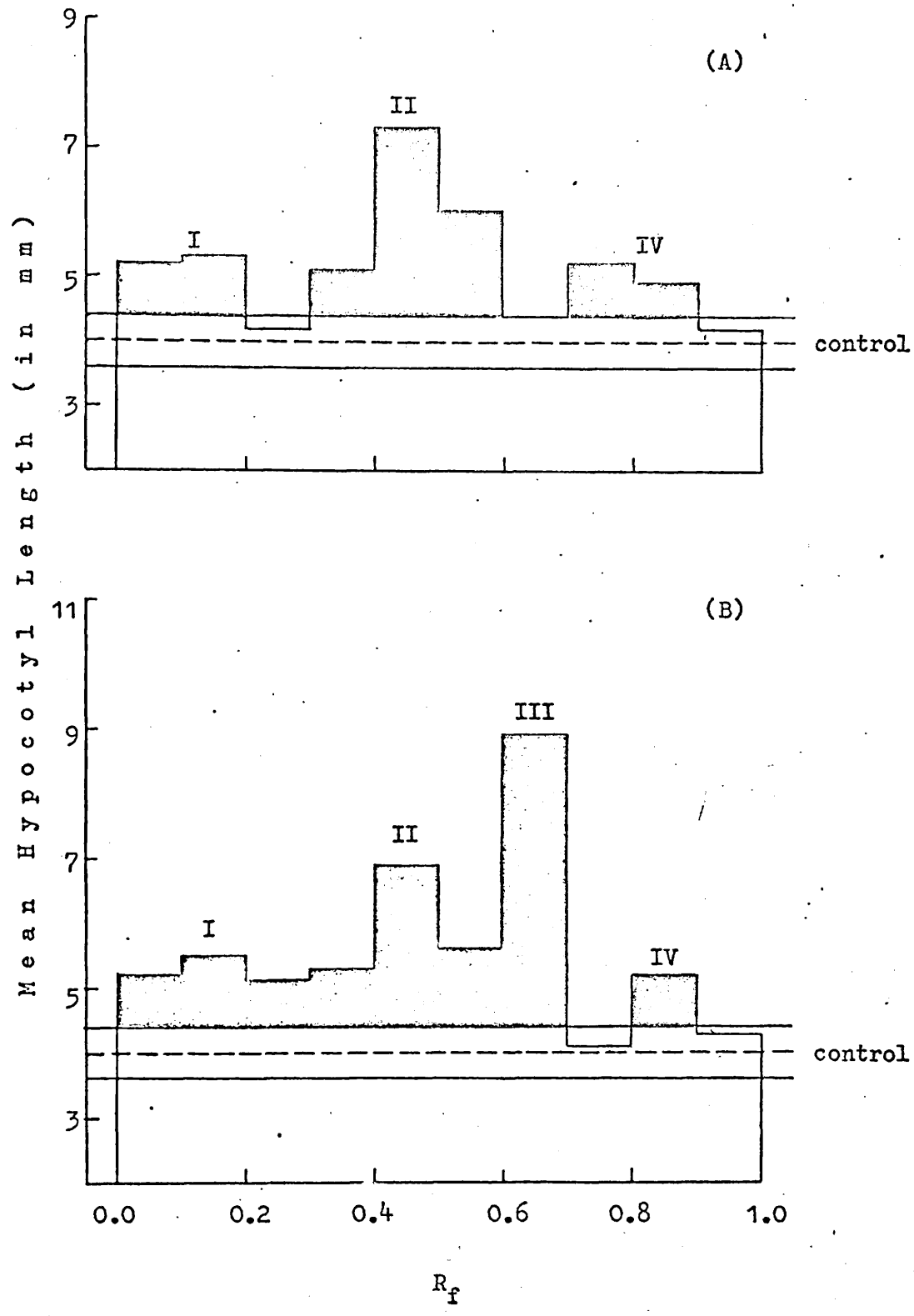

(A)
(B)
I
II
III
IV
control
control
9
7
5
3
11
9
7
5
3
0.0
0.2
0.4
0.6
0.8
1.0
R_f
Mean Hypocotyl Length (in mm)

Figure 43

Lettuce hypocotyl bioassay (43A) and α-amylase bioassay (43B) of eluates from TLC loaded with the free gibberellins of the plumule tissue extracts (replicate No. 3), after their recovery from the PVP column effluents. The plate was developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

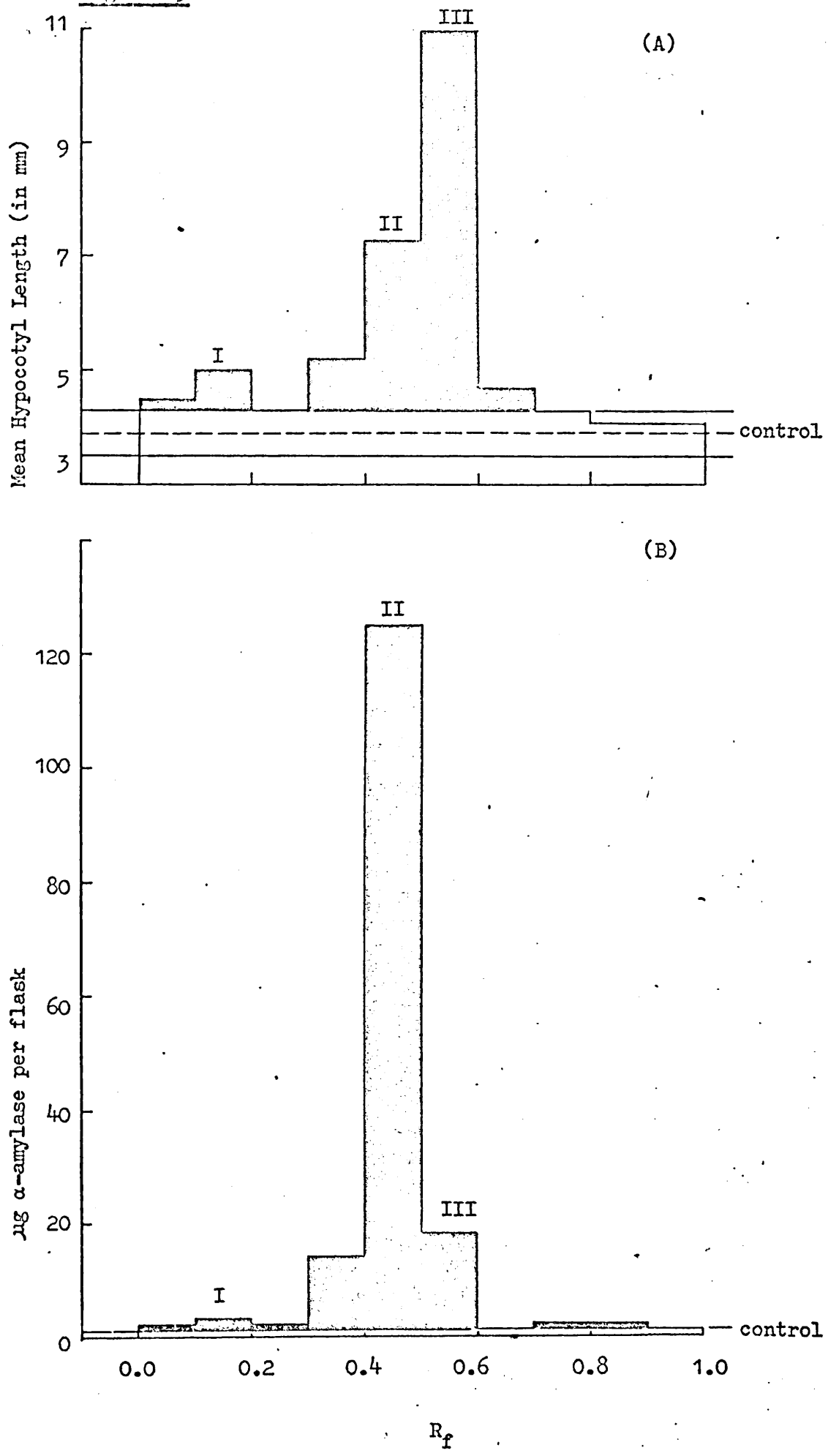
(15:5:1 v/v)

and eluted with wet ethyl acetate.

The plate was loaded with an extract from 1470 gm fresh weight of tissue and an amount of the thin-layer eluates equivalent to 176 gm and 17.6 gm fresh weight of tissue were used for the lettuce assay and the α-amylase assay tests respectively.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 43


(A)
Mean Hypocotyl Length (in mm)
11
9
7
5
3
I
II
III
control
(B)
µg α-amylase per flask
120
100
80
60
40
20
0
I
II
III
control
0.0
0.2
0.4
0.6
0.8
1.0
R_f

C. Free Gibberellin Content of the Cotyledons Extract

The cotyledons of 350 selected 10-days old etiolated broad bean seedlings (ca 1000 gm fresh weight) were extracted, and their extract was purified as described with the radicle and plumule tissue extracts. The free gibberellins recovered from the PVP column chromatography effluent, as described by Glenn et al. (1972), were separated by TLC using solvent system (1). The plates were air dried for 2-3 hours at room temperature, divided into 10 equal zones, and each zone was eluted with 6 ml of wet ethyl acetate (for full details see the Materials and Methods Section). The TLC eluates were tested for gibberellin-like activity with the lettuce hypocotyl assay and the α-amylase assay test, the results of which are represented in Figure 44A and 44B respectively.

With the lettuce hypocotyl bioassay test, the thin-layer eluates of the cotyledons extract showed gibberellin-like activity in at least four peaks when an amount of 0.8 ml of each TLC zone eluate (equivalent to 133.3 gm fresh weight of tissue) was used (peaks marked I-IV, Fig. 44A).

With the α-amylase bioassay test on the other hand, only one peak was detected (peak marked II), when a volume of the TLC eluates equivalent to 13.3 gm fresh weight of tissue (0.08 ml) was used for the assay (Fig. 44B).

From the results obtained earlier with the free gibberellin content of the radicle and plumule extracts (Figs. 40-43 A and B), it appears that the cotyledons tissue extracts contain all the peaks detected in the radicle and plumule tissue extracts.

It was not possible to state whether each of these peaks (in the case of each organ) was composed of a single biologically active substance or several such substances. Identification of gibberellins on the basis of their R_f's on TLC can only be very tentative even when adequate standards are available. However, by comparison to the R_f data of the

Figure 44

Lettuce hypocotyl bioassay (44A) and α-amylase bioassay (44B) of eluates from TLC loaded with the free gibberellins of extracts from the cotyledons tissue, after their recovery from the PVP column effluents. The plate was developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

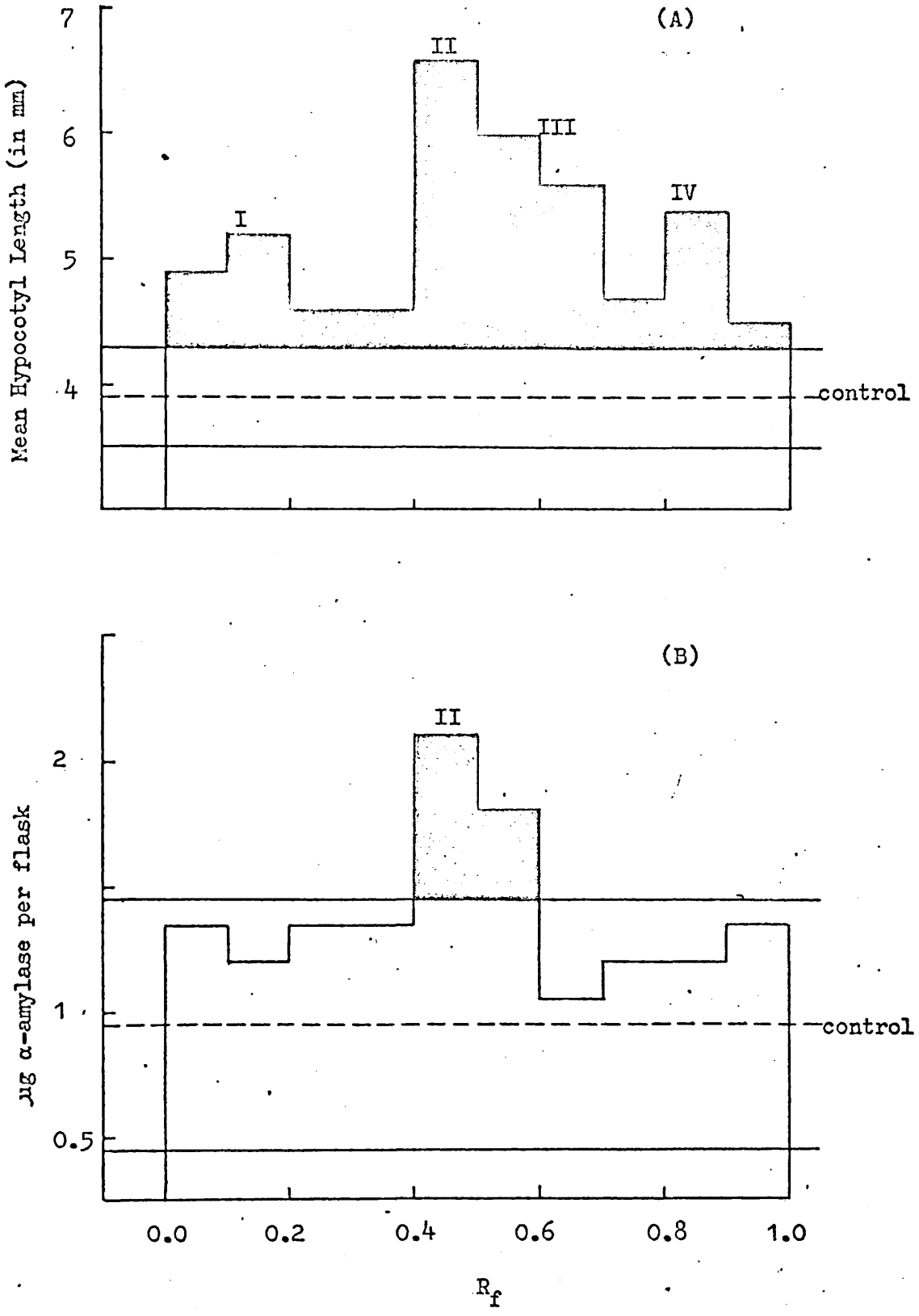
(15:5:1 v/v)

and eluted with wet ethyl acetate.

The plate was loaded with an extract from 1000 gm fresh weight of tissue and an amount of the thin-layer eluates equivalent to 133.3 gm and 13.3 gm fresh weight of tissue were used for the lettuce assay and α-amylase assay tests respectively.

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 44


(A)
Mean Hypocotyl Length (in mm)
7
6
5
4
I
II
III
IV
control
(B)
µg α-amylase per flask
2
1
0.5
II
control
0.0
0.2
0.4
0.6
0.8
1.0
R_f

available standards (Table 2), and by reference to the R_f data of Cavell et al. (1967), it is possible to suggest that the peak with R_f 0.0-0.2 (peak marked I) contains GA_8; the peak marked (II) could contain gibberellins A_1, A_3, A_{13}, or A_{19} at R_f 0.3-0.5; the peak marked (III) could contain gibberellins A_4, A_5, A_7, or A_{14} at R_f 0.5-0.6, and gibberellins A_9, A_{11}, A_{12}, or A_{15} at R_f 0.7-0.8; no known gibberellins would correspond to the peak marked (IV) at R_f 0.8-1.0.

1.2. Acidic Butanol-Soluble Gibberellins

Before it was decided definitely to separate acidic butanol--soluble gibberellins from the extracts prior to the hydrolysis of water-soluble or bound-gibberellins, a simple experiment was carried out to check for the occurrence of such types of gibberellins in the tissues under investigation. In this simplified experiment, 50 gm fresh weight of each organ from ten-days old etiolated broad bean seedlings were extracted separately and the extracts fractionated into an acidic ethyl acetate-soluble fraction and an acidic butanol--soluble fraction as shown in the Flow Diagram (Fig. 5). The butanol was evaporated under vacuum at 40°C, the gummy residue was dissolved in redistilled absolute methanol and any undissolved residue was filtered off through a sintered glass funnel; the filtrate was evaporated to dryness under vacuum at 35°C, the residue containing acidic butanol-soluble gibberellins was dissolved in the least possible amount of redistilled absolute ethanol, and streak loaded quantitatively on the starting line of 5 x 20 cm thin-layer plates of Kieselgel 60 F_{254} which were developed three times with solvent system (3) (see the Materials and Methods Section for full details).

The chromatograms were air dried at room temperature, divided into 10 x 1.5 cm zones which were treated as described in the Materials

Figure 45

Lettuce hypocotyl assay of TLC loaded with acidic butanol-soluble gibberellins from the different organs of etiolated broad bean seedlings.

Fifty gms fresh weight equivalent were loaded on the plate which was developed three times with solvent system (3) which consisted of:

Acetone - acetic acid

(97:3 v/v)

and eluted with distilled water.

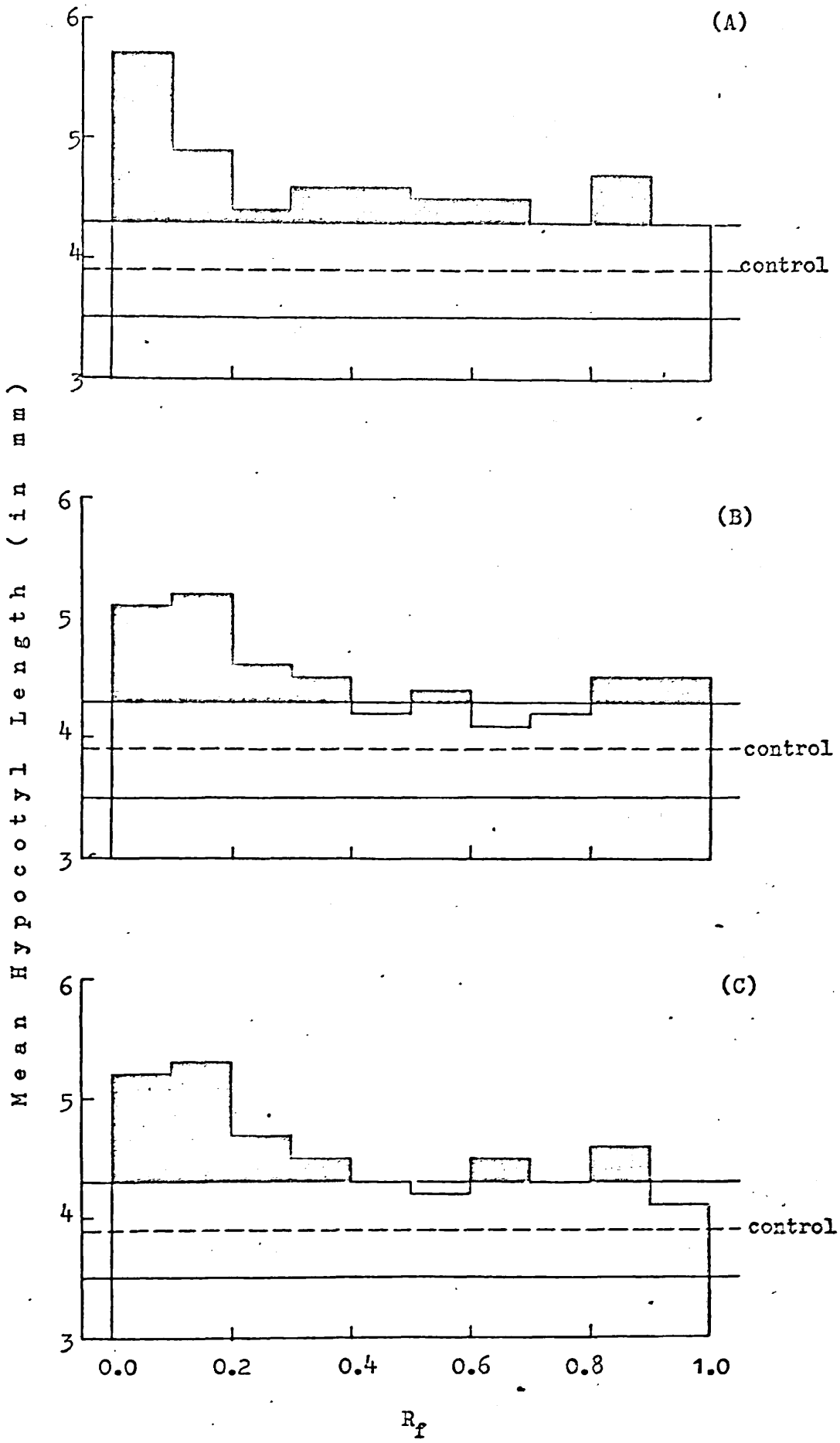
A. acidic butanol-soluble fraction of the radicle tissue extracts

B. ditto of plumule tissue extract

C. ditto of cotyledon tissue extract

Shaded parts of the histogram represent promotion significant at the 5% level of probability.

Figure 45


(A)
(B)
(C)
control
control
control
6
5
4
3
0.0
0.2
0.4
0.6
0.8
1.0
Mean Hypocotyl Length (in mm)
R_f

and Methods Section, and their gibberellin-like activity was determined with the lettuce hypocotyl bioassay test. The results of the bioassay are represented in histogram form in Figure 45A,B, and C for the acidic butanol-soluble gibberellins of the radicle, the plumule, and the cotyledons respectively.

The results show the presence of a barely mobile fairly polar component at R_f 0.0-0.2, under the conditions employed, in all the organs of the etiolated seedling. This component represented the main acidic butanol-soluble gibberellin of the tissue under investigation. Less polar components appeared to occur in at least three zones of the TLC in each tissue extract (Fig. 45 A-C).

The results obtained with this experiment prompted separation of the acidic butanol-soluble gibberellins on a comparatively larger scale; and as this fraction of the extracts could contain gibberellin-glucosides, it was decided to hydrolyse these components. It was also decided to add a purification step before the TLC separation; thus, the acidic butanol-soluble fraction was obtained separately for each organ of the etiolated seedling, purified by means of PVP slurries, and the gibberellins recovered from the slurry filtrates were either resolved by TLC using solvent system (3), or hydrolysed by 0.4N HCl in a water bath at 40°C for 60 minutes. The free gibberellins thus released were then extracted and resolved by TLC using solvent system (1). For full details see the Materials and Methods Section, and the Flow Diagram of Extraction and Purification Technique, Figure 5.

The full details concerning the extracted samples of the different organs of the etiolated seedlings are illustrated in the following Table:

Tissue Extracted	Fresh weight used (obtained from 350 seedlings)	Weight used for glucosides or hydrolysis	Weight eq. for lettuce assay	Weight eq. for α-amylase assay
Radicle	725.0	362.5	72.5	1.45
Plumule	1250.0	625.0	125.0	2.50
Cotyledon	1000.0	500.0	100.0	2.00

It should be pointed out here that in the case of the acidic butanol-soluble gibberellins each TLC zone was eluted with 5 ml of 40% aqueous ethanol, and in the case of the free gibberellins released by acid hydrolysis of the gibberellin-glucosides, each TLC zone was eluted with 5 ml of wet ethyl acetate as described in the Materials and Methods Section. One ml and 0.02 ml of each TLC zone eluate were used for testing the activity of these zones with the lettuce hypocotyl bioassay and the α-amylase bioassay tests respectively (see the Materials and Methods Section for full details of bioassay tests). The results are represented in histogram form in Figures 46 (A-C), 47 (A-C) for the lettuce hypocotyl bioassay and the α-amylase bioassay tests respectively of the acidic butanol-soluble gibberellins, and in Figures 48 (A-C), 49 (A-C) for the lettuce hypocotyl bioassay and the α-amylase bioassay tests respectively of the free gibberellins released by acid hydrolysis.

The results of the lettuce hypocotyl bioassay of the acidic butanol-soluble gibberellins show the presence of substances with different activity and different polarity in the extracts of all the tissues under investigation, especially in the radicle tissue extract where activity was detected in most of the TLC zones (Fig. 46A), in the plumule extracts substances of low mobility constitute the majority of the acidic butanol-soluble substances of the tissue (Fig. 46B), while in the case of the cotyledons extracts the main acidic butanol-soluble gibberellins are fairly polar (Fig. 46C).

Figure 46

Lettuce hypocotyl bioassay of the eluates from TLC loaded with acidic butanol-soluble gibberellins of the different organs from etiolated broad bean seedlings. The plates were developed three times with solvent system (3) which consisted of:

Acetone - acetic acid

(97:3 v/v)

and eluted with 40% aqueous ethanol.

A. Radicle extract:

An amount of the eluate equivalent to 72.5 gm fresh weight of tissue was used in the assay.

B. Plumule extract:

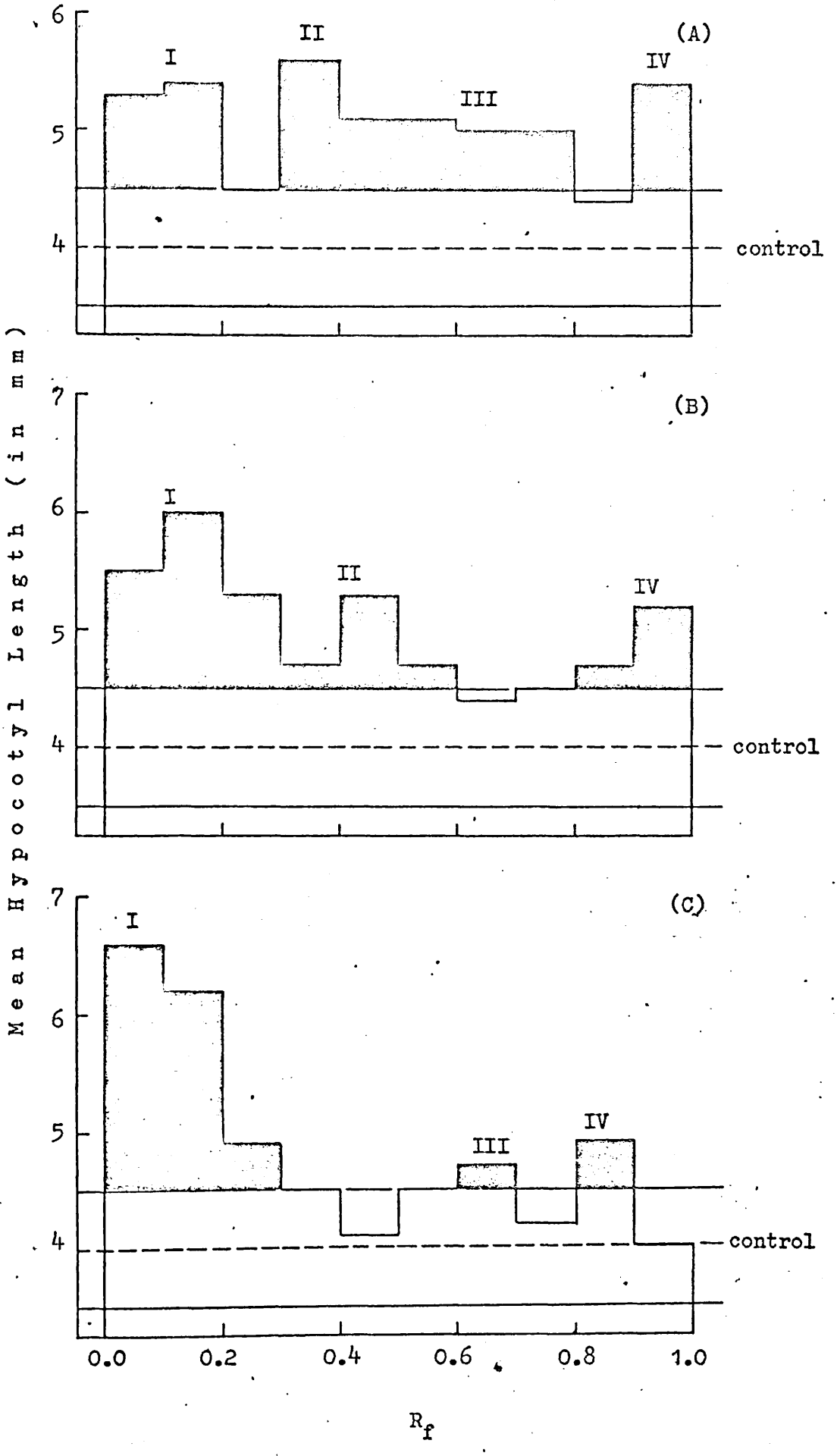
An amount of the eluate equivalent to 125.0 gm fresh weight of tissue was used in the assay.

C. Cotyledons extracts:

An amount of the eluate equivalent to 100.0 gm fresh weight of tissue was used in the assay.

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 46


(A)
I
II
III
IV
control
(B)
I
II
IV
control
(C)
I
III
IV
control
Mean Hypocotyl Length (in mm)
6
5
4
7
6
5
4
7
6
5
4
0.0
0.2
0.4
0.6
0.8
1.0
R_f

The results of the α-amylase bioassay test of the acidic butanol--soluble gibberellins show remarkably high activity of the least polar peak of the radicle tissue extracts (Fig. 47A), this peak is smaller in the case of the plumule tissue extracts (Fig. 47B). In the case of the cotyledon tissue extracts, the presence of only one slightly mobile component was detected with the α-amylase bioassay test (Fig. 47C).

Acid Hydrolysis of Gibberellin-Glucosides: This resulted in a remarkable increase in the activity detected by bioassay tests as acid hydrolysis release free gibberellins which are more active than their glucosides (Yokota et al., 1971a; Hiraga et al., 1974a; Sembdner et al., 1976). The results of the bioassays are represented in histogram form in Figures 48 (A-C), and 49 (A-C) for the lettuce hypocotyl bioassay and the α-amylase bioassay tests respectively.

With the lettuce hypocotyl assay, free gibberellins released by acid hydrolysis of the gibberellin-glucosides extracted from the radicle tissue were resolved into at least four peaks: peak marked (I), peak marked (II), peak marked (III) and peak marked (IV) (Fig. 48A); those released from hydrolysis of the plumule gibberellin-glucosides showed activity in at least three peaks: peak marked (II), peak marked (III) and peak marked (IV) (Fig. 48B); while acid hydrolysis of the gibberellin-glucosides of the cotyledonary tissue extracts resulted in the release of free gibberellins which showed activity in at least four peaks (peaks marked I- IV, Fig. 48C) after their separation by thin-layer chromatography.

All the peaks detected with the lettuce hypocotyl bioassay in the case of the hydrolysed gibberellin-glucosides of the radicle and the plumule tissue extracts were also detected with the α-amylase bioassay test (Fig. 49A,B), while only one peak was detected in the case of hydrolysed gibberellin-glucosides of the cotyledon's tissue extracts

Figure 47

α-Amylase bioassay of the eluates from TLC loaded with acidic butanol-soluble gibberellins of the different organs of etiolated broad bean seedlings. The plates were developed three times with solvent system (3) which consisted of:

Acetone - acetic acid

(97:3 v/v)

and eluted with 40% aqueous ethanol.

A. Radicle extract:

An amount of the eluate equivalent to 1.45 gm fresh weight of tissue was used in the assay.

B. Plumule extract:

An amount of the eluate equivalent to 2.50 gm fresh weight of tissue was used in the assay.

C. Cotyledons extracts:

An amount of the eluate equivalent to 2.00 gm fresh weight of tissue was used in the assay.

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 47

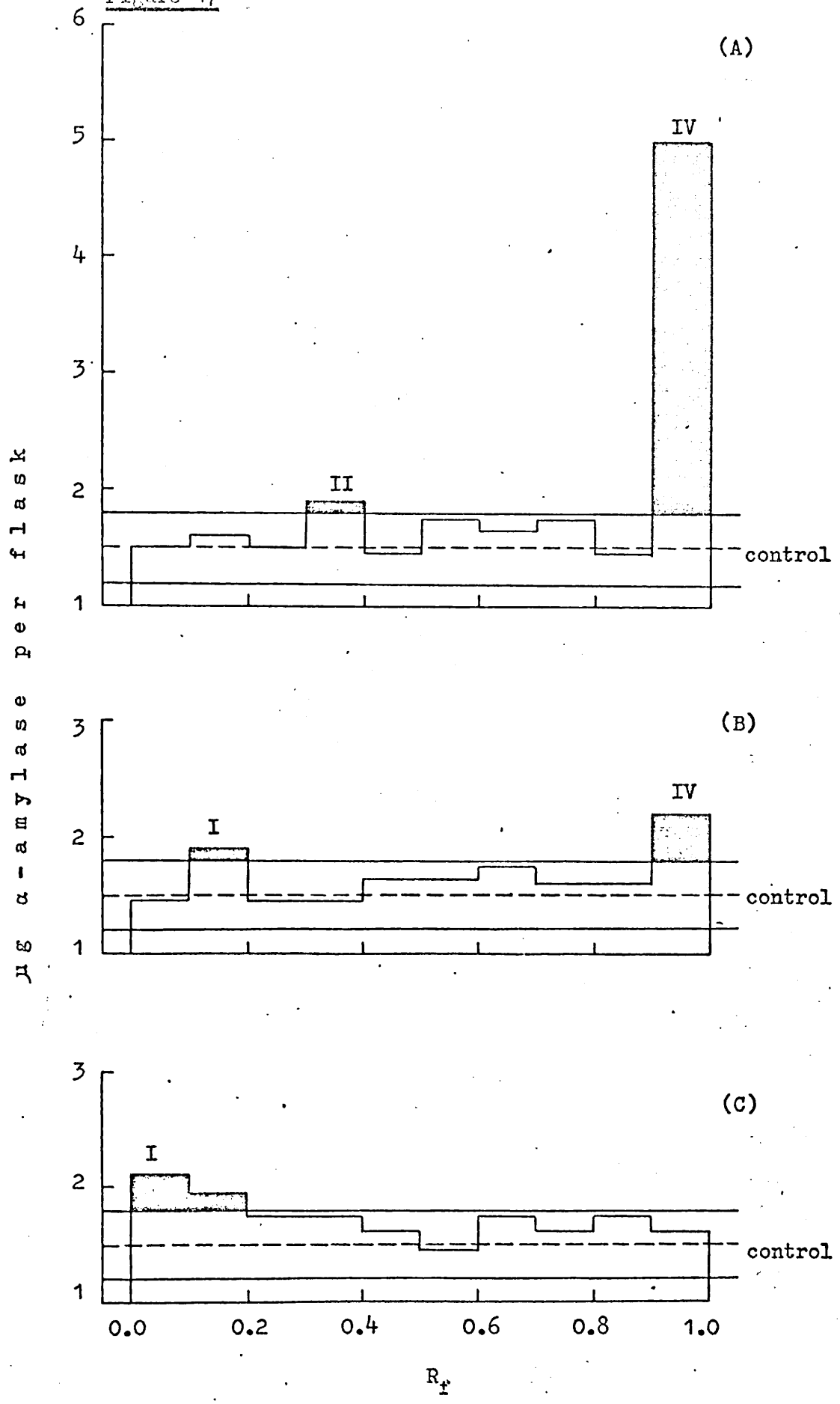

(A)
IV
II
control
(B)
IV
I
control
(C)
I
control
6
5
4
3
2
1
0.0
0.2
0.4
0.6
0.8
1.0
µg α-amylase per flask
R_f

Figure 48

Lettuce hypocotyl bioassay of the eluates from TLC loaded with the free gibberellins released by acid hydrolysis of the gibberellin-glucosides from the different organs of etiolated broad bean seedlings. The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

A. Radicle extract:

An amount of the eluate equivalent to 72.5 gm fresh weight of tissue was used in the assay.

B. Plumule extract:

An amount of the eluate equivalent to 125.0 gm fresh weight of tissue was used in the assay.

C. Cotyledons extracts:

An amount of the eluate equivalent to 100.0 gm fresh weight of tissue was used in the assay.

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 48

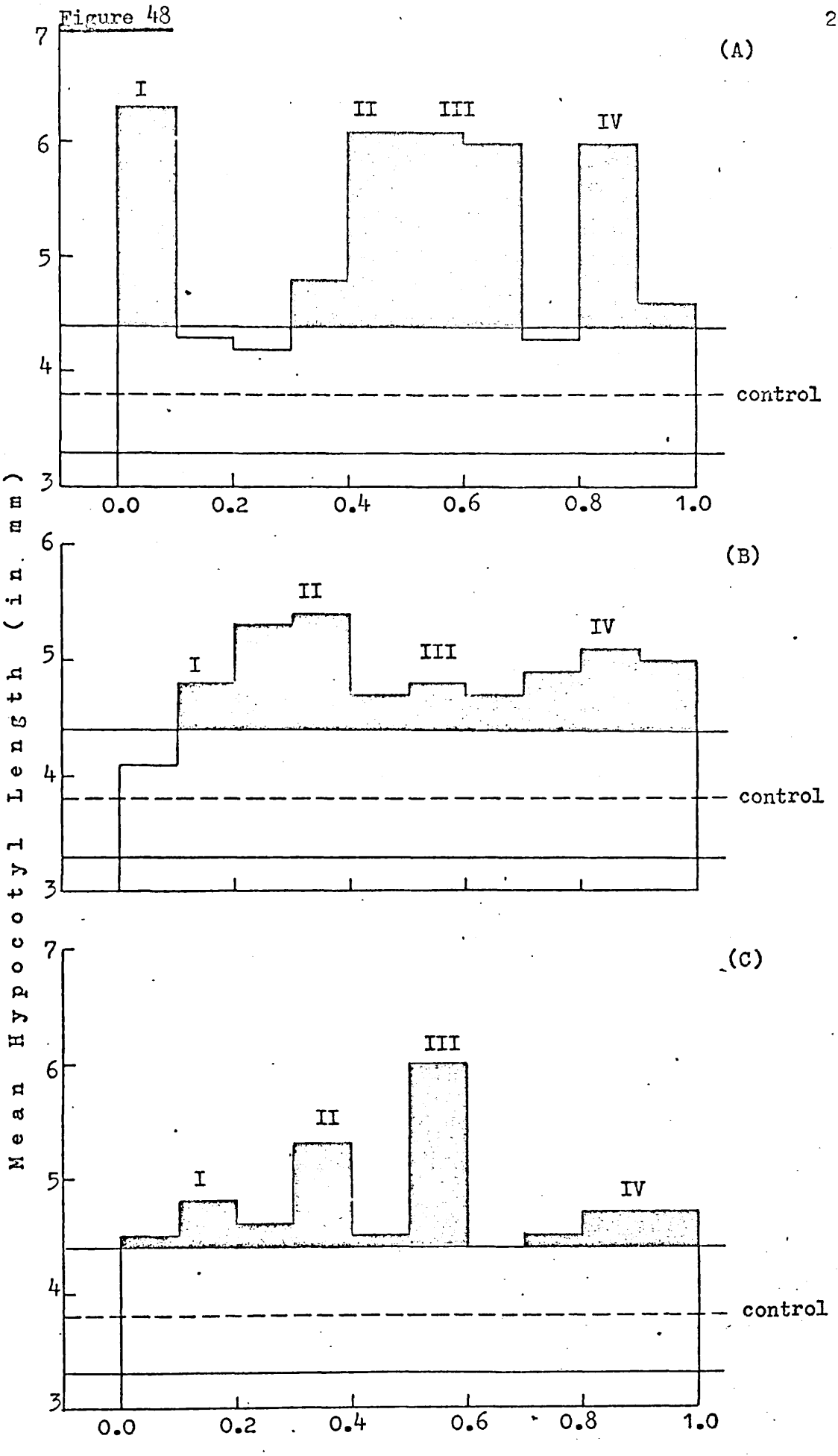

(A)
I
II
III
IV
control
(B)
I
II
III
IV
control
(C)
I
II
III
IV
control
Mean Hypocotyl Length (in. mm)
R_f
0.0
0.2
0.4
0.6
0.8
1.0
3
4
5
6
7

Figure 49

α-Amylase bioassay of the eluates from TLC loaded with the free gibberellins released by acid hydrolysis of the gibberellin-glucosides from the different organs of etiolated broad bean seedlings. The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

and eluted with wet ethyl acetate.

A. Radicle extract:

An amount of the eluate equivalent to 1.45 gm fresh weight of tissue was used in the assay.

B. Plumule extract:

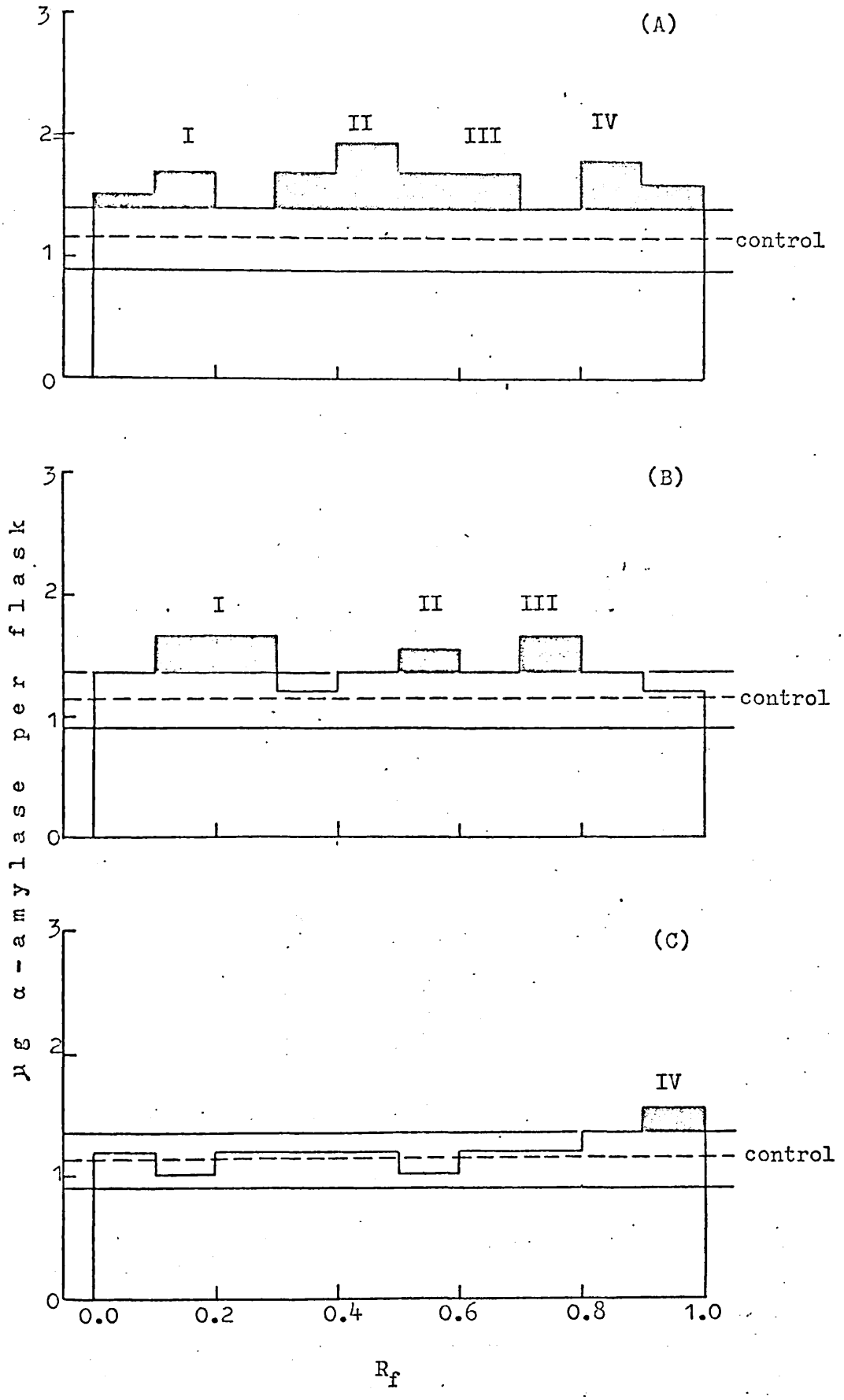
An amount of the eluate equivalent to 2.50 gm fresh weight of tissue was used in the assay.

C. Cotyledons extracts;

An amount of the eluate equivalent to 2.00 gm fresh weight of tissue was used in the assay.

Shaded parts of the histograms represent promotion significant at the 5% level of probability.

Figure 49


(A)
I
II
III
IV
control
(B)
I
II
III
control
(C)
IV
control
μg α-amylase per flask
0
1
2
3
0.0
0.2
0.4
0.6
0.8
1.0
R_f

(Fig. 49C). It was not possible to state whether each of the previous peaks was composed of biologically active substance or several such substances.

1.3. Water-Soluble or Other "Bound" Gibberellin-Like Substances

After fractionating the extracts of the different organs of the etiolated broad bean seedling into an acidic ethyl acetate fraction and an acidic n-butanol fraction as described earlier, a sufficient amount of 5N HCl was added to the remaining aqueous phase of each organ to make the final strength of the solution up to 0.4N (pH 0.08), and the water-soluble or other "bound" gibberellin-like substances were hydrolysed in a water bath at 60°C for 60 minutes (Barendse et al., 1968). The free gibberellins released were then extracted with redistilled de-acidified ethyl acetate at pH 2.5, purified by PVP slurries, PVP column chromatography, and examined by TLC/bioassay (see the Materials and Methods Section for full details).

According to the results obtained in the previous Chapter (Chapter IV) with PVP column chromatography of the free gibberellins released by acid hydrolysis of bound-gibberellins, the columns effluents in the following experiments were collected for gibberellin separation as follows:

a. In the case of the radicle extracts, the first 50 ml were collected together with the next 400 ml.

b. In the case of the plumule extracts, the first 50 ml were discarded, and the next 600 ml were collected together.

c. In the case of the cotyledon extracts the first 50 ml were collected together with the next 200 ml.

Gibberellins were recovered from the columns effluents as described in the Materials and Methods Section, and separated by TLC using solvent

system (1). The plates were divided into 10 equal zones, each of which was eluted with 6 ml of wet ethyl acetate at 2°C overnight (Aung & DeHertogh, 1968); a known volume from each TLC zone was used for testing gibberellin-like activity of the extracts with the lettuce hypocotyl bioassay, and eluates of the zones which showed considerable biological activity were analysed by GLC after their derivatisation. The full details concerning the tissue extracted, as well as extracts are represented in the following Table :

Tissue Extracted	No. seedlings used	Fresh Weight of tissue (in gms)	Vol. of TLC eluate used for bioassay	Fresh Weight equivalent (in gms)
Radicle	475	925	1.0 ml	154.2
Plumule	415	1470	0.8 ml	176.0
Cotyledon	350	1000	0.8 ml	133.3

The results of the bioassay are represented in histogram form in Figure 50 A,B, and C, for the radicle, the plumule, and the cotyledons respectively.

As a general observations, separation of acidic butanol-soluble gibberellins from extracts of the tissues under investigation prior to the hydrolysis of water-soluble or bound-gibberellins in this experiment resulted in a decrease in the number as well as the size of the peaks detected by bioassay test in comparison with those obtained in the previous Chapter (Chapter IV), in which tissue extracts were fractionated into acidic ethyl acetate-soluble gibberellins (free gibberellins), and water-soluble (or bound) gibberellins only. The decrease was observed particularly in the case of the cotyledon extracts, followed by the radicle extracts (Fig. 50A,C). This indicates that most of the water-soluble gibberellins of the cotyledon and the radicle

tissue extracts are conjugated ones; and as they were separated from the extracts with n-butanol at low pH, they appear to be glucosides. In the case of the plumule extracts on the other hand, the number of peaks detected by bioassay test of the free gibberellins released by acid hydrolysis of bound-gibberellins (after separation of acidic butanol-soluble gibberellins from the extract) was comparatively less affected (Fig. 50B). This indicates that the water-soluble gibberellins of the plumule tissue extracts are either conjugated compounds which could not be separated from the aqueous phase with n-butanol at low pH (i.e. they are glucosyl esters; Takahashi, 1974) or they are bound to other unidentified molecules of the tissue constituents.

The results of the bioassay illustrated in Figure 50A, B, and C show that the free gibberellins released by acid hydrolysis of bound--gibberellins of the different organs from the etiolated seedling when purified by PVP slurries, PVP column chromatography followed by TLC of the column effluents and bioassay of the TLC eluate were separated into at least two peaks in the case of the radicle and the cotyledons extracts, and four peaks in the case of the plumule extracts, one of which is a just significant peak (peak marked IV, Fig. 50B). One of the peaks was detected in the extracts of each organ (peak marked II, Fig. 50 A-C); another peak was found to occur in the plumule as well as the cotyledon extracts (peak marked I, Fig. 50B,C). A small peak was detected in the radicle extracts (peak marked III, Fig. 50A), this peak represents the major components in the case of the plumule tissue extracts (Fig. 50B). A last peak was detected in the cotyledons as well as the plumule extracts (peak marked I, Fig. 50B,C).

The constituents of the different peaks in the case of free gibberellins released by acid hydrolysis of gibberellin-glucosides, and/or bound-gibberellins could be the same as those peaks referred to in the case of acidic ethyl acetate-soluble gibberellins according to the R_f data of the gibberellins published by Cavell et al. (1967).

Figure 50

Lettuce hypocotyl bioassay of eluates from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins from the different organs of etiolated broad bean seedling. The plates were developed with solvent system (1) which consisted of:

Ethyl acetate - chloroform - acetic acid

(15:5:1 v/v)

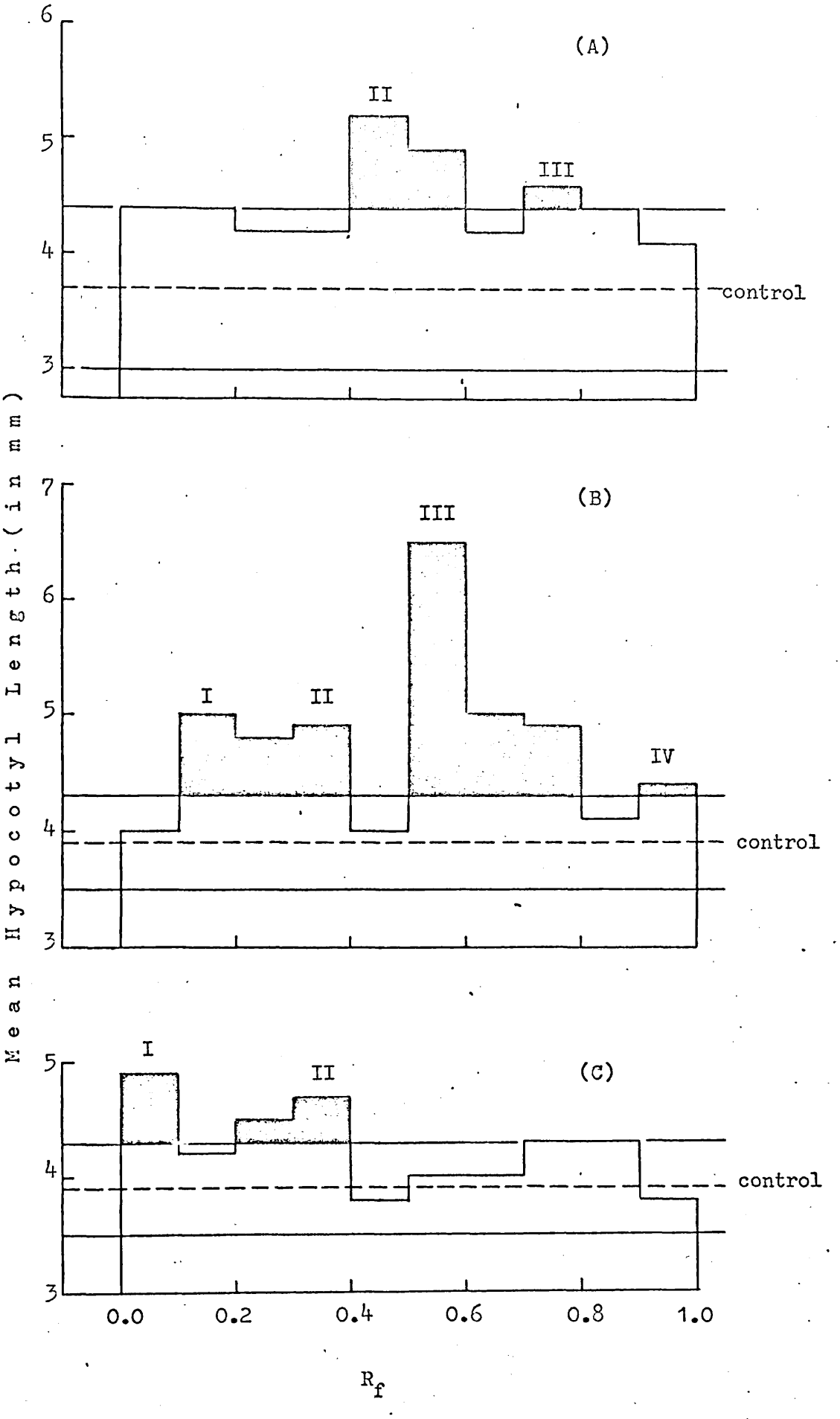
and eluted with wet ethyl acetate.

A. Radicle extract: a volume of the eluate equivalent to 154.2 gm fresh weight was used in the assay

B. Plumule extract: a volume of the eluate equivalent to 176.0 gm fresh weight of tissue was used in the assay

C. Cotyledons extract: a volume of the eluate equivalent to 133.3 gm fresh weight of tissue was used in the assay

Shaded parts of the histogram represent promotion significant at the 5% level of probability.


(A)
II
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(B)
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IV
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(C)
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control
Mean Hypocotyl Length (in mm)
6
5
4
3
7
6
5
4
3
5
4
3
0.0
0.2
0.4
0.6
0.8
1.0
R_f

Distribution of Endogenous Gibberellins in Etiolated Broad Bean (Vicia Faba) Seedling

2. Gibberellin Content as Determined by GLC Analysis

Eluates of the zones from the TLC loaded with the free gibberellins (acidic ethyl acetate-soluble gibberellins), the free gibberellins released by acid hydrolysis of gibberellin-glucosides (acidic butanol--soluble gibberellins) and/or the free gibberellins released by acid hydrolysis of other bound-gibberellins (water-soluble gibberellins), and developed with solvent system (1), that showed considerable activity in the bioassay tests were derivatised for GLC analysis. The development of the TMS ethers proved to be difficult and unsuccessful on the 2% QF-1 column for which reason analysis of the methyl esters only was achieved during this season. Part of the difficulties in the analysis of the TMS ethers by the GLC was the chloride which precipitate on the addition of the silylisating reagents; this precipitate was difficult to sediment in the relatively small volume of the reaction mixture and caused blocking of the syring's needle many times. Preparing the TMS ethers, as described by Dr. MacMillan (personal communication), by treating the residue of the methyl ester with a mixture of trimethyl silyl chloride and hexamethyl disilizan (in the ratio of 1:1 v/v) in GLC grade dry pyridine (8 parts) solved the problem of the precipitate, but when the samples were injected, interfering substances caused a very high background and prevented clear peak detection. This is probably due to the selective properties of the 2% QF-1 column, and perhaps satisfactory separation of the TMS ethers might occur with other sorts of column, e.g. SE-33 (silicon gum rubber E 303) that could be able to eliminate the interfering substances.

An amount of the TLC eluates (0.5 ml in the case of the free gibberellins, 1.5 ml in the case of the free gibberellins released by acid hydrolysis of the gibberellin -glucosides and 1.0 ml in the case of the free gibberellins released by acid hydrolysis of the other bound--gibberellins) was used for the preparation of the methyl ester as described in the Materials and Methods Section. The methylating solvent was evaporated to dryness, by blowing on a stream of oxygen-free nitrogen at the top of the reaction vial which was held on a water bath at 40°C, and the residue was dissolved in an amount of redistilled ethyl acetate ranging from 20-50 µl of which a volume ranging from 2-5 µl was injected for GLC analysis. Each sample was injected at least twice to ensure the reproducibility of the recorded peaks. The retention time of the GLC peaks was then measured and compared to the retention times calculated under the conditions involved for the column used as shown in Table 4 for the identification of the gibberellins.

It should be mentioned here that attempts at identification of the gibberellins by GLC could not go further than the first twenty-four gibberellins (GA_1-GA_{24}) for which retention times on the 2% QF-1 column and under conditions similar to those involved here are available in the literature (MacMillan & Pryce, 1968a) and this permitted calculation of retention times of these gibberellins for the column used according to the retention time obtained with the standard GA_{14} as recommended by Cavell et al. (1967).

2.1. Acidic Ethyl Acetate-Soluble Gibberellins (Free Gibberellins)

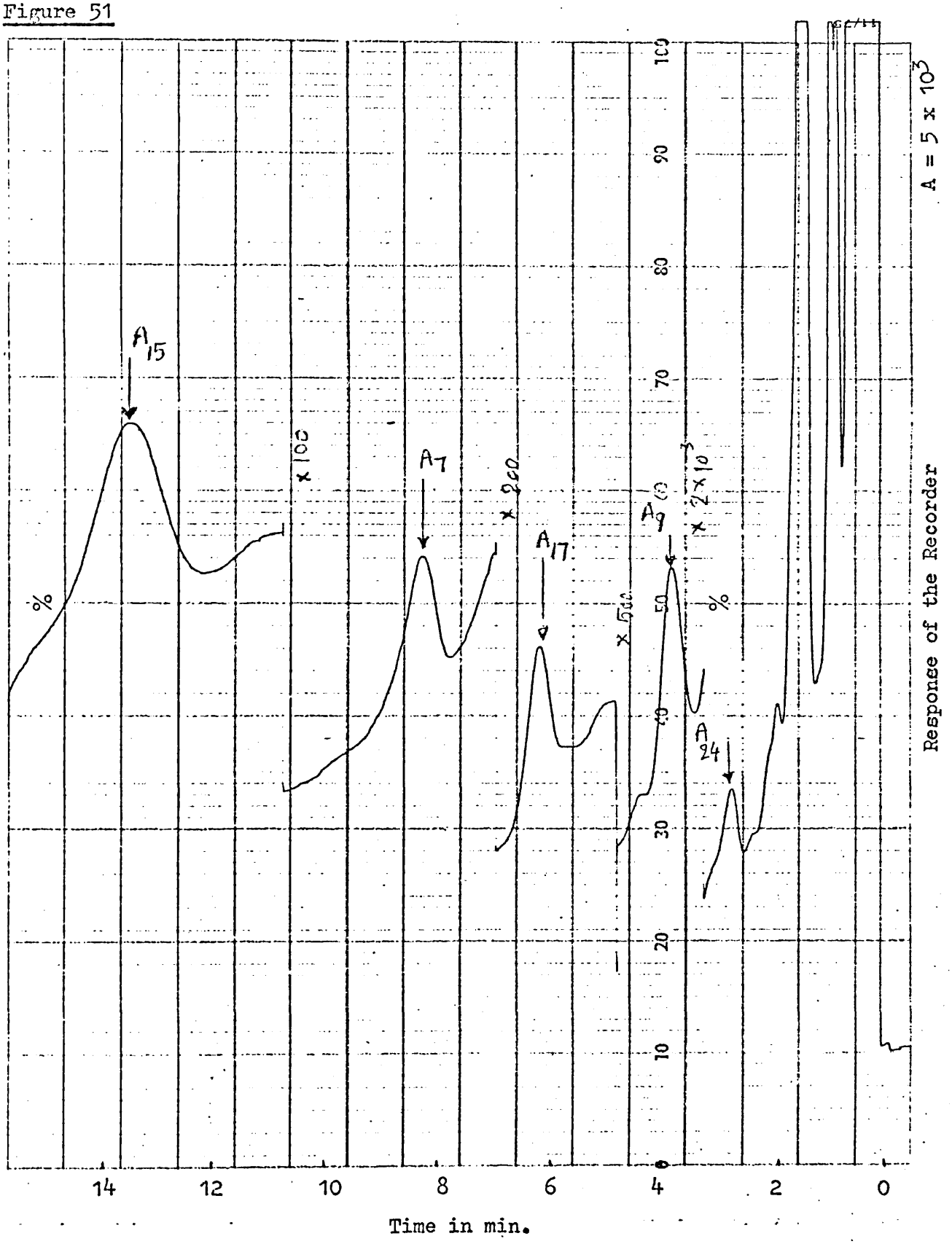
Using retention times relevant to the standards as shown in Table 4, the presence of peaks corresponding to the methyl derivatives of gibberellins A_7, A_9, A_{15}, A_{17}, and A_{24} (Fig. 51) were detected in the eluate of the TLC zone at R_f 0.6-0.7 (peak marked III in Fig. 41A) of

Figure 51

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.6-0.7 from TLC loaded with the free gibberellins of the radicle tissue extracts and developed with solvent system (1).

Half-ml of the TLC eluate was derivatised and the ester dissolved in 50 µl of redistilled ethyl acetate of which 5 µl were injected into the GLC column.

Figure 51


A15
A7
A17
A9
A24
%
x 100
x 200
x 500
x 2x10³
%
100
90
80
70
60
50
40
30
20
10
0
14
12
10
8
6
4
2
0
Time in min.
A = 5 x 10³
Response of the Recorder

the radicle tissue extracts. The methyl derivative of the main gibberellin present in the eluate of the zone at R_f 0.4-0.5 (peak marked II, Fig. 41A) has a retention time corresponding to the methyl ester of gibberellin A_{13} (Fig. 52).

In the case of the free gibberellins of the plumule (Fig. 42B), GLC peaks with retention time corresponding to the methyl derivatives of gibberellins A_{12}, A_{14}, A_{20}, and A_{24} (Fig. 53) were detected in the eluate of the TLC zone at R_f 0.6-0.7 (peak marked III); the eluate of the zone at R_f 0.3-0.4 of Figure 42 (B) when methylated and analysed by GLC, peaks with retention time corresponding to the methyl derivative of gibberellin A_{13} was detected (Fig. 54) together with another gibberellin, the methyl ester of which has a retention time of 3.7 minutes. The eluate of the zone at R_f 0.4-0.5 was found to contain gibberellin A_{13} together with iso-gibberellin A_{13} (Fig. 55) as determined by the retention time of the peaks obtained with the methyl derivatives of this zone. On the other hand, the eluate of the zone at R_f 0.1-0.2 (peak marked I, Fig. 42B) was found to contain at least three gibberellins (Fig. 56) when its methyl ester was analysed by GLC but none of these gibberellins appear to be any of the first twenty-four gibberellins, and their methyl esters have the following retention times: 12.0, 10.3, and 5.0 minutes.

In the case of the free gibberellins of the cotyledons (Fig. 44A), peaks with retention time corresponding to the methyl ester of gibberellins A_{12} and A_{15} (Fig. 57) were detected in the eluate of the zone at R_f 0.6-0.7 (peak marked III, Fig. 44A); the methyl ester of the TLC eluate at R_f 0.4-0.5 (peak marked II) gave peaks with retention times corresponding to the methyl esters of gibberellins A_3, A_5, A_6, A_{13}, and iso-A_{13} (Fig. 58), while the zone at R_f 0.5-0.6 gave three peaks which have retention times of 13.0, 14.6 and 18.2 minutes (Fig. 59).

Figure 52

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.4-0.5 from TLC loaded with the free gibberellins of the radicle tissue extracts and developed with solvent system (1).

Half-ml of the TLC eluate was derivatised and the ester dissolved in 40 µl of redistilled ethyl acetate of which 5 µl were injected into the GLC column.

Figure 52

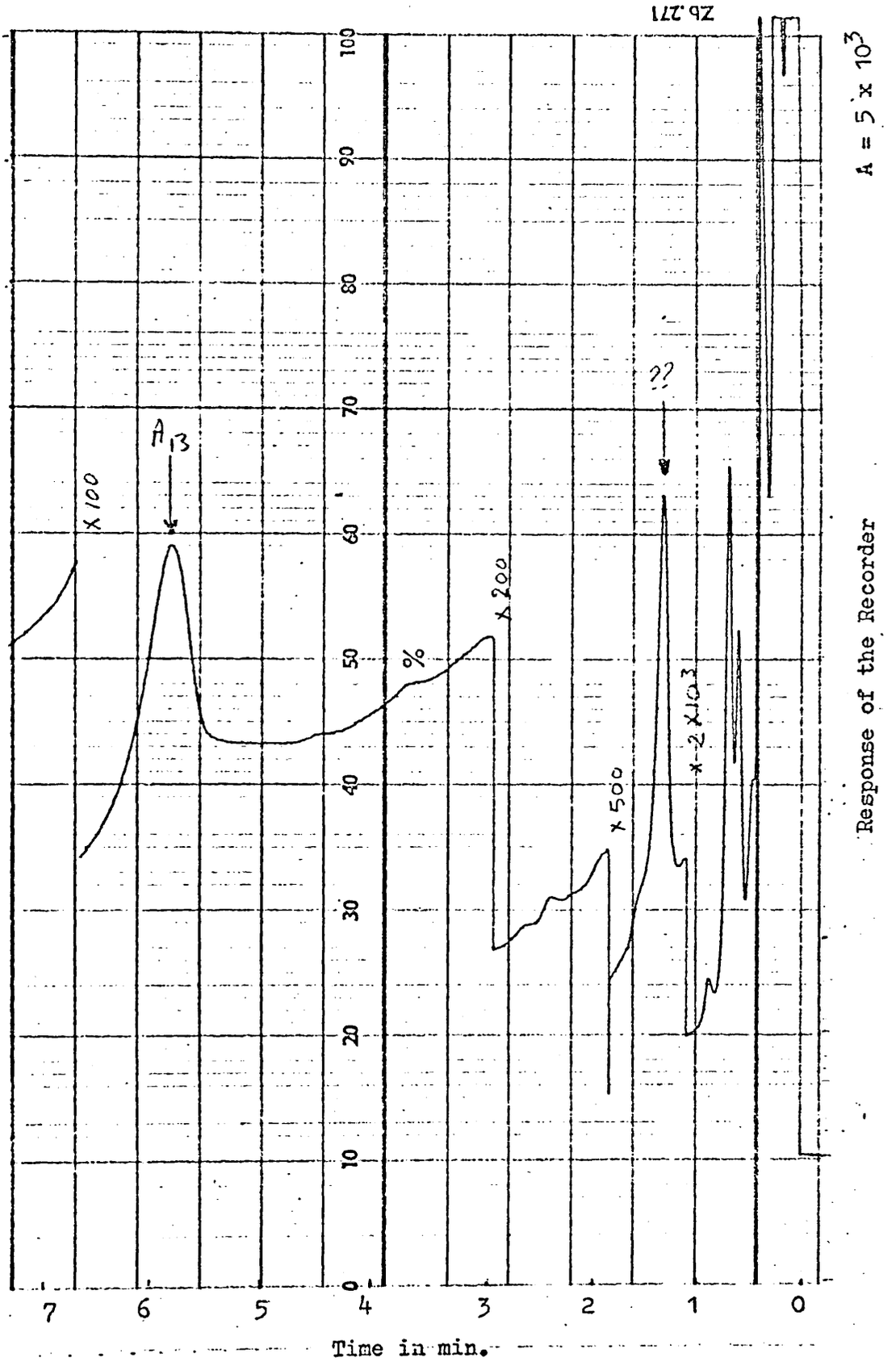

A = 5 x 10³
Response of the Recorder
Time in min.
A13
x 100
x 200
x 500
x 2 x10³
??
%
7
6
5
4
3
2
1
0
10
20
30
40
50
60
70
80
90
100

Figure 53

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.6-0.7 from TLC loaded with the free gibberellins of the plumule tissue extracts and developed with solvent system (1).

Half-ml of the TLC eluate was derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 5 µl were injected into the GLC column.

Figure 53

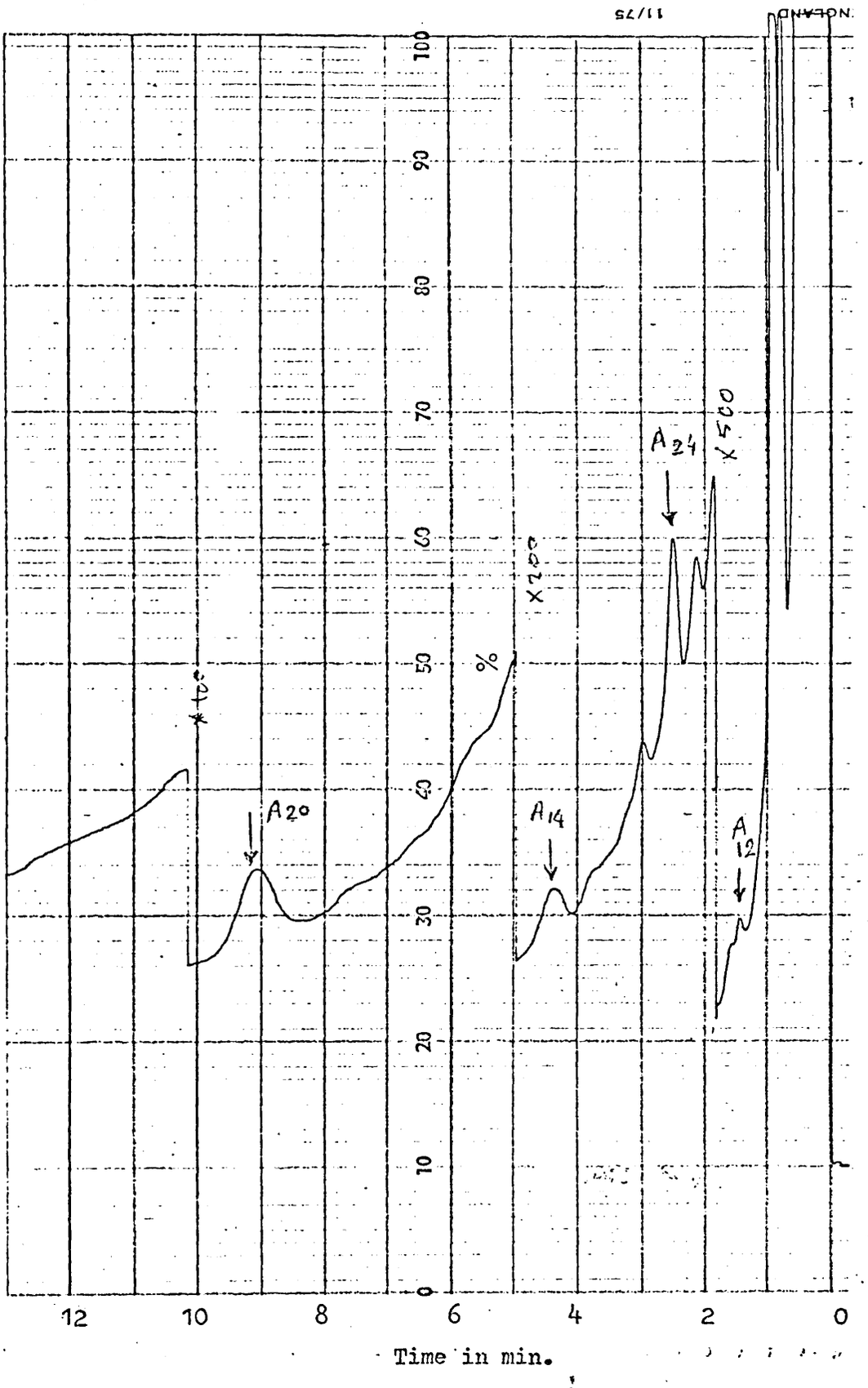

A = 2 x 10^-3
Response of the Recorder
Time in min.
12
10
8
6
4
2
0
A20
A14
A12
A24
x200
x500
%

Figure 54

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.3-0.4 from TLC loaded with the free gibberellins of the plumule tissue extracts and developed with solvent system (1).

Half-ml of the TLC eluate was derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 3 µl were injected into the GLC column.

Figure 54

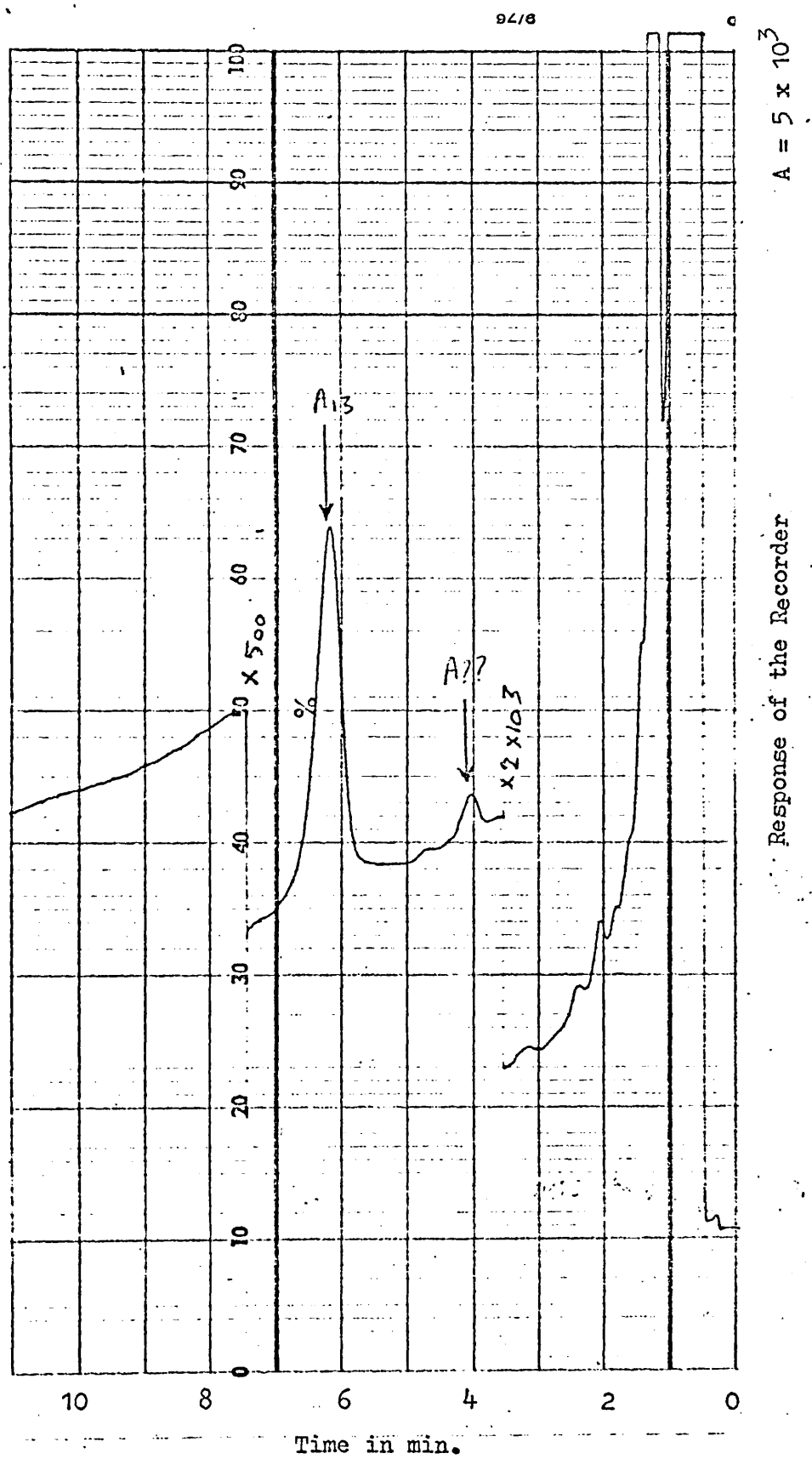

A = 5 x 10³
Response of the Recorder
A13
A??
x 500
x 2 x10 3
%
100
90
80
70
60
50
40
30
20
10
0
10
8
6
4
2
0
Time in min.

Figure 55

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.4-0.5 from TLC loaded with free gibberellins in the plumule tissue extract and developed with solvent system (1).

Half-ml of the TLC eluate was derivatised and ester dissolved in 30 µl of redistilled ethyl acetate of which 3 µl were injected into the GLC column.

Figure 55

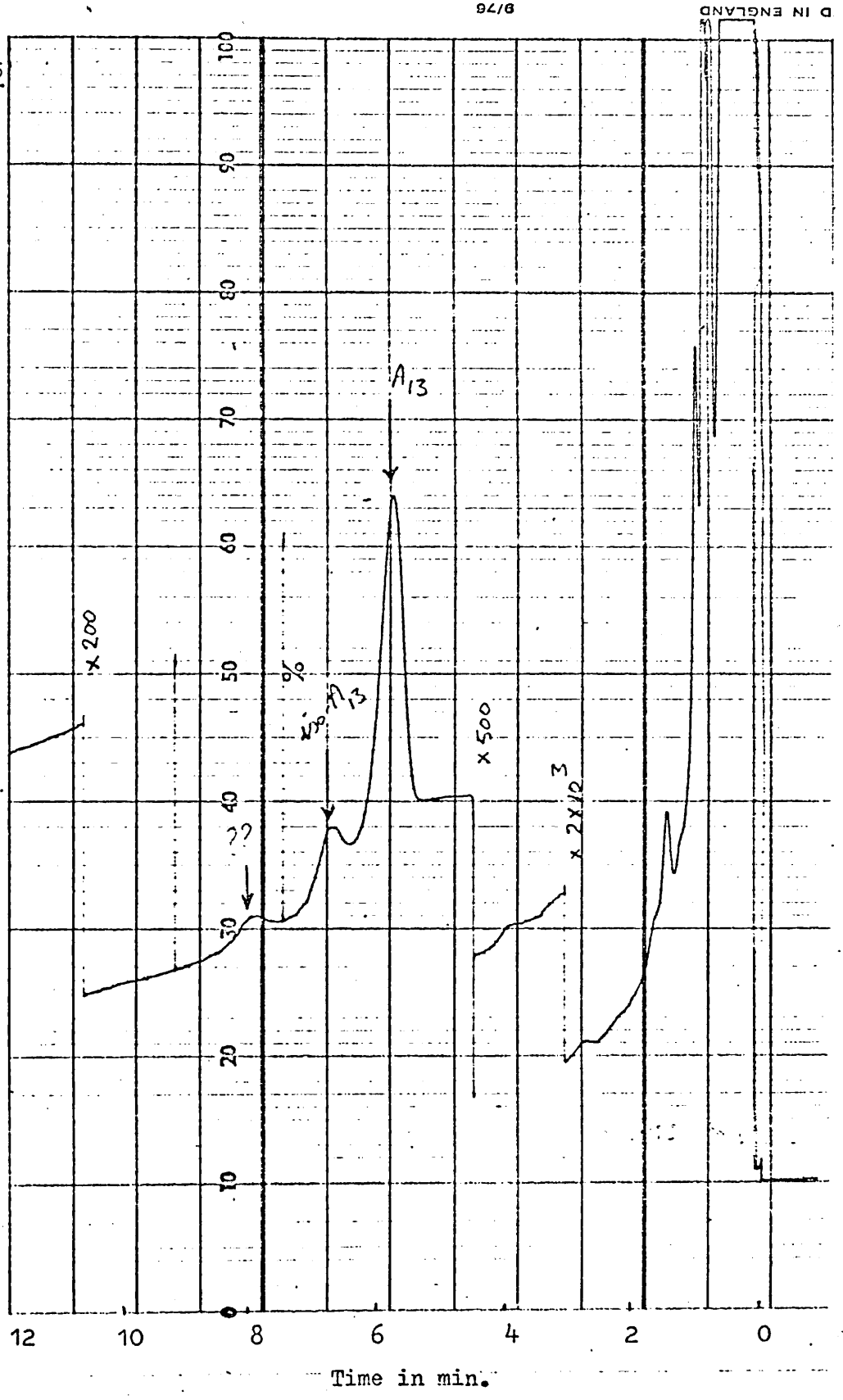

9/76
D IN ENGLAND
A = 5 x 10^3
Response of the Recorder
100
90
80
70
60
50
40
30
20
10
0
A13
x 200
x 500
x 2 x 1/2
12
10
8
6
4
2
0
Time in min.

Figure 56

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.1-0.2 from TLC loaded with the free gibberellins in the plumule tissue extracts and developed with solvent system (1).

Half-ml of the TLC eluate was derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 5 µl were injected into the GLC column.

Figure 56

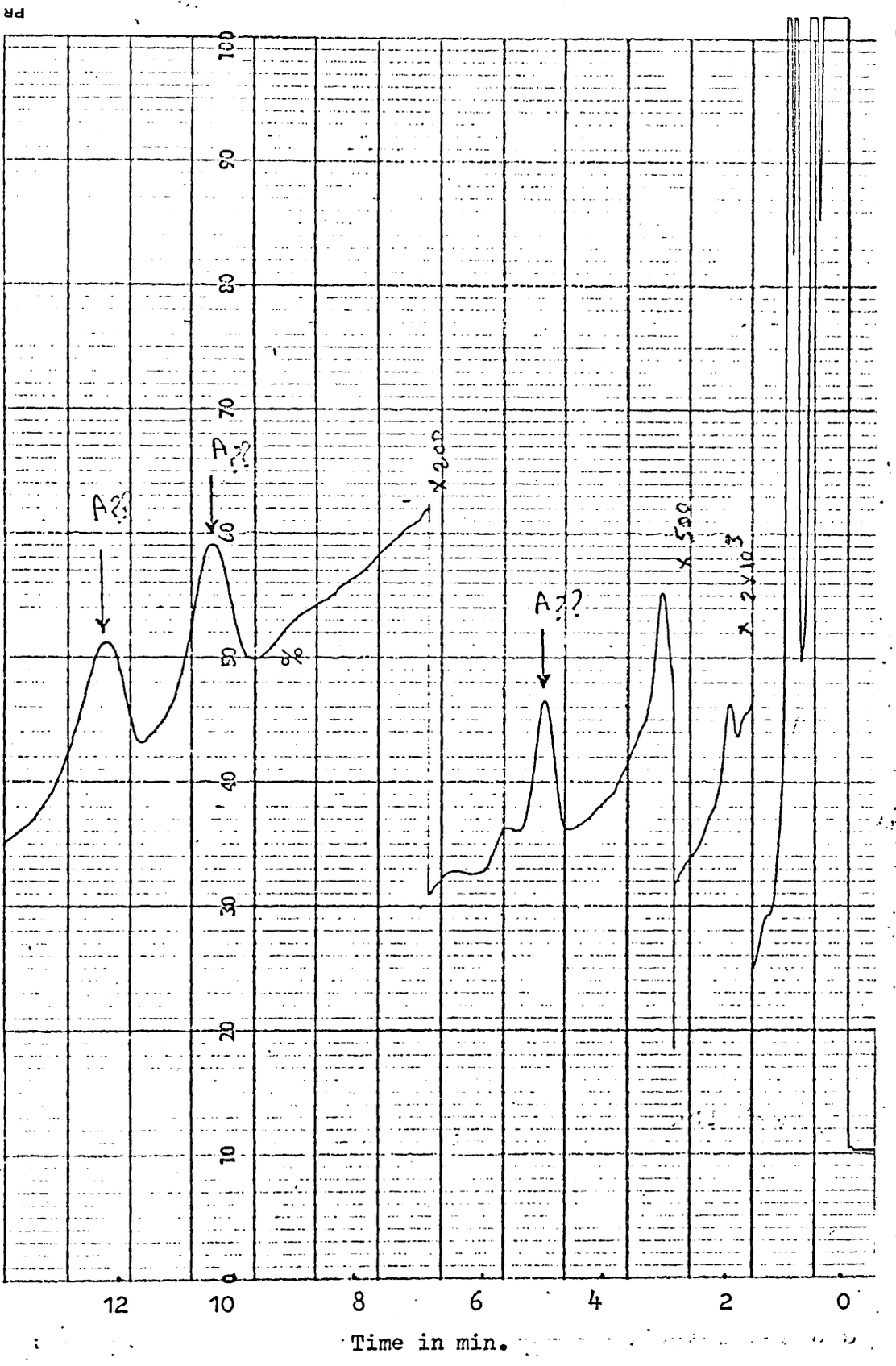

PR
A = 5 x 10^3
Response of the Recorder
100
90
80
70
60
50
40
30
20
10
0
%
A??
A??
A??
x 200
x 500
x 2x10^3
12
10
8
6
4
2
0
Time in min.

Figure 57

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.6-0.7 from TLC loaded with the free gibberellins in the cotyledons tissue extracts and developed with solvent system (1).

Half-ml of the TLC eluate was derivatised and the ester dissolved in 40 µl of redistilled ethyl acetate of which 2 µl were injected into the GLC column.

Figure 57

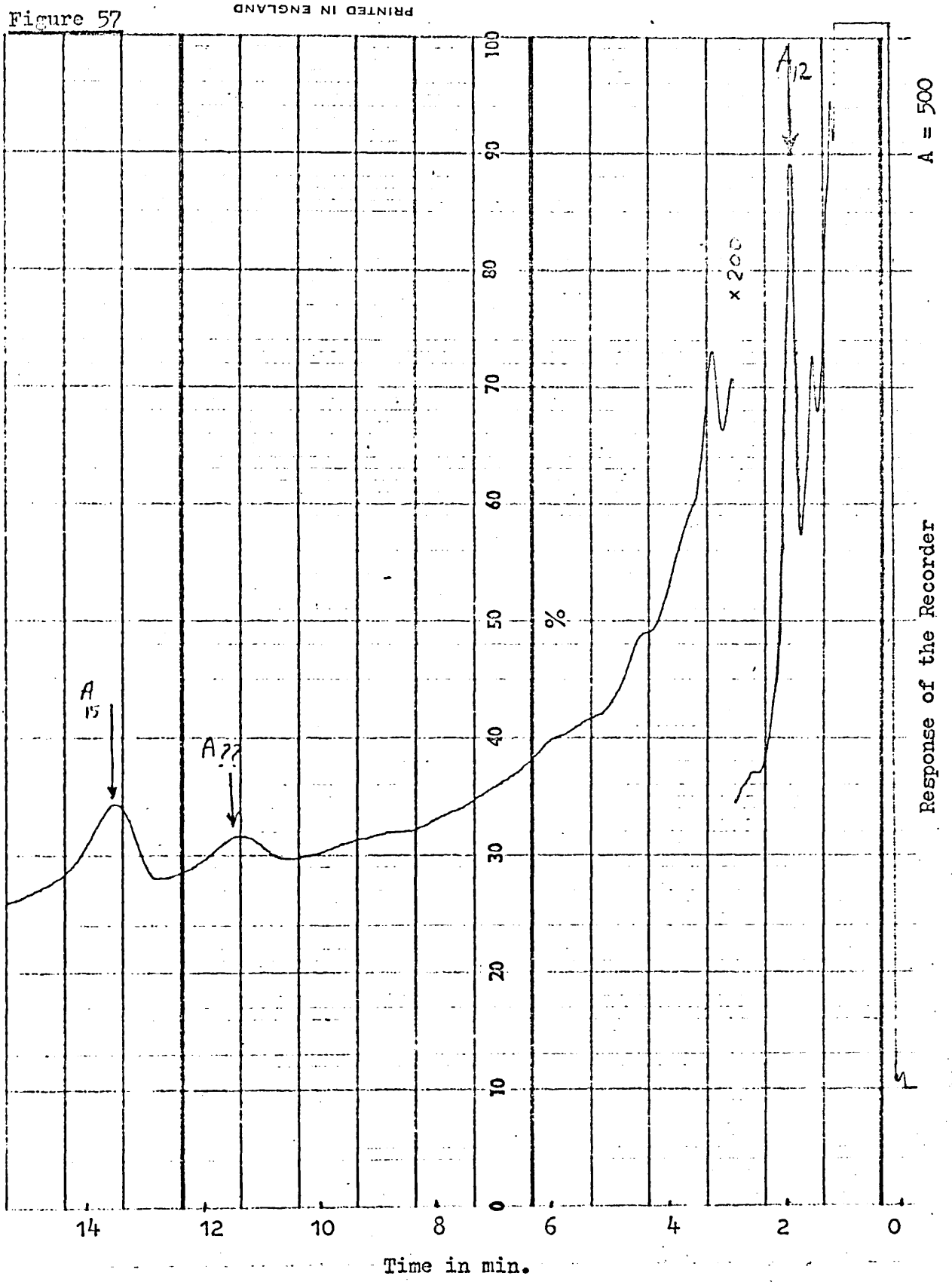


Figure 58

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.4-0.5 from TLC loaded with the free gibberellins in the cotyledons tissue extract and developed with solvent system (1).

Half-ml of the eluate was derivatised and the ester dissolved in 40 ul of redistilled ethyl acetate of which 2 ul were injected into the GLC column.

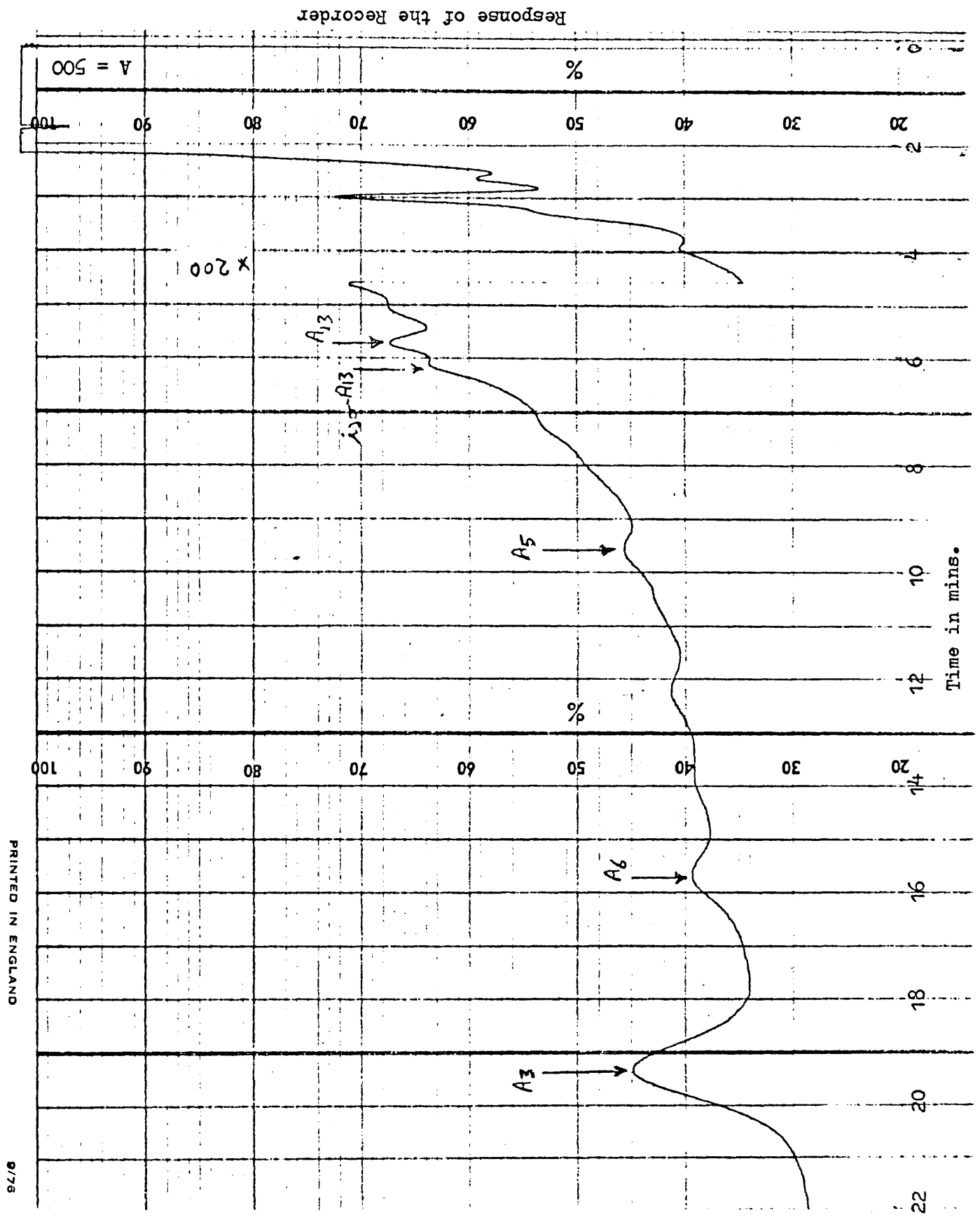


Figure 58

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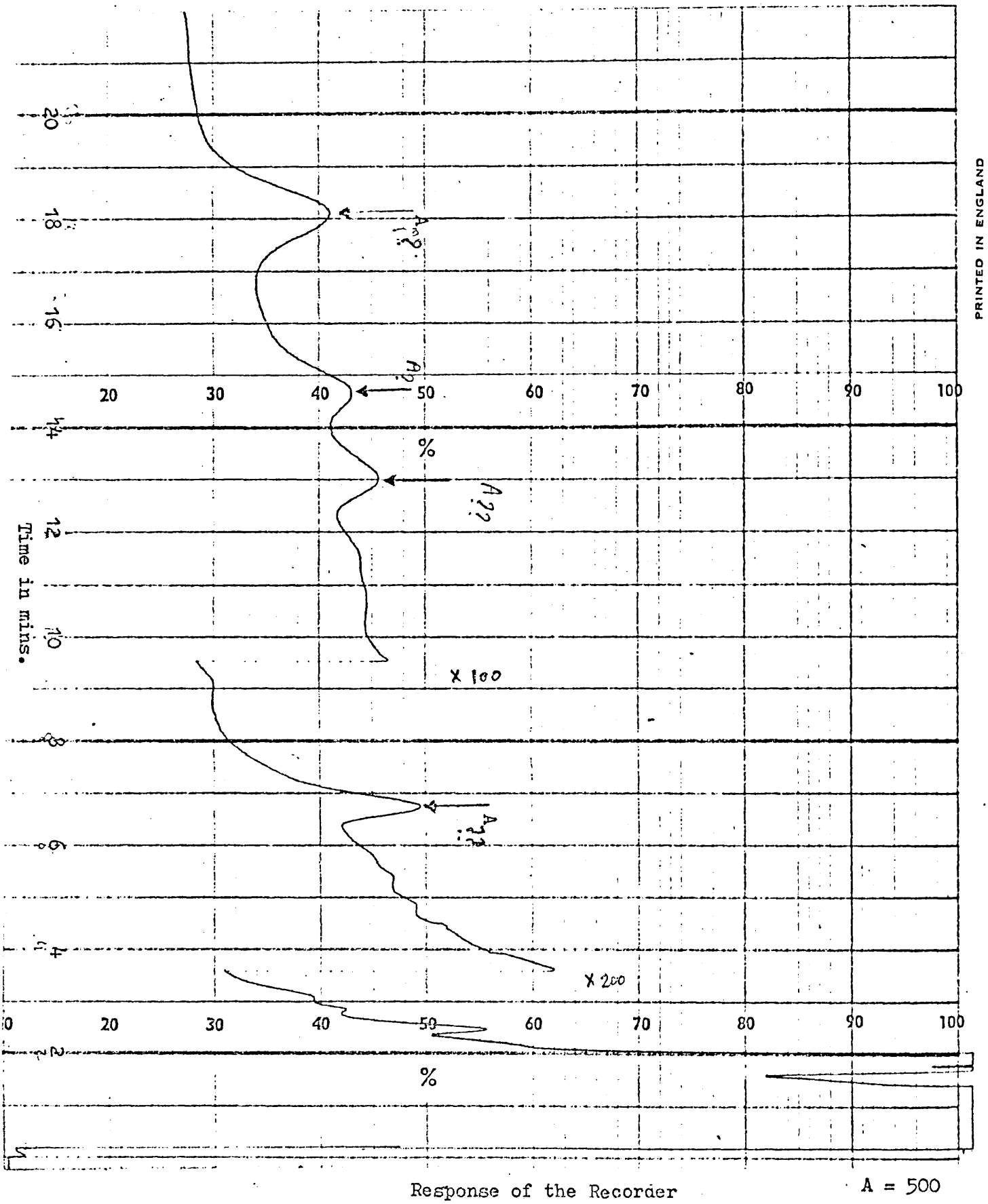
Figure 59

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.5-0.6 from TLC loaded with the free gibberellins in the cotyledons tissue extract and developed with solvent system (1).

Half-ml of the eluate was derivatised and the ester dissolved in 40 ul of redistilled ethyl acetate of which 2 ul were injected into the GLC column.

Figure 59.



Response of the Recorder

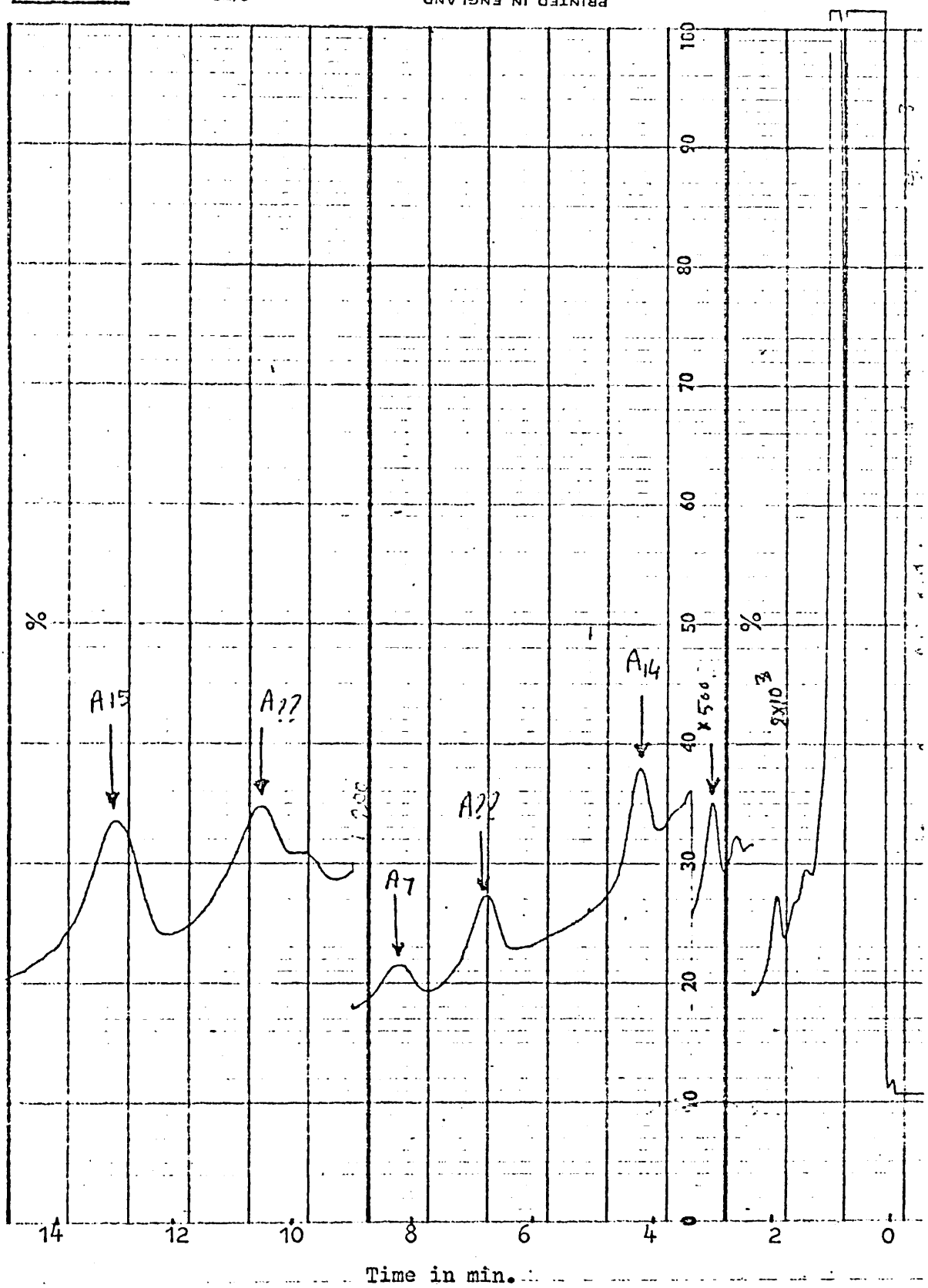
A = 500

Figure 60

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.8-0.9 from TLC loaded with the free gibberellins in the cotyledons tissue extracts and developed with solvent system (1).

Half-ml of the eluates was derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 3 µl were injected into the GLC column.

Figure 60


A15
A??
A7
A??
A14
x 500
2x10^3
%
%
A = 5 x 10^3
Response of the Recorder
Time in min.
14
12
10
8
6
4
2
0

From the peak marked (IV) (R_f 0.8-0.9) on the other hand, GLC peaks with retention times corresponding to the methyl ester of gibberellin A_7, A_{14}, and A_{15} were detected (Fig. 60) together with three other peaks having retention times of 3.1, 7.0 and 10.6 minutes.

2.2. The Free Gibberellins Released by Acid Hydrolysis of the Gibberellins-Glucosides

Derivatives of the eluates from the zones of the TLC loaded with the free gibberellins released by acid hydrolysis of the gibberellin--glucosides in extracts from the different organs of the etiolated broad bean seedlings that showed considerable activity in the bioassay tests used (Figs. 48 A-C, 49 A-C) were injected into the GLC and retention times relevant to the methyl esters of the standard gibberellins as shown in Table 4 were used. The following results were obtained:

In the case of the hydrolysed gibberellins-glucosides of the radicle (Figs. 48A, 49A), the zone at R_f 0.6-0.7 (peak marked III) gave peaks with the GLC having retention times corresponding to the methyl derivatives of gibberellins A_9, A_{12}, A_{14}, A_{20}, and A_{24} (Fig. 61), while the zone at R_f 0.5-0.6 gave peaks with retention times corresponding to the methyl derivatives of gibberellins A_{13} and A_{14} (Fig. 62).

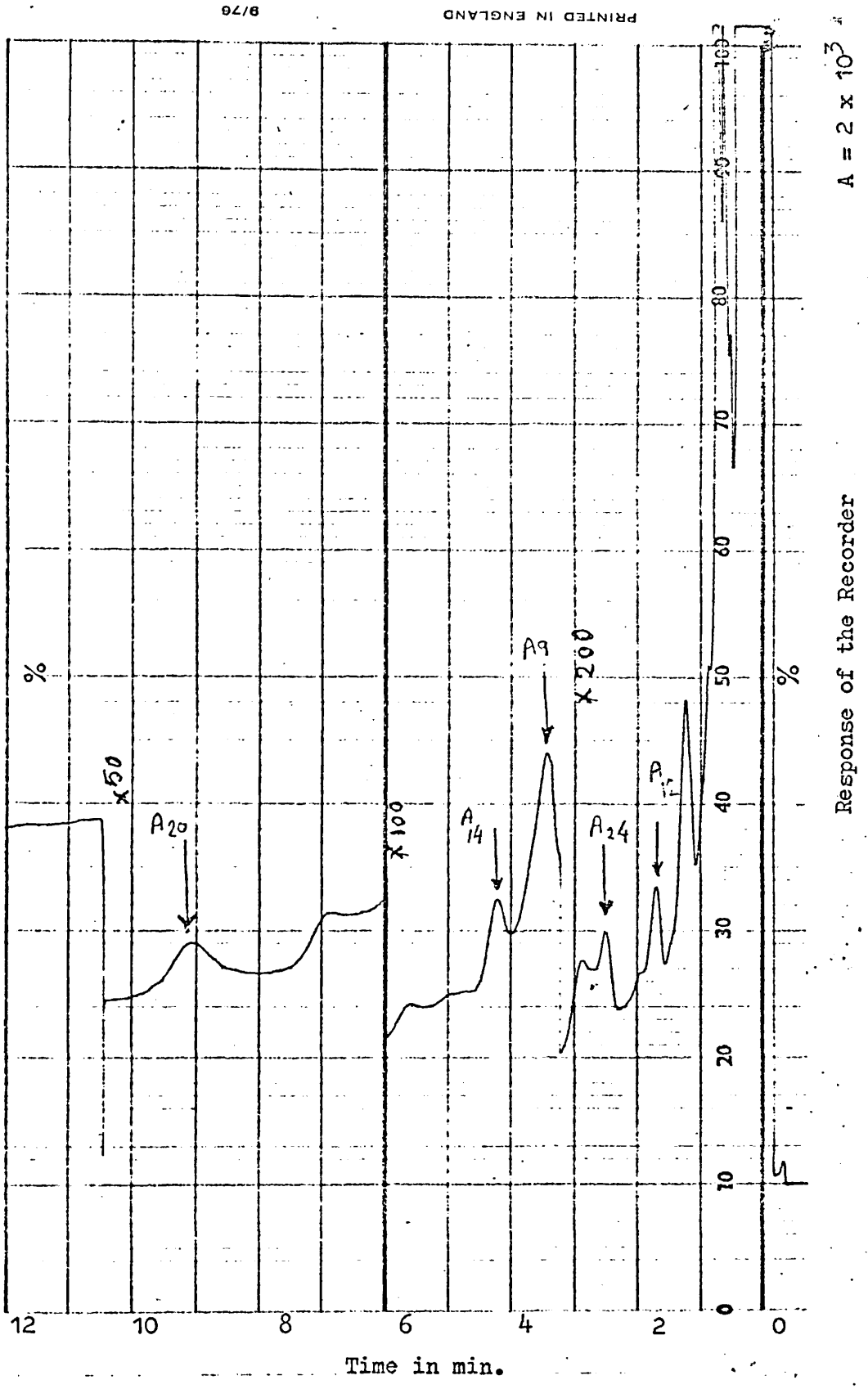
In the case of the hydrolysed gibberellins-glucosides of the plumule (Figs. 48B, 49B), the zone at R_f 0.1-0.2 (peak marked I) gave peak with the GLC the retention time of which corresponded to the methyl ester of gibberellin A_8. The zone at R_f 0.7-0.8 (peak marked IV) gave peaks with retention times corresponding to the methyl derivatives of gibberellins A_{14} and A_{24}, together with three peaks with the following retention times: 27.9, 34.1 and 41.4 minutes. The zone at R_f 0.8-0.9 of the same peak gave three peaks with the GLC (Fig. 63), one of which

Figure 61

GLC analysis of the gibberellins in eluates of the zone at R_f 0.6-0.7 from TLC loaded with the free gibberellins released by acid hydrolysis of the gibberellin-glucosides in the radicle tissue extracts and developed with solvent system (1).

1.5 ml of the eluate were derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 2 µl were injected into the GLC column.

Figure 61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9/76
A = 2 x 10^-3
Response of the Recorder
%
%
x50
x100
x200
A20
A14
A9
A24
A
0
10
20
30
40
50
60
70
80
90
100
12
10
8
6
4
2
0
Time in min.

Figure 62

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.5-0.6 from TLC loaded with the free gibberellins released by acid hydrolysis of the gibberellin-glucosides in the radicle tissue extracts and developed with solvent system (1).

1.5 ml of the eluate were derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 4 µl were injected into the GLC column.

Figure 62

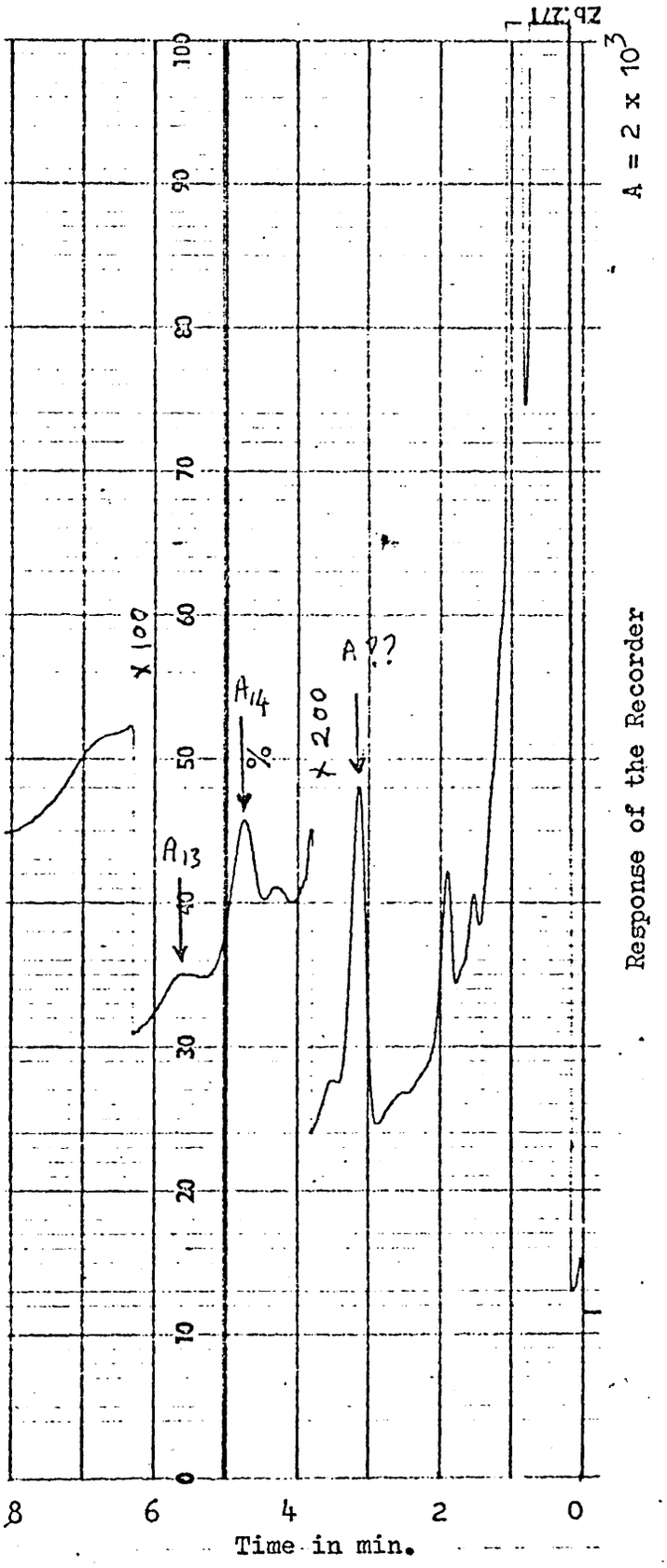

A = 2 x 10^-3
Response of the Recorder
Time in min.
8
6
4
2
0
100
90
80
70
60
50
40
30
20
10
0
x 100
A14
%
x 200
A ??
A13

Figure 63

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.8-0.9 from TLC loaded with the free gibberellins released by acid hydrolysis of the gibberellins-glucosides in the plumule tissue extracts and developed with solvent sytem (1).

1.5 ml of the eluates were derivatised and the ester dissolved in 40 µl of redistilled ethyl acetate of which 4 µl were injected into the GLC column.

Figure 63

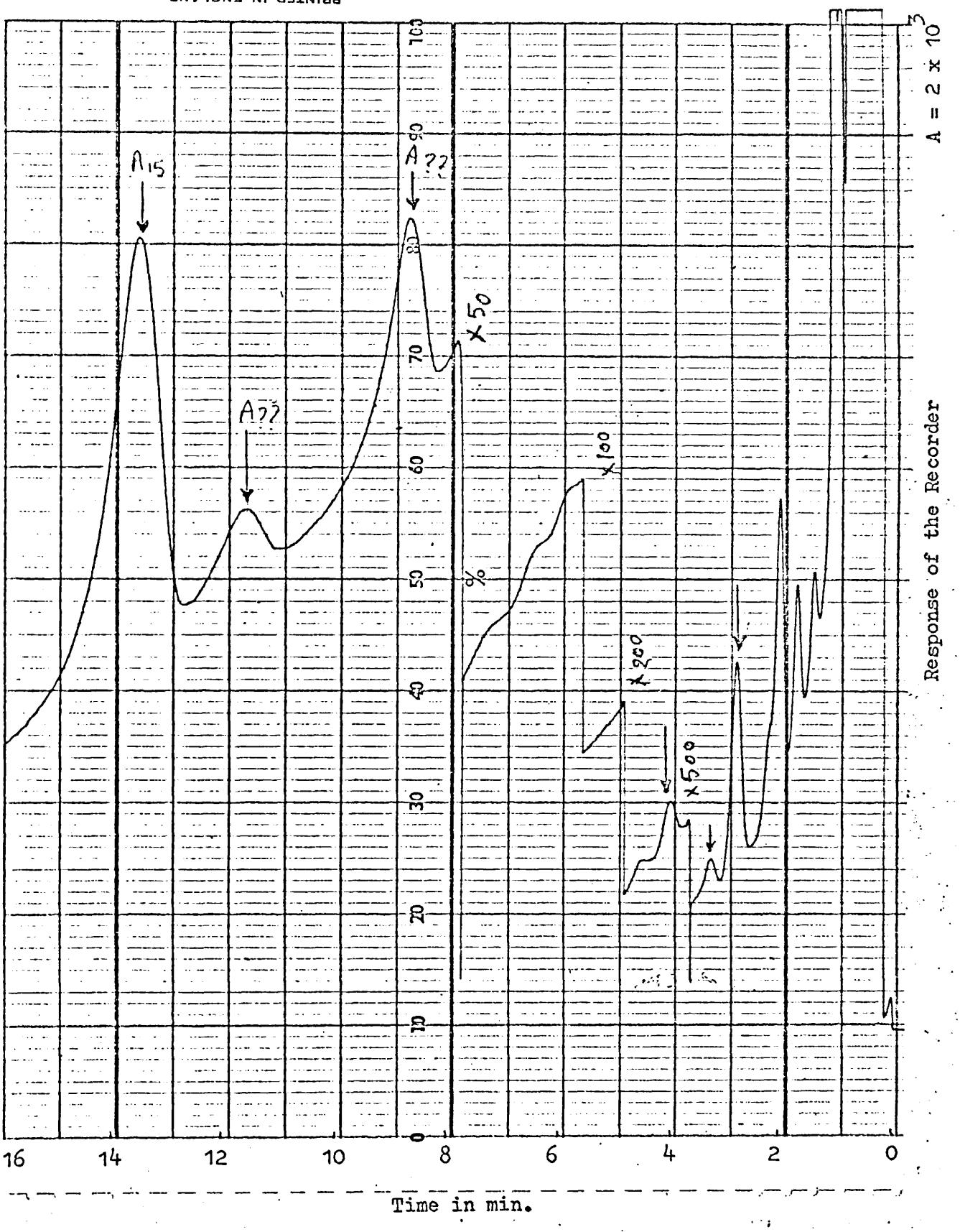
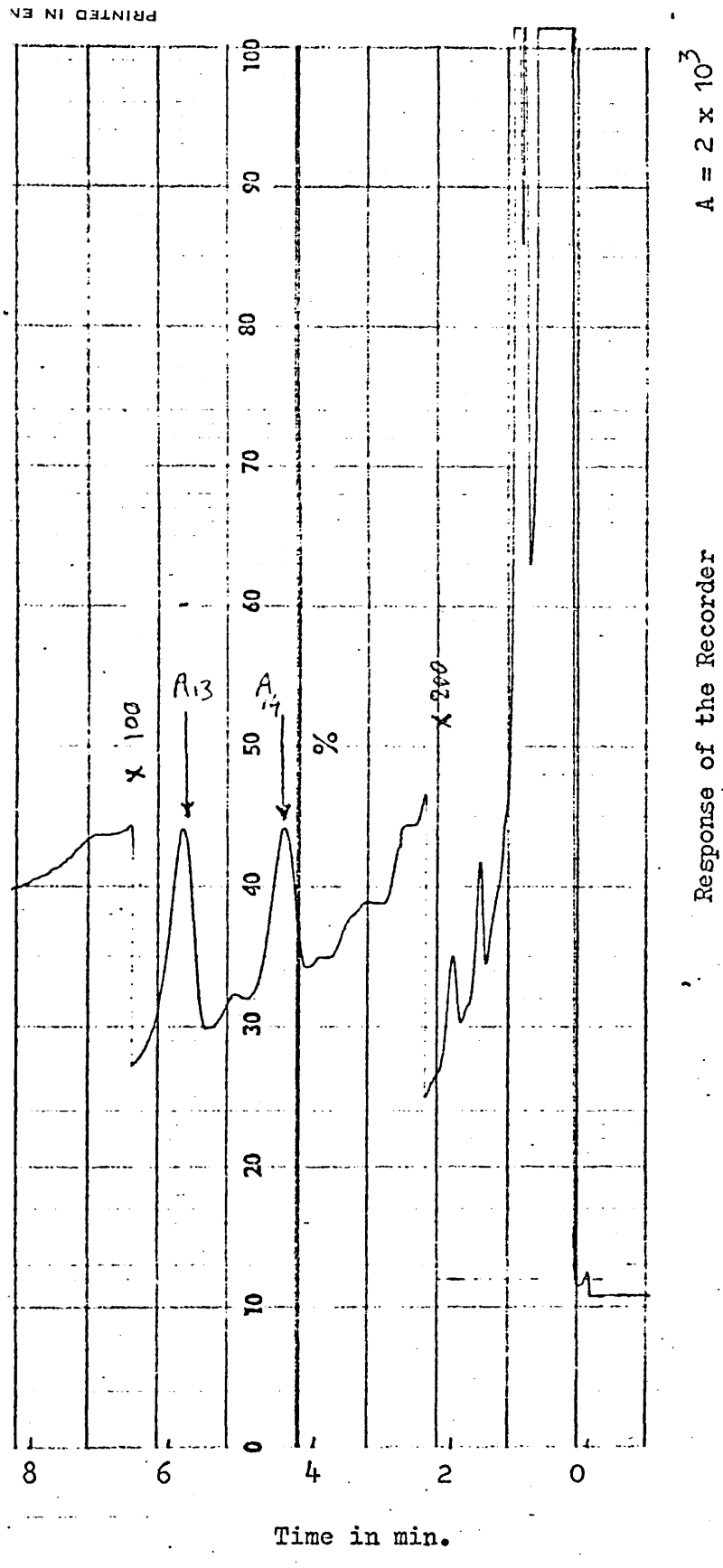


Figure 64

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.5-0.6 from TLC loaded with the free gibberellins released by acid hydrolysis of the gibberellins-glucosides in the cotyledons tissue extracts and developed with solvent system (1).

1.5 ml of the eluate were derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 3 µl were injected into the GLC column.

Figure 64


PRINTED IN EN
A = 2 x 10^3
Response of the Recorder
x 100
A13
A14
%
x 200
0
10
20
30
40
50
60
70
80
90
100
8
6
4
2
0
Time in min.

has a retention time corresponding to the methyl ester of gibberellin A_{15}, and the other two have retention times of 8.7 and 11.6 minutes which do not correspond to the methyl ester of any of the first twenty--four gibberellins represented in Table 4.

In the case of the hydrolysed gibberellin -glucosides of the cotyledons (Figs. 48C, 49C), the zone at R_f 0.5-0.6 (peak marked III) gave peaks with the GLC having retention times corresponding to the methyl esters of gibberellins A_{13} and A_{14} (Fig. 64).

2.3. The Free Gibberellins Released by Acid Hydrolysis of Other Bound-Gibberellins

Eluates of the zones of the TLC, loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins of the radicle, the plumule and/or the cotyledons which showed considerable activity in the bioassay test, when derivatised and analysed by GLC the following results were obtained:

A. In the case of the radicle extracts (Fig. 50A), eluates from the TLC zone at R_f 0.7-0.8 gave peaks with retention times corresponding to the methylesters of gibberellins A_7, A_9, A_{12}, A_{17}, and A_{24} (Fig. 65), while the zone at R_f 0.4-0.5 was found to contain a gibberellin, the retention time of its methyl ester being 3.4 minutes (Fig. 66), which does not correspond to the retention time of any of the gibberellins represented in Table 4.

B. In the case of the plumule extracts (Fig. 50B), eluates from the TLC zone at R_f 0.1-0.2 gave a peak with a retention time corresponding to the methyl ester of gibberellin A_8; the eluates of the TLC zone at R_f 0.2-0.3 was found to contain a gibberellin, the retention time of its methyl ester being 6.4 minutes (Fig. 67). This gibberellin was found in the eluates of the TLC zone at R_f 0.3-0.4 of Figure 50(B).

Figure 65

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.7-0.8 from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins in the radicle tissue extracts and developed with solvent system (1).

One ml of the eluate was derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 5 µl were injected into the GLC column.

Figure 65

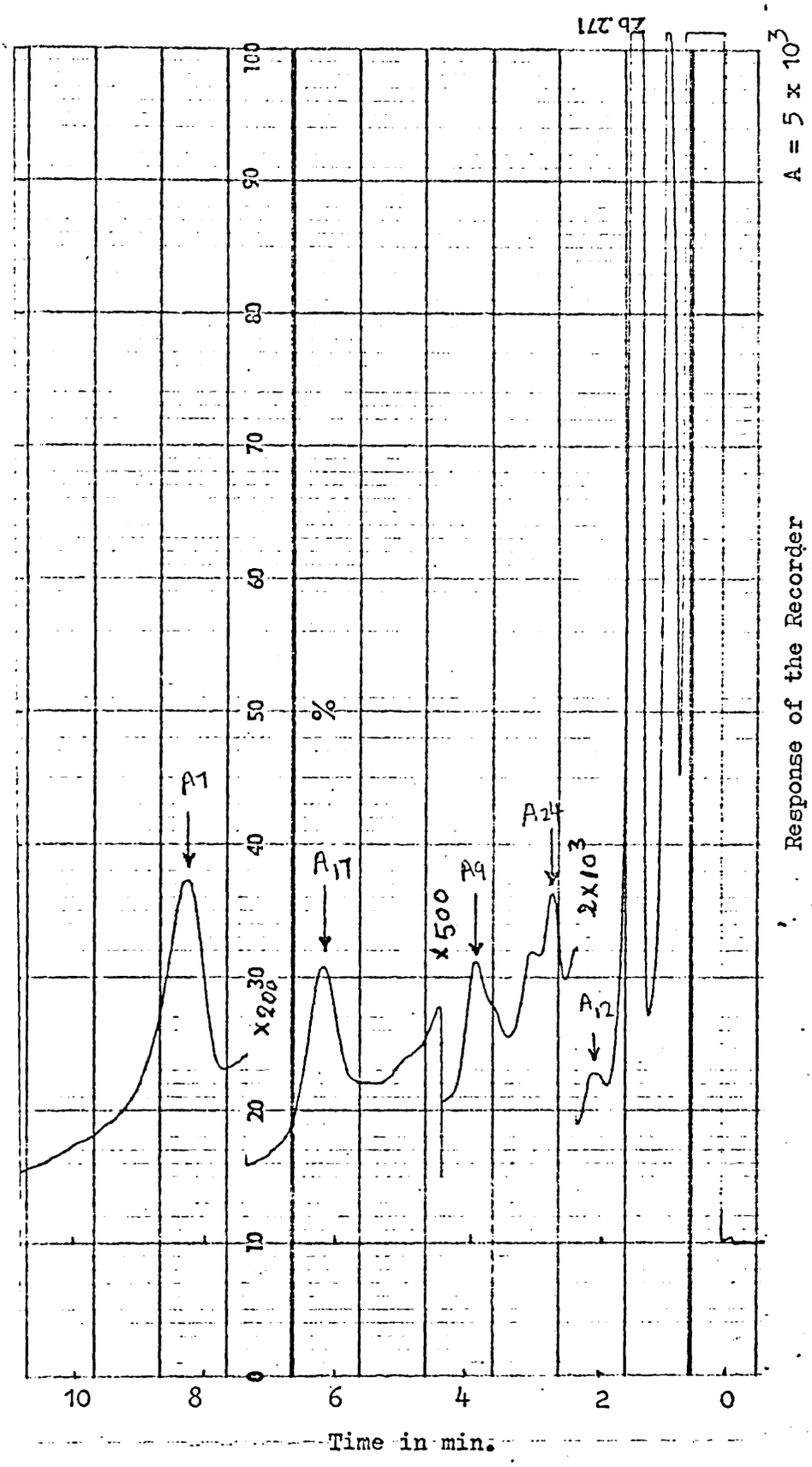

Zb.271
A = 5 x 10^-3
Response of the Recorder
100
90
80
70
60
50
%
40
30
20
10
0
A1
A11
A9
A24
A12
x500
x200
2x10^-3
10
8
6
4
2
0
Time in min.

Figure 66

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.4-0.5 from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins in the radicle tissue extracts and developed with solvent system (1).

One ml of the eluate was derivatised and the ester dissolved in 50 µl of redistilled ethyl acetate of which 5 µl were injected into the GLC column.

Figure 66

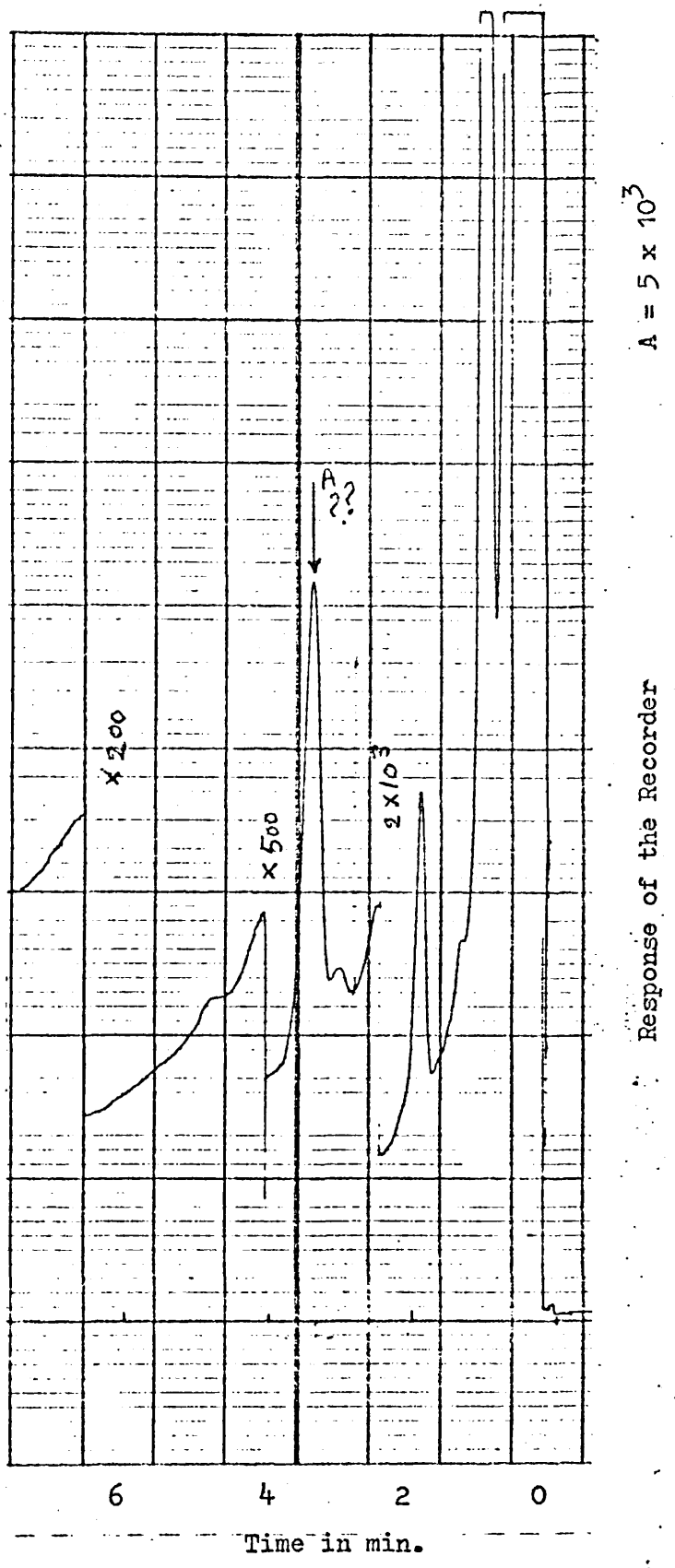
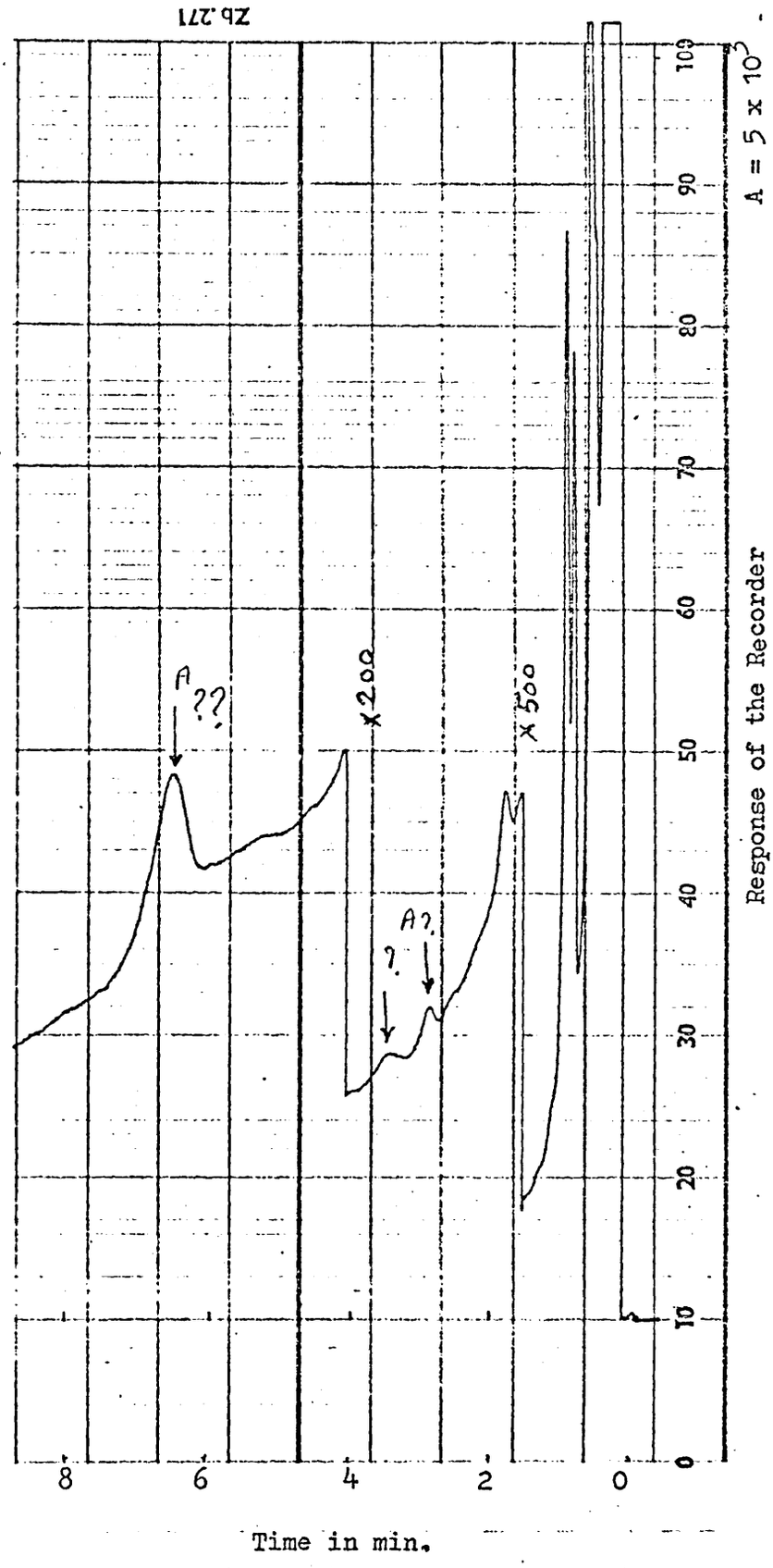

A = 5 x 10^-3
A ??
x 200
x 500
2 x 10^-3
6
4
2
0
Time in min.
Response of the Recorder

Figure 67

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.2-0.3 from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins in the plumule tissue extracts and developed with solvent system (1).

One ml of the eluate was derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 3 µl were injected into the GLC column.

Figure 67


Zb.271
A = 5 x 10⁻³
Response of the Recorder
A ??
x200
x500
A ?
?
0
10
20
30
40
50
60
70
80
90
100
8
6
4
2
0
Time in min.

The methyl ester of the eluate from the TLC zone at R_f 0.5-0.6 gave peaks with retention times of 2.4 and 4.1 minutes (Fig. 68); that of the TLC zone at R_f 0.6-0.7 gave peaks with retention times corresponding to the methyl derivatives of gibberellins A_9, A_{12}, A_{24}, together with the gibberellin found in the zone at R_f 0.5-0.6 (Fig. 68) with 4.2 minutes retention time (Fig. 69); while the eluate of the TLC zone at R_f 0.7-0.8 gave peaks with retention times corresponding to the methyl ester of gibberellins A_7, A_{12} and A_{24} (Fig. 70).

C. In the case of the cotyledon extracts (Fig. 50C), the eluate of the TLC zone at R_f 0.3-0.4 gave a peak with a retention time corresponding to the methyl ester of gibberellin A_{13} (Fig. 71).

Figure 68

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.5-0.6 from TLC loaded with the free gibberellins released by acid hydrolysis of the plumule bound-gibberellins and developed with solvent system (1).

One ml of the eluate was derivatised and the ester dissolved in 30 µl of redistilled ethyl acetate of which 3 µl were injected into the GLC column.

Figure 68

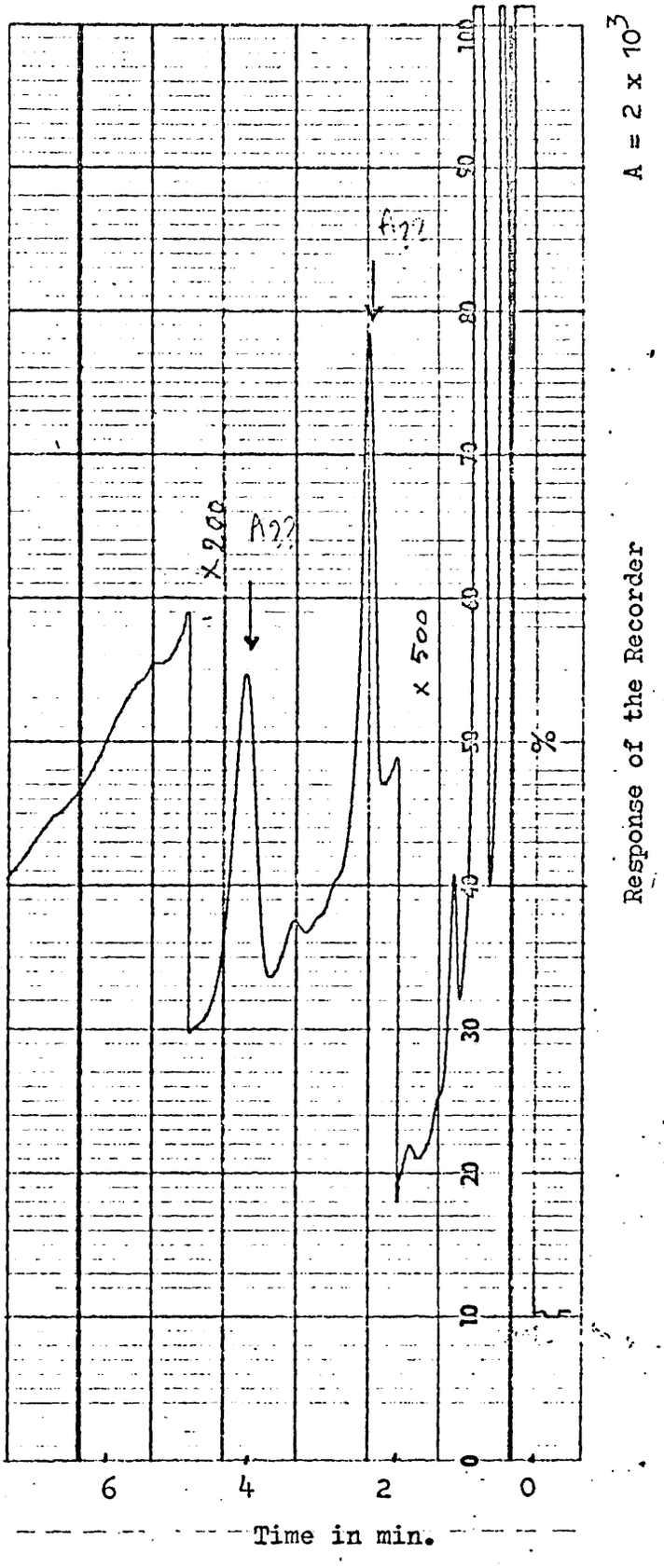

A = 2 x 10³
Response of the Recorder
%
100
90
80
70
60
50
40
30
20
10
0
A22
x 200
x 500
6
4
2
0
Time in min.

Figure 69

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.6-0.7 from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins in the plumule tissue extracts and developed with solvent sytem (1).

One ml of the eluate was derivatised and the ester dissolved in 40 ul of redistilled ethyl acetate of which 4 ul were injected into the GLC column.

Figure 69

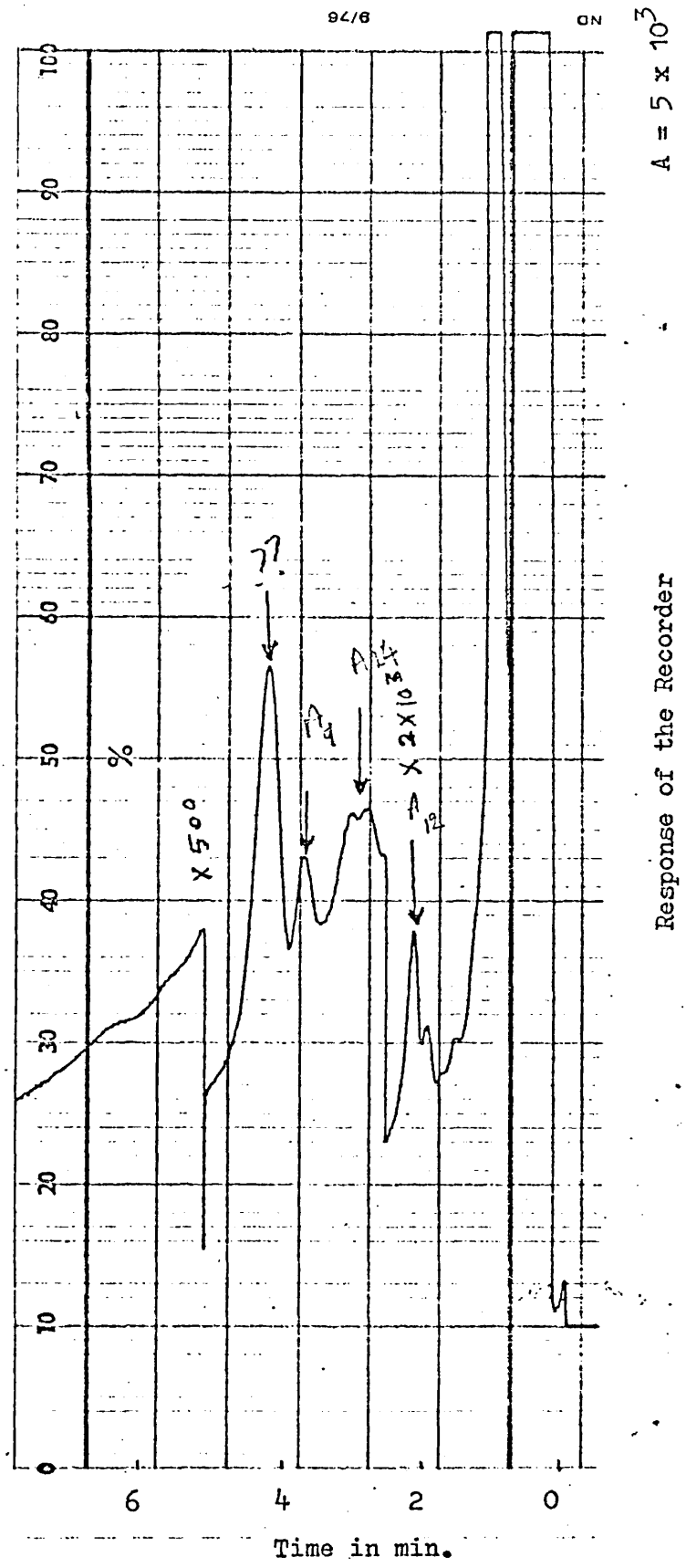

A = 5 x 10^-3
Response of the Recorder
%
X 500
X 2X10^-3
100
90
80
70
60
50
40
30
20
10
0
6
4
2
0
Time in min.

Figure 70

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.7-0.8 from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins in the plumule tissue extracts and developed in solvent system (1).

One ml of the eluate was derivatised and the ester dissolved in 40 µl of redistilled ethyl acetate of which 4 µl were injected into the GLC column.

Figure 70

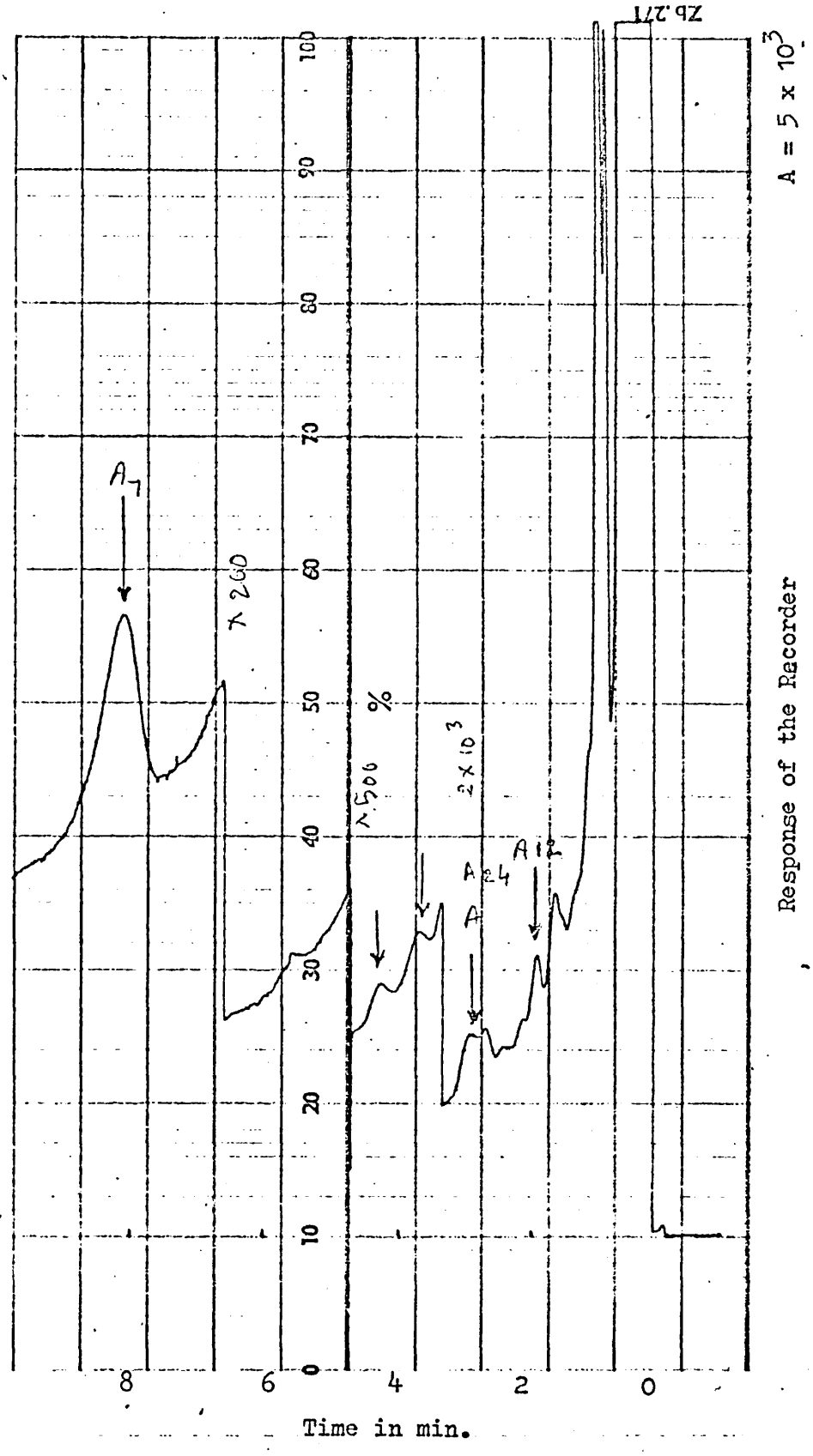
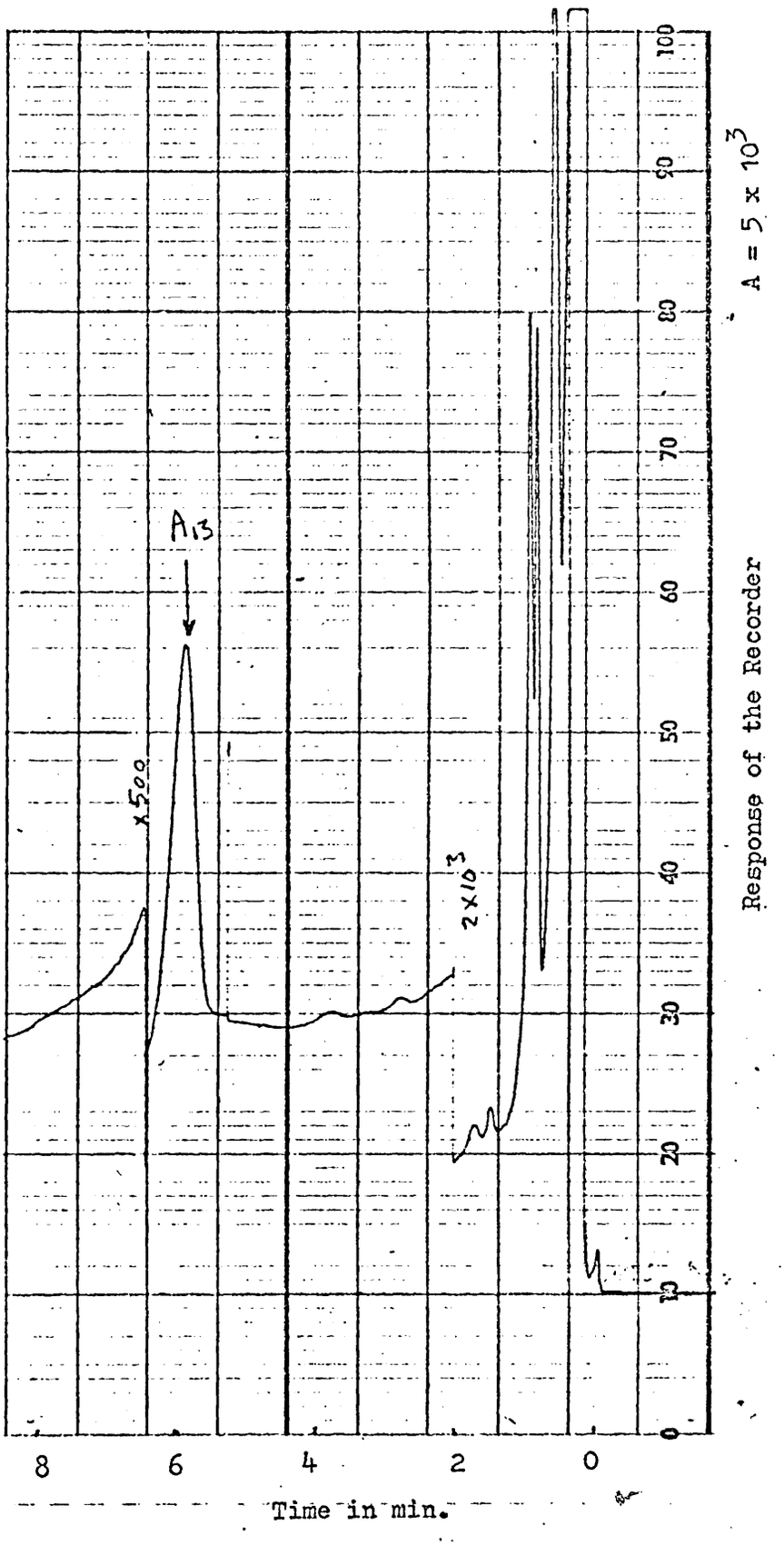

Zb.27
A = 5 x 10^-3
Response of the Racorder
100
90
80
70
60
50
40
30
20
10
0
A7
λ 260
λ 506
%
2 x 10^3
A24
A
A12
8
6
4
2
0
Time in min.

Figure 71

GLC analysis of the gibberellins in the eluate of the zone at R_f 0.3-0.4 from TLC loaded with the free gibberellins released by acid hydrolysis of the bound-gibberellins in the cotyledons tissue extracts and developed with solvent system (1).

One ml of the TLC eluate was derivatised and the ester dissolved in 40 ul of redistilled ethyl acetate of which 3 ul were injected into the GLC column.

Figure 71


A13
x500
2x10^3
A = 5 x 10^3
Response of the Recorder
100
90
80
70
60
50
40
30
20
10
0
8
6
4
2
0
Time in min.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

Occurrence of the Gibberellins in Plant Tissues

It has been pointed out in the Introduction that endogenous gibberellins can exist in plant tissues in three forms as follows:

i. Free gibberellins, which can be easily separated from the tissue extracts by partitioning against ethyl acetate at low pH (e.g. Hayashi & Rappaport, 1966; Crozier & Reid, 1971; Yokota et al., 1971b; Williams et al., 1974). They occur as 19-carbon or 20-carbon mono-, di-, or tri-carboxylic acids, and they are not associated by any detectable form of bonding to other substances.

ii. Conjugated-gibberellins, which have been referred to in the early literature as water-soluble, or more commonly, bound-gibberellins; they can be readily separated from tissue extracts by partitioning against n-butanol at low pH in the case of the gibberellin-glucosides and at high pH in the case of the glucosyl-esters (e.g. Hashimoto & Rappaport, 1966b; Sembdner et al., 1968; Yokota et al., 1971c; Hiraga et al., 1974b; Takahashi, 1974). Conjugated-gibberellins can be hydrolysed by acid or alkali to give the free gibberellin and glucose.

iii. Bound-gibberellins, with the possible exception of GA_{28} and GA_{32}, they are more polar than the free gibberellins (Lang, 1970) but their structure is yet unidentified although some authors have suggested the existence of protein-bound gibberellins (e.g. Hayashi & Rappaport, 1962; Jones, D.F., 1964; Pegg, 1966; Reinhard & Sacher, 1967; Dale, 1969). Bound-gibberellins can be separated from tissue extracts by ethyl acetate at low pH after their hydrolysis by dilute hydrochloric acid.

The work involved in this thesis revealed the existence of acidic ethyl acetate-soluble gibberellins, acidic n-butanol-soluble gibberellins as well as water-soluble or other bound-gibberellins in the different organs of the etiolated broad bean (Vicia faba) seedling. Since the fractionation procedure employed here did not include partitioning against n-butanol at high pH, and since no experiment has been carried out to check specifically for the occurrence of gibberellin glucosyl--esters in the tissues under investigation, so the water-soluble or other bound-gibberellins referred to here might have contained gibberellin glucosyl-esters as well. The term "bound-gibberellins" used does not imply that I have any proof of binding to specific molecules or cell particles but only that these gibberellin forms do not appear either in the acidic ethyl acetate fraction or in the acidic n-butanol fraction, but are rendered soluble in ethyl acetate at low pH and active in the bioassay tests upon acidic hydrolysis.

The results achieved with dilute hydrochloric acid in releasing gibberellins from conjugation and/or the "bound" form should be treated with a certain amount of caution. Although the effect of mild acid treatment on the gibberellins has not been investigated thoroughly, acid hydrolysis of 2-O-β-glucosyl gibberellin A_3 resulted in rearrangement of the released gibberellin A_3 to gibberic acid (Tamura et al. 1968a). Also, incubation of gibberellin A_{21} (Canavalia gibberellin-I) under conditions similar to those described by Barendse, Kende and Lang (1968) caused Wagner-Meerwein type rearrangements of the molecule to produce three biologically inactive compounds (Tamura et al., 1968b).

Separation of Endogenous Gibberellins from the Different Organs of Etiolated Broad Bean Seedlings by TLC and Their Identification by Bioassay Tests and GLC Analysis

The gibberellin content of immature seeds, which are a rich source of this group of hormones, and their changes during the course of seed development and maturation has been reported by many investigators (e.g. Cavell et al., 1967; MacMillan & Pryce, 1968a, 1968b; Durley, MacMillan & Pryce, 1971; Eeuwens, Gaskin & MacMillan, 1973; Frydman et al., 1974), while those of germinating seedlings, where gibberellins are thought to participate in the process of germination, has been scarcely reported (Gotoh, 1970; Crozier et al., 1971; Bowen et al., 1973; Hiraga et al., 1974c, 1974d).

Eluates from the TLC loaded with extracts from the different organs of the etiolated broad bean seedlings (the extracts being purified by PVP slurries and PVP column chromatography in the case of free gibberellins and the free gibberellins released by acid hydrolysis of water-soluble gibberellins; and purified by PVP slurries only in the case of the acidic butanol-soluble gibberellins and the free gibberellins released by acid hydrolysis of the gibberellin-glucosides) and developed with solvent system (1) (Table 2), were tested biologically with the lettuce hypocotyl bioassay and the α-amylase bioassay tests or with the lettuce hypocotyl bioassay test only (Figs. 29-50). The eluates from the zones that showed considerable activity in the bioassay tests were derivatised for GLC analysis (Figs. 51-71).

It must be always brought in mind that the extracts might have contained gibberellin-like substances that were not particularly active in the bioassay tests used; they might also have contained, in addition to the gibberellins detected by GLC, some other gibberellins which were present in concentrations too low to permit their detection by the GLC

which is sensitive to concentrations up to 100.0 ng [the combined gas chromatography-selected ion current monitoring (GC-SICM) which is sensitive to amounts of gibberellins as little as 1.0 ng (Frydman et al., 1974) but was not available to me]. The peaks detected in the case of each tissue with the bioassay tests used and their relative activities are represented in Table 9:

Table 9 The Reproducible Peaks Detected in Extracts from the Different Organs of Etiolated Broad Bean Seedlings by Bioassay and their Relative Quantity

A. Lettuce Hypocotyl Assay

Peaks Detected	Free GAs			Acidic butanol-sol. GAs			Hydrolysed GAs-gluco-			*Hydro. Bound-GAs		
	R.	P.	C.	R.	P.	C.	R.	P.	C.	R.	P.	C.
I		++	++	++	+++	++++	++	+	+		+	+
II	+++	+++	+++	+++	++		++	++	+	++	++	+
III	++++	+++	+++	++		+	++++	++	++	+	+++	
IV			++	+	+	+	++	+++	++		+	

B. α-Amylase Assay

I		+			+	+	+					
II	++	+++	++	+			++	++				
III	+	+					++	+				
IV				+++	+		+	+	+			

Relative quantity: ++++ high; +++ moderate; ++ low; + very low.

* Hydrolysed bound-GAs tested biologically with the lettuce assay test only.

R.= Radicle , P.= Plumule , C.= Cotyledon

As a general observation from the Table, most of the gibberellins detected (either naturally occurring free or released free by acid hydrolysis of conjugated and/or other bound-gibberellins) were found to be more active in the lettuce hypocotyl bioassay test than in the α-amylase bioassay test, as judged by the number of peaks detected.

In addition to the peaks illustrated in Table 9, some peaks detected in the radicle and the plumule extracts on some occasions were found to be not reproducible in all the samples; these peaks are peak marked (IV) in Figure 40A,B, and peak marked (I) in Figure 41A in the case of the free gibberellins from the radicle tissue extracts; and peak marked (IV) Figure 42A,B, in the case of the free gibberellins from the plumule tissue extracts. Since these peaks were not detected in all the samples, it could be possible that their potential response was masked by inhibitors in the extracts in which they were not detected.

In 1967, Cavell et al. catalogued the thin-layer chromatographic properties of gibberellins A_1-A_{15}, A_{18} (which was known as Lupinus gibberellin-I at that time) and A_{19} (which was known as Bamboo-gibberellin at that time) in six solvent systems, one of which is the solvent system (1) which has been used here. This makes it possible to compare the results obtained with the R_f values of Cavell et al. (1967). This comparison is presented in Tables 10, 11 and 12 for the free gibberellins, the free gibberellins released by acid hydrolysis of the gibberellin -glucosides and the free gibberellins released by acid hydrolysis of other bound-gibberellins respectively. It should be always borne in mind that the R_f values of the gibberellins on TLC are not reproducible (Cavell et al., 1967) so the comparison here is only made to give a rough identification of the gibberellins contained in the different TLC zones. This identification was supported by GLC analysis which depends on the measurements of the retention time of

the GLC peaks obtained with the derivatised extracts and comparing this with the retention times of the standard gibberellins as shown in Table 4. This method is recommended for the identification of gibberellins in plant tissue extracts and the retention times were found to be reproducible (Cavell et al., 1967; MacMillan & Pryce, 1968a, 1968b).

However, there still remained a few fractions with high gibberellin activity, called here "unknown gibberellins" whose identity could not be indicated either by their R_f on TLC, as recorded by Cavell et al. (1967) or by their retention times on GLC as reported by MacMillan and Pryce (1968a).

In the case of the free gibberellins from the radicle tissue extracts, GLC analysis of the peak marked (III) in Table 9 suggested the presence of gibberellins A_7, A_9, A_{15}, A_{17}, and A_{24} (Table 10). This is in agreement with the results of the bioassay tests which showed more activity in this peak with the lettuce hypocotyl assay than with the α-amylase assay test as gibberellin A_{15} is active in the first assay and not active in the second one, and gibberellins A_7 and A_9 are more active in the lettuce hypocotyl assay than in the α-amylase assay test (see Table 1). An unexpected result was the GLC analysis of the zone at R_f 0.8-0.9 (peak marked IV in Fig. 40B), which was found not to be reproducible in all the extracts and a suggested explanation was made for this on the basis of the bioassay results on page 261. This GLC analysis showed a peak with retention time corresponding to the methyl ester of gobberellin A_9. This gibberellin was one of the gibberellins detected at peak (III) and this could mean that peak (IV) is just an extension of the peak marked (III). Another unexpected result was obtained when eluate of the peak marked (I) Figure 41A was derivatised and the ester injected into the GLC column. Interfering

substances which caused a high background precluded GLC identification. This peak (I) could be a trace of the major gibberellins of the peak marked (II), Table 9. An inhibiting impurity running at say R_f 0.15 could suppress the bioassay response in this segment and give the impression of two gibberellin peaks where only one broad one existed.

The peak marked (III), Figure 48A,B, of the hydrolysed gibberellin-glucosides appeared to contain gibberellins A_9, A_{12}, A_{14}, A_{20}, and A_{24} (Table 11). This is in agreement with the values of the R_f of these gibberellins on TLC and would explain the higher activity of the peak in the lettuce hypocotyl assay than in the α-amylase assay test, since GA_{20} is highly active in the first assay and has a low activity in the second assay. The zone at R_f 0.7-0.8 in the case of the free gibberellins released by acid hydrolysis of the bound-gibberellins appear to contain gibberellins A_7, A_9, A_{12}, A_{17} and A_{24} (Table 12).

The results of the GLC analysis of eluates from the TLC peaks that showed considerable activity in the bioassay tests, in the case of the free gibberellins, after their derivatisation are shown in Table 10. From the Table we notice that both zones at R_f 0.3-0.4 and at R_f 0.4-0.5 appear to contain gibberellin A_{13}. This broad peak could be due to overloading of the thin-layer plate. Overloading was also noticed in the zones at R_f 0.7-0.8 and 0.8-0.9 in the case of the hydrolysed glucosides (Table 11) and in the zones at R_f 0.6-0.7 and 0.7-0.8 in the case of the gibberellins released by acid hydrolysis of bound-gibberellins (Table 12). In the case of the free gibberellins in the cotyledons tissue extracts, the number of peaks detected by the lettuce hypocotyl assay was found to be more than that detected by the α-amylase assay test (Table 9). GLC analysis of the peak marked (III) suggested the presence of gibberellin A_{15} as a constituent of this peak (Table 10) and this could explain why this peak was not detectable with the α-amylase assay test as GA_{15} is active with the lettuce hypocotyl assay test and inactive in the α-amylase assay test.

Table 10 The Free Gibberellins in the Different Organs of Etiolated Broad Bean Seedlings as Identified by TLC and GLC

TLC peaks and their R_f	Gibberellin content according to Cavell et al.	GLC		
		Radicle	Plumule	Cotyledon
I 0.0-0.2	A_2 A_8		3 unknown	
II 0.3-0.4	A_1 A_3 A_{10} A_{18}		1 unknown A_{13}	
III 0.4-0.5	A_{13} A_{19}	A_{13}	A_{13} iso-A_{13}	A_3 A_5 A_6 A_{13} iso-A_{13}
III 0.5-0.6	A_5 A_6			3 unknown
III 0.6-0.7	A_4 A_7 A_{14}	A_7 A_9 A_{15} A_{17} A_{24}	A_{12} A_{14} A_{20} A_{24}	A_{12} A_{15}
III 0.7-0.8	A_9 A_{11} A_{12} A_{15}			
IV 0.8-0.9 0.9-1.0	?? ??			

Table 11 The Free Gibberellins Released by Acid Hydrolysis of the Gibberellin-Glucosides in the Different Organs of Etiolated Broad Bean Seedlings as Identified by TLC and GLC

TLC peaks and their R_f	Gibberellin content according to Cavell et al.	GLC		
		Radicle	Plumule	Cotyledon
I 0.0-0.2	A_2 A_8		A_8	
II 0.3-0.4	A_1 A_3 A_{10} A_{18}			
II 0.4-0.5	A_{13} A_{19}		3 unknown	
III 0.5-0.6	A_5 A_6	A_{13} A_{14}		A_{13} A_{14}
III 0.6-0.7	A_4 A_7 A_{14}	A_9 A_{12} A_{14} A_{20} A_{24}		
III 0.7-0.8	A_9 A_{11} A_{12} A_{15}		A_{24} 3 unknown	
IV 0.8-0.9 0.9-1.0	?? ??		A_{15} 2 unknown	

Table 12 The Free Gibberellins Released by Acid Hydrolysis of the Other Bound-Gibberellins in Extracts from the Different Tissues of Etiolated Broad Bean Seedlings as Identified by TLC and GLC

TLC peaks and their R_f	Gibberellin content according to Cavell *et al.*	GLC		
		Radicle	Plumule	Cotyledon
I 0.0-0.2	A_2 A_8		A_8	
II 0.3-0.4	A_1 A_3 A_{10} A_{18}		1 unknown	A_{13}
II 0.4-0.5	A_{13} A_{19}	1 unknown		
III 0.5-0.6	A_5 A_6		2 unknown	
III 0.6-0.7	A_4 A_7 A_{14}		A_9 A_{12} A_{24} 1 unknown	
III 0.7-0.8	A_9 A_{11} A_{12} A_{15}	A_7 A_9 A_{12} A_{17} A_{24}	A_7 A_{12} A_{24}	
IV 0.8-0.9 0.9-1.0	?? ??	A_{15} 2 unknown		

The fraction containing the acidic n-butanol-soluble gibberellins showed activity with the bioassay tests used in a number of zones in each tissue extract (Figs. 45-47, A-C). Acid hydrolysis of this fraction resulted in an increase in the number and the size of the peaks as detected by bioassay tests (Figs. 48,49, A-C) because acid hydrolysis results in the release of the free gibberellins which are more active than their conjugated forms (Yokota et al., 1971a; Hiraga et al., 1974a; Sembdner et al., 1976). It should be mentioned here that most of the GLC results obtained for the free gibberellins released by acid hydrolysis of conjugated-gibberellins were not of great value and the TLC eluates were found to contain impurities which precluded the detection of peaks with the GLC in most of the zones detected by bioassay. The reason for this is that the purification carried out with this fraction (PVP slurries only) was enough to give clear TLC eluates to permit biological analysis but it was not enough to permit GLC analysis. Furthermore the amount of tissue used was not enough to give measurable peaks on GLC. For future work, two conditions should be ensured for this fraction of the gibberellins:

i. An increase of the amount of tissue to be extracted.

ii. Addition of further purification steps; in this case PVP column chromatography could be successful as it was in the case of the free gibberellins and the free gibberellins released by acid hydrolysis of the water-soluble gibberellins.

The Site of Gibberellin Biosynthesis

In barley, the gibberellin which is concerned in the control of developmental changes of the aleurone layer of the endosperm during germination, is synthesised in the embryo. In broad bean, however, which has no endosperm, the site of gibberellin synthesis might be in the cotyledons, which are the main storage organs, or in the embryonic axis or in both. Evidence supporting the hypothesis that the cotyledons are the sites of synthesis might be if all the peaks with gibberellin-like activity and also the gibberellins detected by GLC analysis in the extracts from the radicle and the plumule tissues, also occurred in extracts from the cotyledons tissue. This was in fact found to be largely true (see Tables 10, 11, 12).

On the other hand, roots are certainly known to synthesis gibberellins (e.g. Jones, R.L. & Phillips, 1966; Sitton, Richmond & Vaadia, 1967) and to export considerable amounts of these hormones to the shoot (e.g. Phillips & Jones, R.L., 1964; Carr & Reid, 1968) although their role is unclear. Studies on endogenous gibberellins in sunflower suggested that roots synthesised the same type of gibberellins as the shoots (Jones, R.L., 1965). Recent evidence suggests that the sites of gibberellin biosynthesis in shoots and roots may be closely interlinked in that gibberellin precursors produced in the shoot may be converted into other gibberellins in the roots which are then translocated back to the shoot (e.g. Crozier & Reid, 1971). However, it is well known that translocation of applied gibberellins in plants occur very rapidly (e.g. McCombe, 1964; Clor, 1967; Barendse, 1975), so, the comparison of extractable gibberellins in the various organs of the etiolated broad bean seedling cannot indicate the sites of synthesis of gibberellins in a particular organ, as levels could be due to translocation and accumulation of gibberellins produced elsewhere into this organ. The endogenous gibberellins in extracts from the

different organs of the etiolated broad bean seedlings, as identified by GLC analysis are presented in a diagram form in Figure 72.

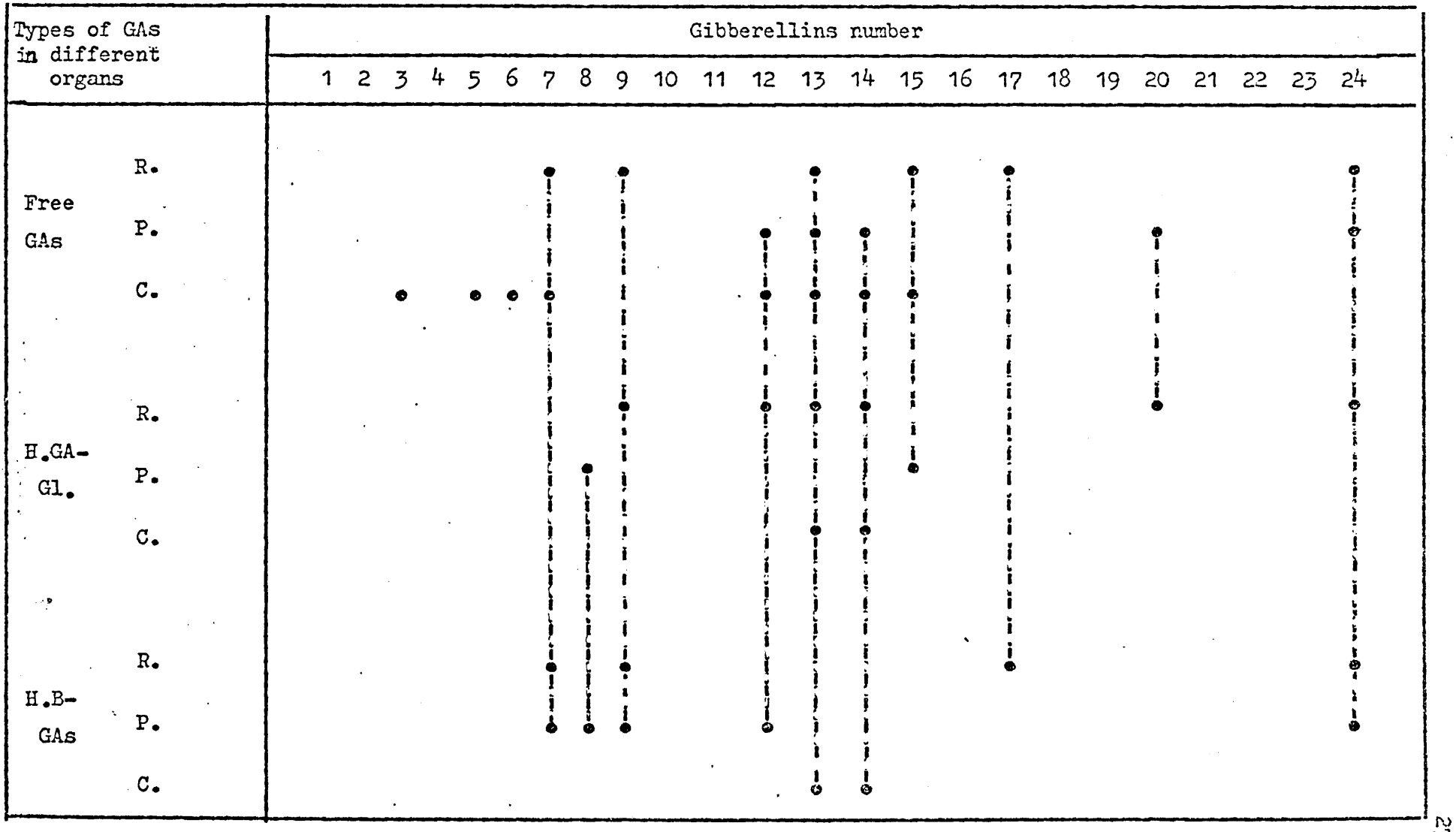
There is considerable evidence that immature seeds are a site of gibberellin biosynthesis (e.g. Graebe et al., 1965; Zeevaart, 1966; Banerjee, 1968; Eeuwens, Gaskin & MacMillan, 1973; Hiraga et al., 1974b), and it is possible that the gibberellins present in the immature seeds of broad bean are temporarily inactivated by being bound to larger molecules. However, to date, no study of the endogenous gibberellins in immature broad bean seed is known to allow comparison with the results obtained in this study. This suggestion is supported by the results obtained by Hashimoto and Rappaport (1966a) who showed that during the maturation of Phaseolus vulgaris seeds, the ethyl acetate-soluble gibberellins initially increased and then almost disappeared as the seed reached maturity, while neutral gibberellin-like substances and acidic butanol-soluble gibberellins continually increased and were present in fairly large amounts in mature seeds. The same conclusion was reported by Frydman, Gaskin and MacMillan (1974) on studying the gibberellins of Pisum sativum seeds throughout its maturation.

The stored bound-gibberellins in the mature seeds could then be released during germination into free forms rather than be synthesised de novo, at least in the early development of the seedlings. Support for this hypothesis lies in the fact that most of the free gibberellins released by acid hydrolysis of conjugated and/or other bound-gibberellins in this study are identical with the acidic ethyl acetate-soluble (or free) gibberellins present in the tissues as detected by GLC analysis (see Fig. 72). The other free gibberellins which were not found in a conjugated and/or a bound form (i.e. A_3, A_5, and A_6) could be synthesised from gibberellin A_{13} which was found in all tissue and in all forms and is known to be a precursor of all the other gibberellins (e.g. Lang, 1970). In agreement with this are the findings of Barendse et al. (1967)

and Barendse and Lang (1966) with Pharbitis and Silene seedlings. It is also supported by the postulate made by Paleg (1965) which suggests that seedling growth in the dark may not necessarily be promoted by de novo synthesis of gibberellins but it could result from a utilisation of the seedling's conjugated and bound-gibberellin reserves. This postulate was later confirmed by the findings of Dale and Felippe (1968); they treated Phaseolus vulgaris seeds with the growth retardant CCC (2-chloro-ethyl trimethyl ammonium chloride), which is known to inhibit the synthesis of gibberellins in a number of systems (e.g. Kende, Ninnemann & Lang, 1963; Harada & Lang, 1965; Cross & Myers, 1969) and detected soluble gibberellins in the Phaseolus seedlings; this proves that gibberellins were liberated from a bound state during seedling growth and were not synthesised de novo.

An interesting results is that gibberellins A_3, A_5, and A_6 were detected in the free form in extracts from the cotyledonary tissue only. The presence of gibberellin A_3 could be due to conversion from gibberellin A_5, with gibberellin A_6 as intermediate, i.e. $A_5 \longrightarrow A_6 \longrightarrow A_3$. This suggestion is supported by the results of Durley et al. (1973) who found that radioactive gibberellin A_5 (^{3}H-GA_5) when applied to seedlings of dark grown dwarf pea (P. sativum L. cv. Meteor), was converted to two acidic compounds, one of which is GA_3 and the other is an unknown compound chromatographically similar to GA_3. Gibberellic acid could be endogenous in Vicia faba as it has been detected in geotropically stimulated Vicia faba roots by El-Antably and Larsen (1974), so if it is indeed endogenous to V. faba it is possible that gibberellin A_{20} is its precursor (Lang, 1970) and this suggestion explains why gibberellin A_{20} was detected in the plumule and the radicle tissue extracts, but not in the cotyledons tissue extracts; probably because its consumption is faster than its formation.

Figure 72 A Diagram Representing the Distribution of the Gibberellins Identified by GLC in the Different Organs of the Etiolated Broad Bean Seedlings



From the diagram presented in Figure 72 we find that gibberellin A_{12} appeared to occur in the free form in extracts from the plumule and the cotyledonary tissues and in bound form in extracts from the radicle and the plumule tissues. This gibberellin was found by Graebe et al. (1974) to be completely converted by a cell-free system from Cucurbita maxima (in the absence of Mn^{2+}) to more highly oxidised gibberellins which when fractionated and separated by TLC were found to be gibberellins A_{15}, A_{24}, A_{36}, and A_{37}. However, of these gibberellins, gibberellin A_{15} and gibberellin A_{24} appeared to occur in the radicle tissue extracts and this could mean complete conversion of gibberellin A_{12} in the radicle tissue; and since no information is available about the chromatographic properties of gibberellin A_{36} and gibberellin A_{37} with the solvent system used in the case of TLC and with the 2% QF-1 column as their methyl esters, these gibberellins could be translocated from the radicle to the plumule tissue. In this case, the unknown free gibberellins in the plumule tissue extracts could include gibberellins A_{36} and A_{37}. The identification of gibberellin A_{14} in a conjugated form in the cotyledons and the radicle tissue extracts and in free form in the plumule and the cotyledons tissue extracts (Fig. 72) could be interpreted as follows:

Gibberellin A_{14} is one of the native hormones of the broad bean seed which is released free during seed germination and hence translocated to the radicle and the plumule tissues. Gibberellin A_8 was determined in the plumule tissue extracts only, this gibberellin and the disappearance of gibberellin A_{14} from the radicle tissue extracts could be correlated as Durley, Railton and Pharis (1974) have found that gibberellin A_{14}-[17-^{3}H], when applied to seedlings of dark grown dwarf pea (P. sativum L. cv. Meteor) was converted to GA_1, GA_8, GA_{18}, GA_{23}, GA_{28} and GA_{38} following a sequence of interconversion as follows:

$GA_{14} \rightarrow GA_{18} \rightarrow GA_{38} \rightarrow GA_{23} \rightarrow GA_{1} \rightarrow GA_{8}$ and GA_{8} in the case of broad bean, is translocated to the plumule tissue where it was bound to other molecules.

The ideas expressed in this Chapter and the numerous other possible interpretations of the results presented can be regarded as extremely tentative hypotheses. When there is more detailed information on the identity of the unknown gibberellins, the biosynthesis, sites of biosynthesis and sites of action of native gibberellins of etiolated broad bean seedlings will be more clear.

SUMMARY

1. Methanolic extracts from the radicle, plumule and cotyledonary tissues of etiolated broad bean (Vicia faba) seedlings were fractionated into an acidic ethyl acetate-soluble fraction, which contains most of the free gibberellins, an acidic butanol-soluble fraction, which contains the gibberellin-glucosides and a water-soluble fraction which contains other bound-gibberellins.

2. Both the free gibberellins and those released free by acid hydrolysis of bound-gibberellins (Banerjee et al., 1968) were purified by means of PVP slurries and PVP column chromatography. The gibberellins recovered from these columns effluents (Glenn et al., 1972) were resolved by thin-layer chromatography on activated Keiselgel-60 F_{254} plates which were developed with a solvent system consisting of:

Ethyl acetate-chloroform-acetic acid (15:5:1 v/v; Cavell et al., 1967).

The gibberellins were eluted from the TLC plates by wet ethyl acetate overnight at 2°C (Aung & DeHertogh, 1968).

3. The acidic butanol-soluble gibberellins and the free gibberellins released by acid hydrolysis of the gibberellin-glucosides were purified by PVP slurries. The gibberellins recovered from the filtrate in the first case were separated by TLC on Keiselgel-60 F_{254} plates which were developed three times with a solvent system consisting of:

Acetone-acetic acid (97:3 v/v; Yokota et al., 1971c),

these gibberellins were eluted with 40% aqueous ethanol. In the second case (i.e. the hydrolysed gibberellin-glucosides) the gibberellins released were separated and eluted as those released by acid hydrolysis of the bound gibberellins.

4. Eluates of the TLC were tested biologically for their gibberellin--like activity with the lettuce hypocotyl assay (Frankland & Wareing, 1960; Jones, R.L., 1968) and in most cases by the α-amylase assay test (Jones, R.L. & Varner, 1967).

5. The gibberellin content of each TLC active zone was roughly identified on the basis of their R_f values (Cavell et al., 1967).

6. Eluates of the TLC zones that showed considerable activity in the bioassay tests (with the exception of the acidic n-butanol-soluble fraction) were methylated and their methyl esters were analysed by GLC on a 2% QF-1 column (1.5 m long x 6 mm external diameter x 4 mm inner diameter) using a Pye Series 104 dual column gas chromatograph (Model 24) fitted with a flame ionization detector head. The chromatograms were normally run isothermally at 220°C with Argon carrier gas at a flow rate of 60 ml/min. The column was calibrated (Cavell et al., 1967) and the retention times of gibberellins A_1-A_{24} were calculated from the retention times of authentic GA_{14} using a Table published by MacMillan and Pryce (1968a) giving the relative retention times of gibberellins GA_1 to GA_{24}.

7. The gibberellin content of the active TLC zones were identified by measuring the retention times of the GLC peaks and comparing these with the retention times calculated for the column used.

8. The results obtained here by bioassay tests and GLC analysis revealed that the different organs of the etiolated broad bean seedling contain gibberellins in the free form, the conjugated form, as well as the bound form.

9. The conjugated and/or the bound-gibberellins in the broad bean seeds seem to release free gibberellins on germination. All the tissues investigated appeared to contain gibberellin A_{13} in the free form. This gibberellin is known to be a precursor for most of the other known gibberellins.

10. The plumule and the cotyledonary tissues appeared to contain gibberellin A_{12} in the free form. That this gibberellin was not detected in the radicle tissue extracts could be due to its conversion to other gibberellins.

11. The radicle and the plumule tissues appeared to contain gibberellin A_{20} in the free form. In the case of the cotyledonary extracts, this gibberellin was not detected. This is probably due to its conversion to gibberellin A_3 which was detected in extracts from the cotyledonary tissue only.

12. Identification of gibberellins by GLC analysis is only tentative and some of the peaks may be due to residual impurities in the extracts.

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