The Effect of Low Temperature Storage on the Gibberellins of *Tulipa gesneriana* L. cv. Apeldoorn.

by

Elizabeth M. Kennedy

Thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy.

Department of Botany
University of Glasgow

June, 1988

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGEMENTS

I would like to thank Dr A. Crozier and Mr G. Hanks for their help and supervision, and Mrs A. Sutcliffe (Glasgow University) and Mr S.K. Jones (Institute of Horticultural Research, Littlehampton) for their technical assistance. I also wish to acknowledge Dr R. Atzorn and Dr C.G.N. Turnbull for providing technical help and useful discussion, and Professor E.W. Weiler and Dr J.P. Knox for provision of antiserum. In addition I would like to thank my parents for their unfailing support and encouragement.

CONTENTS

SUMMARY

During 12 weeks dry storage of tulip bulbs cv. 'Apeldoorn' at 5°C and 17°C, growth of the shoot, daughter bulbs and basal plate proceeded slowly. The relative growth rate (RGR) of each component appeared to be slightly higher at 17°C than 5°C. At both temperatures the RGR of the shoot was the highest of all the components, and after an early decrease at 5°C it then increased during the second 6 weeks of storage, although not to the initial level.

Initial analysis of whole bulb extracts by high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) suggested that gibberellin (GA) levels fell during 5°C storage. Three main peaks were found in the 0, 6 and 12 week extracts. One of these was similar to GA_1 while a second peak corresponded to $GA_{8/20}$. Later analysis was hampered by probable decomposition of GAs in the freeze-dried tissue during storage. Analysis of a GA_1-like peak in extracts of the individual bulb components indicated that it was highest in the basal plate and daughter bulb extracts, whilst it was not detected in many of the scale and shoot extracts.

Radiolabelled GA_3 and GA_4, injected into bulbs during cold storage, were both metabolised relatively slowly, but GA_4 was metabolised to a greater extent than GA_3. Metabolism was slower with increasing length of cold storage prior to injection. Analysis by HPLC and gel

permeation chromatography (GPC) indicated that the main metabolite of [^{14}C]GA_3 was a free GA. One of the [^{3}H]GA_4 metabolites seemed likely to be a GA conjugate whilst the other was a free GA.

ABBREVIATIONS

ABA	Abscisic acid
AMO-1618	2-*iso*propyl-4-[trimethyl-ammonium chloride]-5-methylphenyl piperidine-1-carboxylate.
Ancymidol	∝-cyclopropyl-∝-(p-methoxyphenyl) -5-pyrimidine methyl alcohol.
BSA	Bovine serum albumin
B_o	Maximum binding in the absence of unlabelled antigen
CCC	2-chloroethyltrimethyl ammonium chloride.
cpm	Counts per minute
dpm	Disintegrations per minute
DW	Dry weight
ECD	Electron capture detector
EtOAc	Ethyl acetate
FID	Flame ionisation detector
GA_n	Gibberellin A_n
GA-GE	Gibberellin glucosyl ester
GA-Me	Gibberellin methyl ester
GA-O-Gluc	Gibberellin-O-glucoside
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
LD	Long days
LDP	Long day plant

MeOH	Methanol
NAA	Naphthalene-1-acetic acid
N.D.	Not detected
PBS	Phosphate buffered saline
PVP	Polyvinylpolypyrrolidone
RGR	Relative growth rate
RIA	Radioimmunoassay
R_t	Retention time
SD	Short days
SE	Standard error
SIM	Selected ion monitoring
TCY	Tetcyclacis
THF	Tetrahydrofuran
TIBA	2, 3, 5-triiodobenzoic acid
UB	Unspecific binding
nW5°C	n weeks 5°C storage
nW17°C	n weeks 17°C storage

INTRODUCTION

INTRODUCTION

The genus *Tulipa* belongs to the family Liliaceae and there are 100-150 species of tulip (De Hertogh, Aung and Benschop, 1983), but the origin of the garden tulip is unknown (Rees, 1972). Classification of cultivated tulips has divided them into 15 groups, and the cultivar 'Apeldoorn', used in this study and commonly used commercially and experimentally, is a Darwin hybrid tulip, the result of hybridisation between Darwin tulips and *T. fosteriana* (De Hertogh, Aung and Benschop, 1983).

The tulip bulb consists of true scales (not leaf bases), surrounding a central axis which bears leaves and a terminal flower. The scales are separated by very short internodes, and daughter bulbs develop in the scale axils ready to replace the mother bulb, which dies after flowering. The bulb scales are fleshy storage organs rich in carbohydrates necessary for the considerable internal development which takes place while the bulbs are subterranean, and for the rapid shoot growth which occurs in spring (Algera, 1936, 1947; De Hertogh, Aung and Benschop, 1983). The whole bulb is surrounded by a brown, papery 'tunic' which is the exhausted remains of a once fleshy scale, developing during senescence of the above ground parts (Rees, 1972, 1984). The floral stem has 3-5 leaves and the flowers generally have 6 perianth segments, 6 stamens and 1 style with a 3-lobed stigma, and occur in a wide range of colours (De Hertogh, Aung and

Benschop, 1983).

LIFE CYCLE

The tulip is Asiatic in origin and its physiology and life cycle are closely related to its native habitat and climate of hot, dry summers and cold winters. The visible growing season of the tulip is short, lasting only a few months in the spring, after which the leaves, flower stem and mother bulb die, leaving the daughter bulbs to survive underground over the summer and winter. The bulbs have a cold requirement for stem extension and normal flower development (not for floral initiation which takes place in July), ensuring that flowering takes place once the warmer temperatures of spring have arrived and not during adverse winter conditions (Kamerbeek, Beijersbergen and Schenk, 1970; Rees, 1972, 1984).

Looking at the development of a single bulb, initiation occurs within its mother bulb sometime between February and July (Year 1), depending on its position within the mother bulb. The outermost daughter bulb is initiated in February and the process continues inwards until the innermost is initiated in July. Its scales are all initiated by October (Year 1), and the apex is then inactive through the winter until the following May (Year 2). This cold period seems to be necessary for the subsequent growth of the bulb (De Hertogh, Aung and Benschop, 1983; Rees, 1984), and it has been considered

whether it might also be required for floral initiation, which occurs in Year 2 (Rees, 1984).

In February (Year 2) this bulb starts to develop its own daughter bulbs so that there are 3 generations of bulbs present. The first leaf is initiated in May (Year 2) and the apex will only become reproductive once all the leaves (3-5) have been initiated. Non-flowering bulbs produce only 1 leaf (Hartsema, 1961; Rees, 1972, 1984; De Hertogh, Aung and Benschop, 1983). Temperature is very important for floral initiation and the optimum temperature is 17-20°C, but initiation will occur over the range 5-31°C (Rees, 1972, 1984; De Hertogh, Aung and Benschop, 1983). Bulb size also affects initiation and below the critical circumference of 6-9 cm a bulb will remain vegetative (Rees, 1972). If flower initiation does take place it occurs in July, coincident with initiation of the innermost daughter bulb and death after anthesis of the mother bulb (Rees, 1972). Floral organs develop from the perianth segments inwards, and the so-called Stage 'G' is the point when the gynoecium is fully developed and floral differentiation is considered to be complete (A.D.A.S., 1981; De Hertogh, Aung and Benschop, 1983).

The stem internodes are initiated during floral differentiation. When bulbs cv. 'Paul Richter' were stored at 17°C, reaching Stage G in mid-August, the first (lowest) internode was initiated in early August, and the process continued acropetally until the last (top) internode was visible at Stage G (Shoub and De Hertogh,

1975). Most of the first internode cells were produced during a 6 week period of cell division following initiation, and cell division also followed initiation of the last internode, continuing past the end of 17°C storage (Shoub and De Hertogh, 1975).

Root initiation and growth occur in late summer/autumn (Year 2), but the shoot does not emerge above ground until the next spring (Year 3) when it flowers, and the mother bulb then dies (Rees, 1972).

TULIP FORCING

Tulips have been grown in western Europe for four centuries and remain a very important flower crop. The features which suit tulips to their native habitat - no above-ground growth during the hot, dry summer and a cold requirement for proper flowering - are still present in commercially grown bulbs and can be manipulated by growers to control the time of flowering. The principle of bulb forcing, as it is called, lies in replacing low temperatures, which bulbs would receive naturally in winter, with an artificial cold treatment given out of season. The bulbs are then grown on in a heated glasshouse and can be brought into flower as early as December, depending on the cultivar. The forcing season is divided into early, mid- and late season, these being early to late December, early to late January and February to early April respectively. Not all cultivars are

suitable for forcing, but those that are suitable are particularly suited to one or two of these seasons. For example, 'Apeldoorn' is recommended for early/mid season forcing and 'Paul Richter', another cultivar commonly used in experiments, is recommended for early forcing (A.D.A.S., 1981). There are two main methods of forcing - traditional or standard forcing and five-degree or direct forcing (A.D.A.S., 1981).

TRADITIONAL FORCING Bulbs for traditional forcing are lifted from the ground in late June/July, the exact time depending on how soon they are required to flower (A.D.A.S., 1981). At lifting time the flower has not been initiated, and after lifting the bulbs are stored at a high temperature to encourage rapid initiation and differentiation. Exact treatments depend on the season for which flowers are required.

For early forcing bulbs are stored at 34°C for 7 days immediately after lifting, then at 20°C until they reach Stage G (A.D.A.S., 1981). Bulbs for mid-season forcing are stored at 20°C instead of 34°C, prior to cold storage which may begin after Stage G is reached. Bulbs are then stored dry at 9°C for 6 weeks ("Pre"- or "double-cooling"), followed by planting in boxes, and cooling for a further 8-16 weeks, duration dependent on variety. It is best to return the planted bulbs to controlled low temperature stores but they can also be stored outside. If stored outside, the boxes are either stacked on

pallets, surrounded by straw or black polythene, or placed on a standing ground and covered with straw, to keep the temperature down. The outside temperature is obviously more variable so that these methods are less reliable, adequacy of cold treatment being determined by examining the position of the flower bud (A.D.A.S., 1981). After cold storage the bulbs are transferred to the glasshouse, usually at 18°C, for rapid growth and flowering, normally in 3 weeks (A.D.A.S., 1981). Bulbs used for late season forcing do not receive pre-cooling; they are stored at 20°C after lifting until Stage G, then at 17°C till planting, either at 9°C or outdoors (A.D.A.S., 1981).

FIVE-DEGREE FORCING Five-degree or direct forcing is a more recent development. For early forcing the bulbs are lifted early and given 34°C heat treatment, then 20°C storage, in the same manner as for traditional forcing. For mid-season forcing the bulbs are stored at 20°C from lifting till Stage G, then at 17°C. Cold storage is given to dry bulbs at 5°C, and rooting only takes place once the bulbs are in the glasshouse, so that compared with traditional forcing the bulbs are at low temperature for a shorter time, but in the glasshouse for a longer time. The bulbs are stored for 9-12 weeks at 5°C, planted in the glasshouse and grown at 13-18°C for flowering after 6-9 weeks. Flower quality appears to be better if growth is started at a lower temperature and increased to 18°C rather than starting at 18°C immediately (A.D.A.S., 1981).

FLORAL BLASTING Timing of cold storage is important, as flower "blasting" (necrosis of the flower bud) may occur if it is started too early, either before, or too soon after, Stage G (De Munk and Hoogeterp, 1975). Too short a cold period or too low a temperature can also induce blasting (De Munk and Hoogeterp, 1975) as can high temperatures before planting which sometimes occurs during transport and is called "heating in transit" (Rees, 1972). Exposure to ethylene, either during storage or in the greenhouse, also promotes blasting, the effects being manifested during growth in the glasshouse (De Munk, 1973). Injection of gibberellin A_3 (GA_3), $GA_{4/7}$, benzyladenine or kinetin into the flower bud can overcome the ethylene effect whilst abscisic acid (ABA) and auxin appear to induce blasting (De Munk and Hoogeterp, 1975; De Munk and Gijzenberg, 1977).

STORAGE TEMPERATURE The normal temperature for direct forcing is 5°C, but -1°C storage for 6 or 9 weeks resulted in earlier flowering than cooling at 5°C. However, -1°C storage also caused more floral blasting, and after 15 weeks storage the 5°C cooled bulbs flowered earlier (Moe and Wickstrøm, 1979). Rees and Briggs (1975) found that starting cold storage at a low temperature and increasing it during storage was more effective than cooling at a constant temperature.

Unlike tulips Easter lilies require true vernalisation but the cold period can be nullified by subsequent high

temperatures (Rees, 1972). To determine whether this could also happen in tulips, bulbs were stored at high temperatures after 6 weeks at 9°C, and prior to planting and further cooling (Rees, 1973). The cold treatment appeared to be irreversible and flowering was hastened rather than delayed by warm treatment compared to controls (Rees, 1973).

STEM EXTENSION The extent and speed of stem extension seen on transfer to the greenhouse is most affected by the length of the prior cold period. High temperature storage or a short cold period lead to slow stem extension, but increasing lengths of cold storage lead to maximum growth rate being reached earlier in the greenhouse and to shorter time to anthesis (Rees, 1969; Moe and Wickstrøm, 1973). Short cold treatments also lead to poor flower quality, for example, the perianth of 'Paul Richter' flowers had green tips whilst in 'Apeldoorn' the tips or larger areas of the perianth were white (Rees, 1969; Moe and Wickstrøm, 1973).

Most cell division in the stem internodes occurs immediately after initiation (Shoub and De Hertogh, 1975) and rapid stem growth immediately prior to flowering is mainly by cell elongation, but some cell division does seem to take place later, in both forced and field-grown bulbs (Rees, 1969; Gilford and Rees, 1973; Shoub and De Hertogh, 1974, 1975). The last internode of 'Paul Richter' had a period of cell division during cold

storage, two weeks after planting at 9°C (Shoub and De Hertogh, 1975). Then, both the first and last internodes underwent cell division after transfer to the glasshouse, mainly in the epidermal cells, the nodal region and the vascular bundles (Shoub and De Hertogh, 1975). During the glasshouse phase the cells of the first internode increased from 45 μ to 200 μ at anthesis whilst the last internode cells increased from 30 μ to 220 μ (Shoub and De Hertogh, 1975).

The growth pattern of the stem during the greenhouse phase of forcing is sigmoid (De Hertogh and Breg, 1980). The lowest internode began its grand growth period first, followed sequentially by the other internodes until the grand growth period of the top internode began last but continued after anthesis, by which time growth of the other internodes had ceased (De Hertogh and Breg, 1980). Shoub and De Hertogh (1975) found that cell elongation in the first internode began earlier than in the last internode. Growth also appeared to be rhythmic, not continuous, occurring overall for 12-36 hrs then stopping for 12-24 hrs. The plants were grown under the prevailing natural photoperiod and the first internode seemed to grow more during the night, between 2000 hrs and 0800 hrs, than during the day (De Hertogh and Breg, 1980). Okubo and Uemoto (1984a and b, 1985) also observed that first internode elongation was stimulated by darkness and suppressed in the light.

It has been speculated that GAs and other growth regulators may be involved in tulip stem elongation and this had been approached in three ways :-

1)By examining the endogenous growth substances during and after the cold treatment.
2)By studying the effects of applied GAs and determining whether they can replace the cold treatment with respect to stem elongation and flowering.
3)By applying growth retardants and studying their effects on growth and endogenous growth substances.

1)ENDOGENOUS GROWTH SUBSTANCES

The endogenous growth substances of tulips have been the subject of study for some time, but there is still little unequivocal information about their possible relationship with stem elongation and flowering. The main method of analysis has been bioassays which, although useful as a first step in qualitative analysis, have many drawbacks if used as the sole quantitative method. The biological activities of individual GAs vary and also the extracts analysed were often relatively crude and the bioassay response can be inhibited or promoted by inhibitors and contaminants in an extract. These and other problems in the use of bioassays and interpretation of bioassay results, are clearly illustrated by Reeve and Crozier (1980) and Graebe and Ropers (1978). It seems

likely that in many cases the proposed changes in growth substance levels were due to inaccurate estimation rather than real changes.

GIBBERELLINS GA-like substances were first reported in the cultivar 'Elmus' and the results implied that rooting and cooling at 9°C resulted in an increase in their activity in the shoots and scales compared with non-cold-treated bulbs (Aung and De Hertogh, 1967). Dry cooling at 5°C also appeared to produce higher activity in cv. 'Ralph', but to a lesser extent (Aung and De Hertogh, 1968). The cold-induced increase appeared to be mainly due to the free GA-like substances (i.e. ethyl acetate fraction), although there was a small increase in the bound activity (i.e. aqueous fraction, re-extractable into ethyl acetate after acid hydrolysis) under 9°C cooling (Aung and De Hertogh, 1968). In the cultivar 'Elmus' the GA-like substances decreased at the start of low temperature treatment but increased towards the end of treatment (De Hertogh *et al.*, 1971). Van Bragt (1971), however, found that total GA-like substances in 'Apeldoorn' changed little over 12 weeks 5°C dry cooling, whereas after 12 weeks 20°C storage, levels had dropped.

Free and bound GA-like substances have been found in all tulip organs (Aung, De Hertogh and Staby, 1969a; De Hertogh *et al.*, 1971), and it is generally found that shoots contain greater activity than scales, and roots least activity (De Hertogh *et al.*, 1971; Van Bragt, 1971;

Einert, Staby and De Hertogh, 1972; Alpi and De Hertogh, 1975). However, results vary depending on which bioassay is used (De Hertogh *et al.*, 1971; Einert, Staby and De Hertogh, 1972). During 12 weeks storage free GA-like substances in the scales of 'Apeldoorn' appeared to increase at 5°C to a higher level than that found at 20°C, but the bound activity decreased to a similar low level at both temperatures (Van Bragt, 1971). After 12 weeks at either temperature the free activity in the buds had decreased to seemingly almost identical levels but the bound activity was lower in 5°C bulbs (Van Bragt, 1971). When rooted and cooled at three sequentially lower temperatures there was an apparent decrease in GA-like substances in 'Elmus' shoots due to a decline in free activity with bound activity remaining stable, whilst scale and root GA-like substances also decreased (Einert, Staby and De Hertogh, 1972). It was suggested that in the species *T. alberti* Regel. cold treated shoots contained low activity of free GA-like substances but non-cold treated shoots contained mainly bound activity (Rakhimbaev, Syrtanova and Solomina, 1978).

Traditional forcing involves the effect of moisture as well as temperature because the bulbs are rooted, and Aung and De Hertogh (1968) proposed that there was a greater increase in GA-like substances after rooting and cooling compared to dry cooling. Aung, De Hertogh and Staby (1971a) found that wet storage caused dramatic increases in activity of GA-like substances compared with dry

storage at 9°C and 17°C. Removal of the basal plate prior to wet storage appeared to result in a reduction of free and bound GA-like substances (Aung, De Hertogh and Staby, 1971a).

During greenhouse growth of rooted and cooled bulbs cv. 'Elmus' the total GA-like substances appeared to increase initially but then decrease (Einert, Staby and De Hertogh, 1972). The relatively high level in the scales appeared to be primarily due to the developing bulblets. Van Bragt (1971) found that the total GA-like substances in bulbs two weeks after planting at 15°C had decreased to a very low level, whether dry cooled beforehand or not. Bulbs grown at 13°C after 9 weeks 5°C precooling had decreased free GA-like activity and increased bound activity, but at 18°C free activity had increased and bound activity decreased (Aung, De Hertogh and Staby, 1969b).

When rooted and cooled 'Paul Richter' bulbs were grown for 1 week at 9°C or 17°C in continuous light or dark, the level of GA-like substances appeared to be higher in dark grown bulbs, mainly due to activity in the shoots and activity was highest in dark grown 17°C bulbs (Alpi and De Hertogh, 1975).

Field-grown bulbs of cv. 'Apeldoorn' exhibited two peaks of GA-like activity, in December/early January (prior to satisfaction of the cold requirement) and February-April (during rapid shoot extension and flowering). The acidic fraction generally contained the

highest activity but the noted increases also occurred in the basic and bound fractions (Hanks and Rees, 1980a). In the daughter bulb samples there was a main peak of activity in late October/November and a second, much smaller peak in March (Hanks and Rees, 1980a). Aung and Rees (1974) found lower activity in field-grown daughter bulbs than Hanks and Rees (1980a). Apart from the difficulties of comparing results from different studies, they used the dwarf pea bioassay which was also found by others to give low activity in daughter bulbs in comparison to other bioassays (De Hertogh *et al.*, 1971; Einert, Staby and De Hertogh, 1972).

It was suggested that bulbs of *T. alberti* Regel. and *T. ostrowskiana* Regel. (which were presumably field-grown) contained mainly bound GA-like activity during "dormancy" and that free activity increased at the onset of active growth (Syrtanova and Rakhimbaev, 1973).

No unequivocal identifications of GAs have been made in tulips, but tentative identifications have been made using gas chromatography (GC) in conjunction with a flame ionisation detector (FID), although this is not a particularly useful method for endogenous GA analysis. The FID is a non-specific mass detector, and if used for analysis of impure extracts the high background levels and spurious peaks mean that it is uncertain whether peaks detected really are GAs (Reeve and Crozier, 1975). However, these analyses indicated the presence of GA_5, GA_9 and GA_{13} in roots (Aung, De Hertogh and Staby, 1971b; De

Hertogh *et al.*, 1971), GA_1, GA_5, GA_8 and GA_9 in scales (Aung, De Hertogh and Staby, 1971b) and GA_{13} in daughter bulbs (Aung and Rees, 1974). De Hertogh *et al.* (1971) suggested that the shoot contained GA_1, GA_5, GA_8 and GA_9 but Aung, De Hertogh and Staby (1971b) found only GA_1.

INHIBITORS ABA has been unequivocally identified in non-cold-treated bulbs cv. 'Paul Richter' using GC with an electron capture detector (GC-ECD) and combined GC and mass spectrometry (GC-MS) (Terry, Aung and De Hertogh, 1982). ABA was detected in the shoot, scales and basal plate of non-cold-treated bulbs but not in the bulblets. The ABA levels in the basal plate and shoot were high - 3.6 and 2.6 times greater respectively, than that in the scales (Terry, Aung and De Hertogh, 1982).

An inhibitor found in the cultivars 'Golden Melody' and 'Paul Richter' co-chromatographed with ABA when analysed by thin layer chromatography, and had the same R_f as ABA in five different solvent systems (Aung and De Hertogh, 1979). The content of this ABA-like substance increased during dry storage at 5°C or 13°C and subsequent growth at 13°C. The activity was reported to be low in the scales and shoot but relatively high in the basal plate, bulblets and roots. The highest level during 5°C and 13°C storage appeared to be in the bulblets but after 5°C it decreased steadily during growth, whereas after 13°C it remained high. After 5°C storage the level in the basal plate increased after planting and the root content

decreased, whilst after 13°C the ABA-like substance in the basal plate and root increased (Aung and De Hertogh, 1979).

A growth inhibitor was found in *T. alberti* and *T. ostrowskiana* during "dormancy" but not during active growth (Syrtanova, Turetskaya and Rakhimbaev, 1973). In *T. alberti* an inhibitor, found in the apical bud but not the scales, seemed to have greater activity in cold treated bulbs (Rakhimbaev, Syrtanova and Solomina, 1978). The cultivar 'Purrissima', was given a 34°C treatment for rapid floral initiation, after which the ABA-like substances had decreased, but after 7 weeks at 5°C a high content was found in the basal plate (Suh *et al.*, 1983).

AUXINS Low auxin-like activity was detected in the scales and bud of "dormant" bulbs of *T. alberti* and *T. ostrowskiana* and during growth other apparently growth-stimulating substances were reported (Syrtanova, Turetskaya and Rakhimbaev, 1973). A low level of an indole-like compound was found in the apical bud and scales of cold-treated *T. alberti* bulbs whilst slightly higher levels of two indole-like compounds were found in non-cold-treated bulbs (Rakhimbaev, Syrtanova and Solomina, 1978).

CYTOKININS Cytokinin-like activity was found in *T. alberti* and it appeared to increase as the bulbs started active growth (Rakhimbaev and Solomina, 1975). Little

cytokinin-like activity was found in non-cold-treated bulbs of *T. alberti* but cold treatment appeared to increase activity (Rakhimbaev, Syrtanova and Solomina, 1978). A compound isolated from *T. alberti* was reported to be zeatin ribonucleoside (Solomina *et al.*, 1976).

2) EXOGENOUS APPLICATION OF GIBBERELLINS

It was hoped that the second approach of applying exogenous GAs to tulips, would result in the development of a chemical treatment to replace the long period of cold at present required for forcing tulips. The advantages to the growers would be that flowers could be produced even earlier in the season, flower production would be faster resulting in faster turnover and greater production and hopefully, the high costs of the lengthy cold storage would be reduced.

EFFECTIVENESS OF INDIVIDUAL GAs The most commonly used GA has been GA_3 because it is commercially available, but $GA_{4/7}$ has also been used and various other GAs have been tested. In comparison to GA_3, $GA_{4/7}$ generally seems more effective, although to a certain extent this depends on how it is applied. When GA was injected into uncooled bulbs after planting 1 mg $GA_{4/7}$ was as effective as 50 mg GA_3 whilst 1 mg $GA_{4/7}$ was equivalent to 10 or 50 mg GA_3 on bulbs cooled for 6 weeks at 5°C (Van Bragt and Zijlstra, 1971). Hanks and Rees (1980b) found $GA_{4/7}$ ten times as

effective as GA_3 when injected into 'Apeldoorn' bulbs after 8 weeks at 5°C, prior to planting. The cultivar 'Apeldoorn' responded to both GA_3 and $GA_{4/7}$ but 'Paul Richter' only responded to $GA_{4/7}$ (Hanks, 1982a and b). Hanks and Rees (1980b) tested a number of GAs and GA_3 was considerably less effective than the others. Their order of effectiveness in reducing the glasshouse period was as follows:-

$$GA_7 > GA_4 > GA_1 > GA_9 > GA_{13} > GA_5 >> GA_3$$

METHODS OF APPLICATION The methods of GA application tried have included lanolin paste (Rudnicki, Nowak and Saniewski, 1976; Hanks, 1982a), sprays (Cocozza Talia and Caputo, 1980; Hanks, 1982a) and hydroponic application (Hanks, 1982b), but these methods have generally been unsuccessful. Bulb soaks of almost fully cooled bulbs cv. 'London' had some effect (Bylov and Smirnova, 1979), whilst application of a GA solution to the base of the bulb seemed more effective if the tunic was removed and a cut was made in the base of the bulb (Cocozza Talia and Stellacci, 1977, 1979).

Bulb injection using a hypodermic syringe has been the most successful and commonly used method (eg. Van Bragt and Zijlstra, 1971; Rudnicki, Nowak and Saniewski, 1976; Van Bragt and Van Ast, 1976; Hanks, 1982b, 1984). The standard and most effective method of injection involves pushing the needle through the scales and discharging the

solution next to the shoot (Hanks and Rees, 1980b). Radiolabelled GA_3 injected into a bulb appeared to move easily through the bulb and was not rapidly metabolised (Hanks, 1979; Rees and Hanks, 1979).

Therefore injection is the most successful method on a laboratory scale but is impractical when considered commercially (Hanks, 1979, 1982a). Vacuum infiltration is the latest method considered for commercial use. Tymoszuk, Saniewski and Rudnicki (1979) used it successfully although they indicated that spread of infection could be a problem. Hanks and Rees (1983) applied GA_3 and $GA_{4/7}$ by vacuum infiltration and found it effective, whilst bulb soaks for 2-20 hours had little effect on the glasshouse period. With other methods $GA_{4/7}$ was generally more effective than GA_3 but this difference was less noticeable with vacuum infiltration (Hanks and Rees, 1983). There have been problems, mainly with surfactants and fungicides added to the GA solution which increased flower losses (Jones and Hanks, 1984) but this seems a promising method if the conditions can be optimised.

EFFECT OF GA TREATMENT ON TIME TO ANTHESIS Van Bragt and Zijlstra (1971) injected GA_3 into uncooled bulbs, cv.'Apeldoorn', before planting, and the number of days to flowering was reduced compared to controls. However, although the reduction was greater than that achieved by cooling for 6 weeks at 5°C, it was much less than that

achieved by full 12 weeks cooling (Van Bragt and Zijlstra, 1971). GA_3 applications by Van Bragt and Van Ast (1976) consistently reduced the time from planting to flowering within various storage treatments compared with their controls. Their results are not directly comparable to others using uncooled or partly cooled bulbs, because bulbs not stored for 12 weeks at 5°C were stored for 12 weeks at 21°C or 6 weeks at 5°C with another 6 weeks at 21°C either before or after this (Van Bragt and Van Ast, 1976). The number of days to flowering, compared to controls, was reduced by GA_3 treatment in cv. 'Jewel of Spring' (Cocozza Talia and Stellacci, 1977) and cv. 'Gudoshnik' (Cocozza Talia and Stellacci, 1979; Tymoszuk, Saniewski and Rudnicki, 1979), but not to the extent of being able to replace the cold treatment. Cocozza Talia and Caputo (1980) applied GA_3 to bulbs, cv. 'Gander', but the reduction in time to flowering was only significant in cooled bulbs.

GA_3 appears to be able to reduce the glasshouse period over a fairly wide range of temperatures; Hanks (1984) stored bulbs at temperatures between -2°C and 20°C and GA_3 reduced the glasshouse period by 15-25% within each temperature treatment.

Due to the lack of success in replacing the whole cold treatment, supplementary GA treatments used in conjunction with partial or full cold treatments are now considered more realistic. Hanks and Rees (1980b) stored bulbs, cv. 'Apeldoorn', for 8 rather than the full 12 weeks at 5°C

and injected them with GA_3 prior to planting. A 33 day reduction in time to flowering was obtained, and although the glasshouse time was still longer than for fully cooled bulbs, the overall time to flowering, including cold storage, was reduced (Hanks and Rees, 1980b). Repeated injections, given every 2 weeks from the start of the cold treatment till 4 weeks after planting, seemed to be more effective than a single standard injection (Hanks and Rees, 1980b). The glasshouse period of GA-treated, partly cooled bulbs cv. 'Apeldoorn', which have a long cold requirement, approached that of fully cooled bulbs (Hanks, 1982). GA treatment generally appears to be most successful on cultivars with long cold requirements (Rudnicki, Nowak and Saniewski, 1976), and 'Paul Richter', which has a medium cold requirement, was unresponsive compared with 'Apeldoorn' (Hanks, 1982a and b). The cultivar 'London' has a very long cold requirement of 20-22 weeks at 9°C and is not recommended for forcing, but GA treatment could induce satisfactory flowering after only 18 weeks of cold storage (Bylov and Smirnova, 1979). Application of GA to fully cooled 'Apeldoorn' bulbs could reduce the glasshouse period by 5-7 days compared to untreated controls (Jones and Hanks, 1984).

As well as reducing the glasshouse time precooling results in a reduction in the duration of flowering so that flowering within a batch of bulbs is synchronous. However, GA treatments tend to extend the duration of flowering slightly compared to controls (Cocozza Talia and

Stellacci, 1977, 1979; Cocozza Talia and Caputo, 1980; Hanks and Rees, 1980b). A more positive effect of exogenous GA is a reduction in floral blasting (De Munk and Hoogeterp, 1975; De Munk and Gijzenberg, 1977; Hanks and Rees, 1977).

EFFECT OF GAs ON STEM EXTENSION The cold period is not required just for rapid floral maturation, but also for rapid stem extension and synchronous flowering, and GA treatment tends to have adverse effects on both stem length and duration of flowering. GA treatment appeared to hasten floral maturation, but flowering occurred without the rapid upper internode extension normally associated with tulip flowering, although there was less difference in final stem length (Van Bragt and Van Ast, 1976; Tymoszuk, Saniewski and Rudnicki, 1979; Jones and Hanks, 1984). Some researchers found that GA_3 treated plants produced longer stems than non-GA_3-treated controls (Van Bragt and Zijlstra, 1971; Rudnicki, Nowak and Saniewski, 1976; Cocozza Talia and Caputo, 1980) but a reduction in stem length has been the more general finding.

Both upper and lower, but primarily lower, internodes are affected by the length of the cold period (Charles-Edwards and Rees, 1975), so that short cold treatments lead to short stems due to lack of extension of the lower internodes. Hanks (1982a and b) found that no first internode extension occurred after 0 or 4 weeks of the

required 12 weeks at 5°C, only after 8 or 12 weeks. When partly cooled bulbs cv. 'Apeldoorn' were treated with GA the upper internode was reduced resulting in shorter stems at anthesis, but GA_4, GA_7, GA_9 and GA_{13} all stimulated first internode extension (Hanks and Rees, 1980b). At anthesis the stems were longer than those of non-GA-treated controls but shorter than those of fully cooled bulbs (Hanks and Rees, 1980b). Hanks (1982a and b) also found that $GA_{4/7}$ stimulated first internode extension after longer cold treatments, but the stems were still shorter due to reduction in the middle and upper internodes. $GA_{4/7}$ does not appear to reduce stem length to the same extent as GA_9 (Hanks, 1982a and b; Hanks and Rees, 1983).

The upper internode is the last internode to start elongation (De Hertogh and Breg, 1980) and it is thought to be stimulated by auxin from the gynoecium (Hanks and Rees, 1977). Elongation of the top internode is drastically reduced if the whole flower, or the gynoecium alone, is removed but the effect on the lower internodes is much less (Hanks and Rees, 1977; Saniewski and De Munk, 1981). Indole-3-acetic acid (IAA) applied to the cut surface could reverse this effect (Op den Kelder, Benschop and De Hertogh, 1971; Hanks and Rees, 1977; De Munk, 1979; Saniewski and De Munk, 1981), as could naphthalene-1-acetic acid (NAA) but not GA or kinetin (Op den Kelder, Benschop and De Hertogh, 1971). The final stem lengths of GA_9 treated 'Apeldoorn' bulbs were longer than controls

due to upper internode extension after instead of before flowering (Hanks, 1979, 1982b; Rees and Hanks, 1979). The capsule does not normally develop in 'Apeldoorn' as it is a sterile hybrid, but parthenocarpic capsule development takes place in GA-treated plants followed by upper internode extension, possibly stimulated by auxin from the capsule (Hanks, 1979, 1982a; Rees and Hanks, 1979).

3) GROWTH RETARDANTS

The growth retardant ancymidol (α-cyclopropyl-α-(p-methoxyphenyl) -5-pyrimidine methyl alcohol) inhibits GA biosynthesis by inhibiting oxidation of *ent*-kaurene (Coolbaugh and Hamilton, 1976; Coolbaugh, Hirano and West, 1978). It reduced stem length in tulips, the effect being greatest in the lowest internode and lessening acropetally (Op den Kelder, Benschop and De Hertogh, 1971; Shoub and De Hertogh, 1974; Briggs, 1975; Hanks and Rees, 1977). Cold treatment and exogenous GAs mainly affect first internode extension whilst the upper internode appears to be controlled by auxin (Charles-Edwards and Rees, 1975; Hanks and Rees, 1977, see *Effect of GAs on stem extension*), but ancymidol has also been found to reduce upper internode extension indicating the involvement of GAs (Hanks and Menhenett, 1979).

Cell division, normally occurring in the first internode during the first 12 days of glasshouse growth,

was affected by ancymidol and ceased by day 8 (Shoub and De Hertogh, 1974). Cell elongation was also reduced whilst radial expansion was increased (Shoub and De Hertogh, 1974). Application of $GA_{4/7}$, but not GA_3, could overcome the effect of ancymidol, especially if applied simultaneously (Shoub and De Hertogh, 1974; Hanks and Rees, 1977).

Both ancymidol and TIBA (2,3,5,-triiodobenzoic acid), which inhibits basipetal auxin transport, inhibited dark-induced first internode elongation (Okubo and Uemoto, 1985). If the stem was decapitated leaving only the first internode, it was responsive to IAA alone (in the dark) and to IAA combined with GA_3 or $GA_{4/7}$ (light or dark) but not to GA_3 or $GA_{4/7}$ alone (Okubo and Uemoto, 1985). Okubo and Uemoto (1985) also reported that free GA-like and auxin-like activity, detected by bioassay, increased in the dark and decreased in the light whilst changes in the bound activity were the opposite of the free changes and under natural light auxin-like activity was low. Ancymidol appeared to reduce the increase in free GA-like activity during dark treatment and the activity of bound GA-like substances decreased till they were undetectable. TIBA and ancymidol both reduced diffusible auxin-like activity but TIBA did not alter the changes in GA-like activity (Okubo and Uemoto, 1985). Okubo and Uemoto (1985) speculated that auxin may be the primary controller of first internode elongation but that GAs are somehow involved, possibly by modifying auxin transport, synthesis

or action, or the response of the internode to auxin.

THE INVOLVEMENT OF GAs IN OTHER BULBOUS SPECIES

Not all bulbous species have the same type of life cycle as the tulip - three types of dormancy have been identified and there may be more (Kamerbeek, Beijersbergen and Schenk, 1970; Rees, 1984). In 'Tulip-type' dormancy the bulbs are not truly dormant and slow shoot growth continues during the cold period required for subsequent shoot extension. Floral initiation occurs before the cold period starts. In 'Lily-type' dormancy the cold period is required to overcome true dormancy, and the flower is initiated after stem elongation has started. Ohkawa (1976) found that GA-like substances in *Lilium speciosum rubrum* decreased during optimum cold storage but increased at 21°C. Experiments using exogenous GAs and ancymidol suggested that GAs could be involved in stem elongation and floral development but not floral initiation and differentiation. GAs could only hasten flowering in fully cooled bulbs in which flower initiation would take place and therefore could not replace the cold treatment (Ohkawa, 1976).

In 'Iris-type' dormancy the bulbs experience an imposed summer dormancy which can be overcome by more favourable environmental conditions, and they have a cold requirement for floral initiation (Kamerbeek, Beijersbergen and Schenk, 1970; Rees, 1984). GA-like

substances have been found in iris (Rodrigues Pereira, 1964; Aung, De Hertogh and Staby, 1969a) and they appeared to increase at the end of cold treatment, the highest activity being detected in the flower primordia (Alpi *et al.*, 1976). Sano (1975) attempted to replace the cold treatment with GA but, as with tulip and lily, only with partial success.

GAs AND STEM EXTENSION IN NON-BULBOUS SPECIES

Applied GAs can induce flowering in cold-requiring plants (e.g. *Raphanus sativus*, Suge and Rappaport, 1968) and long day plants (LDP) (e.g. *Samolus parviflorus*, Baldev and Lang, 1965) under non-inductive conditions, but only in plants which are rosettes in the vegetative state (Zeevart, 1983). Two processes occur during flowering - floral development and stem elongation - and when environmentally induced they occur simultaneously (Zeevart, 1983). However, exogenous GA generally causes stem elongation first, followed by flower formation or, in some cases, stem elongation alone (e.g. the cold-requiring plant *Lunaria annua*, Zeevart, 1968). Zeevart (1983) concluded that GAs are mainly involved in stem growth, as opposed to floral initiation, and that flowering accompanies stem growth but is a separate process. In tulips of course, the cold requirement is not involved in floral initiation which occurs before the cold period, but in normal flower development and rapid stem extension to

carry the flower out of the bulb and clear of the leaves (Rees, 1972).

Endogenous GAs have been examined in induced and non-induced plants to determine whether there are changes in the total and individual GA levels and in the GAs present (see Zeevart, 1983 and Pharis and King, 1985). Growth retardants which inhibit GA biosynthesis have also been used to try and elucidate the possible role of GAs in stem extension and flowering. Very often these retardants inhibit stem elongation but flowering still occurs. However, there are cases where inhibitors do inhibit flower formation, as in the LDP *Samolus parviflorus* where AMO-1618 (2-*iso*propyl-4-[trimethyl-ammonium chloride]-5-methylphenyl piperidine-1-carboxylate) and CCC (2-chloroethyltrimethyl ammonium chloride) inhibited stem elongation and flower formation, and applied GA_3 could overcome this effect (Baldev and Lang, 1965).

In *Raphanus sativus* the level of GA-like substances in plants grown from vernalised seed appeared to be higher than that in plants grown from non-vernalised seed and a similar situation was found in plants which had or had not been vernalised (Suge, 1970). CCC reduced the level of GA-like substances and reduced stem elongation in *R. sativus* but did not prevent flowering (Suge and Rappaport, 1968; Suge, 1970). Inductive conditions also produced an increase in extractable GA-like activity in the LDP *Silene armeria* and activity was higher in the shoot tips than in the leaves and stem (Cleland and Zeevart, 1970). When *S.*

armeria was treated with AMO-1618 the endogenous GA levels appeared to decrease and stem elongation was inhibited, but flowering still took place (Cleland and Zeevart, 1970).

Stem elongation in the LDP *Agrostemma githago* was reduced by AMO-1618 but this could be overcome by simultaneous application of GA_{20} (Jones and Zeevart, 1980a). The growth retardant TCY (tetcyclacis) also reduced GA levels and suppressed stem elongation, and the growth suppression could be overcome by applied GA_1 (Zeevart, 1985). Even after 11 inductive LD AMO-1618 could inhibit stem elongation, implying that continuing GA biosynthesis was required for ongoing extension (Jones and Zeevart, 1980a). During photoperiodic induction there was a transient increase in GAs after 8-10 LD but after 14-16 LD the level had fallen again to a level similar to that under SD (Jones and Zeevart, 1980a). Seven GAs have been identified in *A. githago* by GC-MS: GA_{53}, GA_{44}, GA_{19}, GA_{17}, GA_{20}, GA_1 and 3-epi-GA_1 (Jones and Zeevart, 1980b). Using GC-MS in the selected ion monitoring mode (SIM) transient peaks of GA_{53}, GA_{44}, GA_{19}, GA_{17} and GA_{20} were found after 8 LD whereas GA_1 peaked 4 days later and 3-epi-GA_1 showed similar changes to GA_1 (Jones and Zeevart, 1980b). GC-SIM was used to monitor the individual GAs in AMO-1618-treated plants under LD and SD and the results indicated that the metabolism rates of individual GAs increased under LD compared to SD except for GA_{53} which had a high rate under both (Jones and Zeevart, 1980b).

Under SD GA_{44} and GA_{19} decreased, but more slowly than under LD, whilst there was little change in GA_1 and 3-epi-GA_1. Under LD GA_{44} and GA_{19} decreased rapidly while GA_{17} and GA_{20} were stable for the first day then declined. During the first 2 LD GA_1 increased and then started to decrease, and 3-epi-GA_1 increased during the first day but started to decrease before GA_1 did (Jones and Zeevart, 1980b).

In the LDP Spinach LD seemed to result in increased sensitivity of plants to applied GA, and AMO-1618 experiments indicated that under LD the rate of GA turnover may be increased (Zeevart, 1971). Three zones of GA-like activity were detected by the *d-5* corn bioassay under both SD and LD, and their levels varied according to the photoperiod (Zeevart, 1971). Zones I and II were further identified by GC-MS as GA_{19} and GA_{20} respectively, and GA_{53}, GA_{44}, GA_{17} and GA_{29} were also identified (Metzger and Zeevart, 1980a). By considering the structure of these GAs the following metabolic sequence was proposed (Metzger and Zeevart, 1980a) :-

$$GA_{53} \longrightarrow GA_{44} \longrightarrow GA_{19} \longrightarrow GA_{20} \longrightarrow GA_{29}$$

When measured quantitatively by GC-SIM, GA_{19} decreased under LD whilst GA_{20} increased prior to stem elongation (Metzger and Zeevart, 1980b), agreeing with the results concerning zones I and II from the *d-5* corn assay (Zeevart, 1971). It has been suggested that GA_{20} is

active *per se* in spinach, that the level of GA_{20} may be important for stem elongation and that the proposed conversion of GA_{19} to GA_{20} is photoperiodically controlled (Metzger and Zeevart, 1980b). When deuterium-labelled GA_{53} was fed to spinach, [^{2}H]GA_{19} and [^{2}H]GA_{44} were found under SD, but under LD only [^{2}H]GA_{20} was found (Gianfagna, Zeevart and Lusk, 1983). Plants given 4 SD followed by 2 LD contained all three metabolites but [^{2}H]GA_{19} and [^{2}H]GA_{44} levels were lower than after 6 SD. Although the individual steps in the sequence have not been determined these data demonstrate that both GA_{19} and GA_{20} are metabolites of GA_{53}, and furthermore imply that GA_{19} is the likely precursor of GA_{20} (Gianfagna, Zeevart and Lusk, 1983). Other metabolic studies have shown the conversion of [^{3}H]GA_{20} to the 3-epimer of GA_{1} (not endogenous and thought to be an artefact), and to the 2β-hydroxylated GA GA_{29} (Metzger and Zeevart, 1982). The conversion of GA_{20} to GA_{29} via 2β hydroxylation is thought to be an inactivation step, and [^{3}H]GA_{29} accumulated more rapidly under LD than SD (Metzger and Zeevart, 1982).

Studies such as those described indicate that GAs do have a role in stem elongation, although it is not completely understood. Rapid stem elongation is one of the effects of cold on tulips but, as explained before, GAs cannot completely substitute for the cold treatment in this respect and their involvement is not fully understood (see *Effect of GAs on stem extension*). Most of the information on the endogenous GAs of tulips has been

obtained using bioassays. In this investigation radioimmunoassays were used in an attempt to provide more accurate information on GA levels in whole bulbs of the cultivar 'Apeldoorn', and in the individual bulb components, after 0, 4, 6, 8 and 12 weeks 5°C storage and parallel 17°C storage. The growth of the bulb was also followed at the same stages of storage. GA_3 and GA_4 have often been used to try and replace the cold treatment, and bulbs were injected with radiolabelled GA_3 and GA_4 at the beginning, middle and end of cold storage to determine how applied GAs are metabolised, and whether metabolism changes with increasing prior cold storage.

MATERIALS AND METHODS

MATERIALS AND METHODS

PLANT MATERIAL English-grown bulbs of tulip cv. 'Apeldoorn', graded 12+ cm in circumference, were obtained from O.A. Taylor and Sons, Ltd from the same stock source. On arrival, the bulbs were dipped in 0.2 g l^{-1} benomyl, to protect against fungal diseases, then stored at 20°C during which bulbs were dissected at intervals to determine the stage of floral differentiation (A.D.A.S., 1981). After Stage G (gynoecium visible) was reached the bulbs were transferred to 17°C until cold storage began.

GROWTH ANALYSIS After 0, 4, 6, 8 and 12 weeks at 5°C and 17°C, 10 bulbs were taken for a growth analysis coincident with samples taken for growth substance analysis. Each bulb was divided into scales, shoot, basal plate and daughter bulbs and the fresh weight of each organ was recorded as was shoot length. Dry weights were recorded after the tissue had been dried at 80°C for 2 days.

METHODS FOR RADIOIMMUNOASSAY ANALYSIS

STORAGE TREATMENTS On 12 September 1984 bulbs were sampled immediately, or were stored at 5°C or 17°C for 4, 6, 8, or 12 weeks. After the appropriate storage treatment whole bulb samples were taken, washed following removal of the tunic (dry outermost scale), split in half (through the shoot), frozen in liquid N_2 and freeze-dried.

Further bulbs were dissected into scale, shoot, basal plate and daughter bulbs after removal of the tunic and washing. The component parts were frozen in liquid N_2 and freeze-dried separately.

EXTRACTION AND PURIFICATION The freeze-dried material was extracted x 3 with methanol (MeOH) and the extracts combined. The MeOH was removed *in vacuo* at 35-40°C and the resultant aqueous phase diluted with 0.5 M phosphate buffer, pH 8.0, and partitioned x 3 against $^1/_2$ volumes petroleum spirit (B.R. 40-60°C). The aqueous phase was slurried with insoluble polyvinylpolypyrrolidone (PVP) (50 mg ml^{-1}) for 1 hour. After filtration, the aqueous phase was adjusted to pH 2.5 with 50% sulphuric acid and partitioned against 5 x $^2/_5$ volumes ethyl acetate (EtOAc). The aqueous phase was discarded and the EtOAc fraction was cooled in a deep freeze so that the aqueous residue could be removed by filtration. The EtOAc fraction was further dried with anhydrous sodium sulphate and reduced to dryness *in vacuo* at 35-40°C after the addition of toluene.

ION EXCHANGE CHROMATOGRAPHY The first samples extracted were further purified using the anion exchange material DEAE-52 in the acetate form (Whatman). The extracts were dissolved in 80% MeOH in 0.02 M ammonium acetate buffer, pH 6.5, and eluted from the column with 0.01 M ammonium acetate buffer, pH 6.5 and 0.5 M acetic acid. The pH of the aqueous phase was adjusted to 2.5 prior to

partitioning against 5 x $^{2}/_{5}$ volumes EtOAc. The aqueous residue in the EtOAc fraction was removed and the extract reduced to dryness *in vacuo* as described above.

The ion exchange step and subsequent EtOAc partitioning were not included in later purification procedures as they seemed unnecessary.

C_{18} SEP PAK The C_{18} Sep Pak cartridge (Waters Associates) was wetted with 10 ml 70% MeOH in 20 mM ammonium acetate buffer, pH 3.5. The samples were dissolved and passed through the cartridge in 5 ml 70% MeOH followed by a further 5 ml 70% MeOH to wash the cartridge. These eluates were combined and reduced to dryness *in vacuo* at 35-40°C.

INTERNAL STANDARD An internal standard of [2,3-^{3}H]GA_{9} (specific activity 50 Ci mmol^{-1}, synthesised by Amersham International by the method of Yokota *et al.*, 1976) was added to the MeOH used for extraction prior to maceration of the tissue. Aliquots of extracts were counted at frequent intervals during extraction and purification, using an LKB 1211 Minibeta liquid scintillation counter, so that losses could be estimated. The scintillation cocktail used was either Ecoscint (National Diagnostics) or Triton scintillant consisting of 5 g 2, 5-diphenyloxazole (PPO) plus 0.3 g 1, 4-di-[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl -POPOP) dissolved in 200 ml Triton X-100 and 800 ml toluene.

Counts per minute (cpm) were converted to disintegrations per minute (dpm) using the channels ratio method.

METHYLATION Two of the antisera used in subsequent radioimmunoassays required methylated samples and, where necessary, extracts were dissolved in a small quantity of MeOH and methylated with ethereal diazomethane. Methylation was checked by high performance liquid chromatography (HPLC) of aliquots of the extract to determine conversion of the [^{3}H]GA_{9} (internal standard) to [^{3}H]GA_{9} methyl ester (GA_{9}-Me) as described below. If derivatisation was not complete the samples were re-methylated.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) Solvent was delivered at a flow rate of 1 ml min^{-1} by a Spectra Physics SP8100 liquid chromatograph. Samples were introduced via a pneumatically operated Valco injection valve fitted with a 250 or 1000 µl sample loop. Reversed-phase separations were carried out on a 250 x 5 mm i.d. column packed with 5 µm ODS Hypersil (Shandon) and eluted with varying ratios of MeOH and 1% aqueous acetic acid (pH 3.0).

The degree of conversion of [^{3}H]GA_{9} to [^{3}H]GA_{9}-Me indicated whether methylation of extracts had been successful, and to monitor this isocratic reversed phase HPLC at 70% MeOH was used in conjunction with a

radioactivity monitor. Column eluate was mixed with scintillant (3 ml min^{-1}) and passed to a radioactivity monitor comprising of a Coruflow scintillation counter (ICN Tracerlab) fitted with a 300 µl spiral glass flow cell. The scintillant consisted of 10 g PPO dissolved in 330 ml Triton X-100, 670 ml xylene and 150 ml MeOH. Counting efficiency was *ca.* 15% for ^{3}H.

Prior to immunoassays extracts underwent gradient elution reversed phase HPLC using the following solvent programs:-

a) Non-methylated extracts

0-30 min, 30-100% MeOH; 30-35 min, 100% MeOH.

b) Methylated extracts

0-30 min, 40-100% MeOH; 30-35 min, 100% MeOH.

Fractions were collected every 0.6 min and the fractions were reduced to dryness in a 'Speed Vac' sample concentrator (Savant).

RADIOIMMUNOASSAYS Three different antisera were used for radioimmunoassays (RIA). The procedures used, as described below, were based on the method of Atzorn and Weiler (1983). Any variations in this procedure, in the use of the 398 Pool antiserum and MAC 136 antiserum, are noted subsequently.

GA_1-Me Antiserum. This rabbit antiserum was raised in Glasgow against [7-bovine serum albumen (BSA)]GA_1. The

cross reactivities of this antiserum are shown in Table 1.

The reaction mixtures, consisting of 275 µl 0.1 M phosphate buffered saline (PBS) pH 7.4, 25 µl 1% (w/v) γ-globulin, 3 µl [1,2-^{3}H]GA_1-Me (10 000 cpm, 31 Ci mmol^{-1}), 50 µl GA_1-Me standard or methylated extract and 50 µl GA_1-Me antiserum diluted 1:5, were incubated in 2.0 ml polypropylene, conical bottomed scintillation vials (Sarstedt). After 90 min incubation at room temperature 500 µl 90% saturated ammonium sulphate was added to precipitate proteins. After 20 min the vials were centrifuged at 7800 *g* for 3 min in an MH_2 microcentrifuge (Sarstedt) and the supernatants discarded. One ml 50% saturated ammonium sulphate was added followed immediately by further centrifugation at 7800 *g* for 3 min. The supernatants were discarded, the pellet mixed with 1 ml Minisolve 1 scintillation cocktail (Koch Light Laboratories Ltd). The amount of radioactivity bound to the pellet was estimated in an LKB 1211 Minibeta liquid scintillation counter.

The standards used were 0.03, 0.1, 0.2, 0.5, 0.7, 1.0, 2.0, 5.0, 7.0 and 10.0 pmol GA_1-Me and they were assayed in triplicate whilst samples were assayed in duplicate. The UB (unspecific binding) value was determined by replacing the sample/standard and antiserum in the reaction mixture with 100 µl distilled water. B_o (maximum binding in the absence of unlabelled antigen) was determined by replacing the sample/standard with 50 µl distilled water. The data obtained were transformed to

TABLE 1 % Cross Reactivity of GA methyl esters with rabbit anti-[7-BSA]-GA_1.

GA-Me	% Cross Reactivity
GA_1-Me	100
GA_7-Me	72
GA_3-Me	65
GA_{20}-Me	43
GA_4-Me	38
GA_5-Me	12
GA_6-Me	7
GA_9-Me	2

TABLE 2 % Cross Reactivity of GA methyl ester with 398 pool GA_1 antiserum.

GA-Me	% Cross Reactivity
GA_1-Me	100
GA_4-Me	109
GA_3-Me	41
GA_7-Me	30
GA_8-Me	1.1
GA_{20}-Me	<0.4
GA_{12}-Me	n.d.
GA_{24}-Me	n.d.

logit [B/B_o] values, where B is the labelled tracer bound in the presence of unlabelled antigen, corrected for UB (Rodbard, 1974). A logit/log GA_1-Me standard curve was plotted and amounts of GA in extracts were calculated by reference to this curve.

398 Pool GA_1 Antiserum. This antiserum was received from Professor Weiler, University of Osnabrück, and its cross reactivities are shown in Table 2.

The reaction mixture was the same as in the original method given. One µl [^{3}H]GA_1-Me (10 000 cpm, 32.6 Ci $mmol^{-1}$) was used per vial and the antiserum was diluted 1:200 with PBS for use. The original method was followed until the pellet was dissolved in 300 µl distilled water, mixed with 1 ml Ecoscint scintillation cocktail (National Diagnostics) and counted as before.

Standards of GA_4-Me at 0.03, 0.1, 0.3, 0.6, 1.0, 3.0, 6.0 and 10.0 pmol were used and results were calculated as before.

MAC 136 Antiserum. AFRC MAC 136 cell culture supernatant was received from Dr J.P. Knox, Institute of Animal Physiology, Babraham, Cambridge. It was for use with non-methylated samples and had an estimated affinity for GA_1 of 7.5×10^7 l mol^{-1} and a detection range of 2-100 pmol (Knox *et al.*, 1987). The cross reactivities for this antiserum are given in Table 3.

The reaction mixture consisted of 285 µl 0.1 M PBS,

TABLE 3 % Cross Reactivity of GAs with MAC 136 cell culture supernatant (Knox *et al.*, 1987).

GA	% Cross Reactivity	GA	% Cross Reactivity
GA_{53}	167	GA_{14}	0.7
GA_{16}	115	GA_4	0.4
GA_1	100	GA_7	0.3
GA_3	100	GA_9	0.2
GA_{20}	100	GA_{72}	0.1
GA_{29}	100	GA_1-Me	0.1
GA_6	80	GA_{10}	<0.09
GA_8	53	GA_2	0.07
GA_5	48	GA_{45}	0.05
GA_{60}	4	GA_{61}	0.04

pH 7.4, 15 µl horse serum (Flow Laboratories Ltd), 2 µl [^{3}H]GA_1 (7000 cpm, 32.6 Ci mmol^{-1}, Amersham International), 50 µl non-methylated extract or standard plus 50 µl MAC 136 antiserum diluted 1:3 with PBS. After a 90 min incubation 500 µl 100% saturated ammonium sulphate was added and after 20 min the vials were centrifuged at 13 000 *g* for 5 min. The supernatant was discarded and 1 ml 100% saturated ammonium sulphate added followed by centrifugation at 13 000 *g* for 5 min. After the supernatant had been discarded the pellet was dissolved in 300 µl distilled water, mixed with 1 ml Ecoscint scintillation cocktail and counted as described previously.

GA_3 standards were used at 1, 2, 5, 7, 10, 20, 50, 70 and 100 pmol and the results were calculated as before.

RADIOLABELLED GA FEEDS

STORAGE TREATMENTS Five-degree storage began on 18 October 1984 and the bulbs were stored for 0, 6 and 12 weeks.

SUBSTRATES AND FEED PROCEDURE Bulbs were fed with the following GAs after 0, 6 and 12 weeks at 5°C :-

1) 7.326×10^5 dpm [1,2-^{3}H]GA_4 (specific activity 1.5 Ci mmol^{-1}).

2) 7.326×10^5 dpm [1,7,12,18-^{14}C]GA_3 (specific activity 15 mCi mmol^{-1}, Amersham International).

The substrates were dissolved in 0.25 ml (0 week feed) or 0.2 ml of an aqueous solution containing 0.05% (v/v) Tween 20 and 5% (v/v) ethanol (Jones and Zeevart, 1982). The solutions were injected with a hypodermic syringe, through the bulb scales into the space close to the shoot (Hanks, 1984). The incubation times for each feed are shown in Table 4. After the appropriate incubation time the bulbs were frozen in liquid N_2 and freeze-dried. Controls were frozen in liquid N_2 immediately after injection and freeze dried.

TABLE 4 Number of days incubation of the bulb samples fed [^{3}H]GA_4 or [^{14}C]GA_3 after 0, 6 or 12 weeks 5°C storage.

GA FED	NO. WEEKS 5°C STORAGE	INCUBATION TIME (days)
[^{3}H]GA_4	0W5°C	1 4 7 14
	6W5°C	0 4 7 14 21
	12W5°C	0 4 7 14 33
[^{14}C]GA_3	0W5°C	0 1 4 7 14
	6W5°C	0 4 7 14
	12W5°C	0 4 7 14

EXTRACTION AND PURIFICATION The freeze-dried tissue was extracted with 3 x 100 ml MeOH and the combined methanolic extract was reduced to the aqueous phase *in vacuo* at 35-40°C. The aqueous phase was diluted with 0.5 M phosphate

buffer, pH 8.0, filtered through alpha-floc and partitioned against 3 x $^{1}/_{2}$ volumes petroleum spirit (B.R. 40-60°C). The pH of the aqueous phase was adjusted to 2.5 with 50% sulphuric acid prior to partitioning with 5 x $^{2}/_{5}$ volumes EtOAc. The aqueous residue was removed from the EtOAc phase by freezing and drying with anhydrous sodium sulphate and it was then taken to dryness *in vacuo* at 35-40°C.

HPLC Solvent was delivered at a flow rate of 1 ml min^{-1} by a Spectra Physics SP8100 liquid chromatograph. Samples were introduced off-column via a pneumatically operated Valco injection valve fitted with a 250 µl sample loop. Reversed-phase separations were carried out on a 250 x 5 mm i.d. column packed with 5 µm ODS Hypersil (Shandon) eluted with mixtures of MeOH and 1% aqueous acetic acid (pH 3). For [^{3}H]GA_4 samples the following solvent programs were used :-

1)0-20 min., 40-100% MeOH; 20-25 min., 100% MeOH.

2)0-30 min., 20-100% MeOH; 30-35 min., 100% MeOH.

For [^{14}C]GA_3 samples the following solvent program was used :-

a)0-20 min., 20-50% MeOH; 20-25 min., 50-100%; 25-30 min., 100% MeOH.

The column eluate was mixed with scintillant and analysed with an on-line radioactivity monitor as described previously. Counting efficiency was *ca.* 15% for ^{3}H and *ca.* 60% for ^{14}C.

Unlabelled free GAs and GA conjugate standards were detected with an LC871 UV monitor (Pye Unicam) set at 206 nm.

GEL PERMEATION CHROMATOGRAPHY After analysis of the metabolism extracts by gradient HPLC some of the extracts were combined and subjected to GPC. The combined samples were redissolved in 500 µl MeOH plus 500 µl tetrahydrofuran (THF) and subjected to GPC on a Biobeads SX-4 column (2 m x 25 mm) eluted with THF at a flow rate of 2 ml min^{-1} (Reeve and Crozier, 1976). Six ml fractions were collected from elution volume 250-500 ml.

CHAPTER 1

GROWTH ANALYSIS

Introduction

After 0, 2, 4, 6, 8 and 12 weeks storage at 5°C and 17°C ten bulbs were taken for growth analysis, correspondent with the samples taken for analysis by radioimmunoassay. Measurements were made of the fresh and dry weights of the individual parts of the bulb - the scales, basal plate, shoot and daughter bulbs - plus the shoot length. The patterns of changes were the same whether expressed in fresh or dry weights and so the results are only given in dry weight. However the two sets of results gave an indication of the water content, and in the shoot, basal plate and daughter bulbs it fell during storage, to a greater degree at 5°C than 17°C. The water content of the scales did not undergo any major change at either temperature.

Whole Bulb

The general trend during 5°C storage was of a decrease in average dry weight, apart from a small increase between 4 and 6 weeks (Table 1). However, all the samples had high standard errors and, apart from the 12 week sample, they were not significantly different to the 0 week material. At 17°C the average dry weight fluctuated but the final dry weight was close to the initial value, whereas after 12 weeks at 5°C the average dry weight had decreased by approximately 2 g.

TABLE 1 Dry weight (DW) of the whole bulb at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

STORAGE PERIOD (weeks)	AVERAGE DW +/-SE (g)	
	5°C	17°C
0	15.85 +/-0.99	15.85 +/-0.99
4	15.58 +/-1.15	14.08 +/-0.74
6	15.64 +/-0.38	14.79 +/-0.76
8	14.98 +/-0.93	14.26 +/-0.54
12	13.76 +/-0.79	15.71 +/-0.72

Bulb Composition

Expression of the dry weights of the component parts of the bulb as percentages of the total dry weight indicated that at both temperatures the percentage of the scales reduced with time, whilst that of the basal plate, shoot and daughter bulbs increased (Tables 2a and b). The percentages of the basal plate and shoot increased steadily during storage, but the changes in the daughter bulbs were more erratic. The final percentages of the scales were similar at 5°C and 17°C, whilst the shoot and daughter bulb percentages were higher at 17°C than 5°C and the basal plate percentage was slightly higher at 5°C. During the first 8 weeks of storage the percentage of the basal plate was higher than that of the shoot, but the 12 week values were closer, particularly at 17°C.

TABLES 2a and b Change in the scales, basal plate, shoot and daughter bulbs during 12 weeks storage at 5°C and 17°C, expressed as a percentage of the total dry weight (DW). The first 0 week value is a combined value for the scales and basal plate.

TABLE 2a 5°C Storage

STORAGE PERIOD (weeks)	% OF TOTAL DW			
	SCALE	BASAL PLATE	SHOOT	DAUGHTER BULBS
0	99.66		0.15	0.19
4	97.63	1.39	0.76	0.22
6	97.38	1.62	0.83	0.17
8	96.75	1.92	1.09	0.24
12	95.28	2.24	2.11	0.36

TABLE 2b 17°C Storage

STORAGE PERIOD (weeks)	% OF TOTAL DW			
	SCALE	BASAL PLATE	SHOOT	DAUGHTER BULBS
0	99.66		0.15	0.19
4	97.64	1.54	0.65	0.17
6	97.15	1.65	0.96	0.23
8	96.48	1.82	1.33	0.37
12	95.26	2.15	2.16	0.43

Shoot

The shoot dry weight was higher at 17°C than 5°C at almost every stage during storage (Table 3, Figure 1), and this was reflected in the changes in shoot length (Table 4). At both temperatures the relative growth rate (RGR) was highest during the first 4 weeks of storage, with the 5°C value being slightly greater (Table 5). The main difference between the RGR at the two temperatures was during the 4 - 6 week period, when it fell to a much greater extent at 5°C. The final RGR values were almost identical at 5°C and 17°C, and less than half their respective original values.

TABLE 3 Dry weight (DW) of the shoot at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

STORAGE PERIOD (weeks)	AVERAGE DW +/-SE (mg)	
	5°C	17°C
0	23.5 +/-2.2	23.5 +/-2.2
4	116.1 +/-8.5	90.5 +/-5.1
6	129.4 +/-6.8	141.1 +/-7.2
8	159.1 +/-8.5	189.5 +/-11.7
12	282.4 +/-14.3	336.5 +/-12.6

FIGURE 1

Change in the dry weight of the shoot during 12 weeks storage at 5°C and 17°C.

■ = 5°C storage

□ = 17°C storage

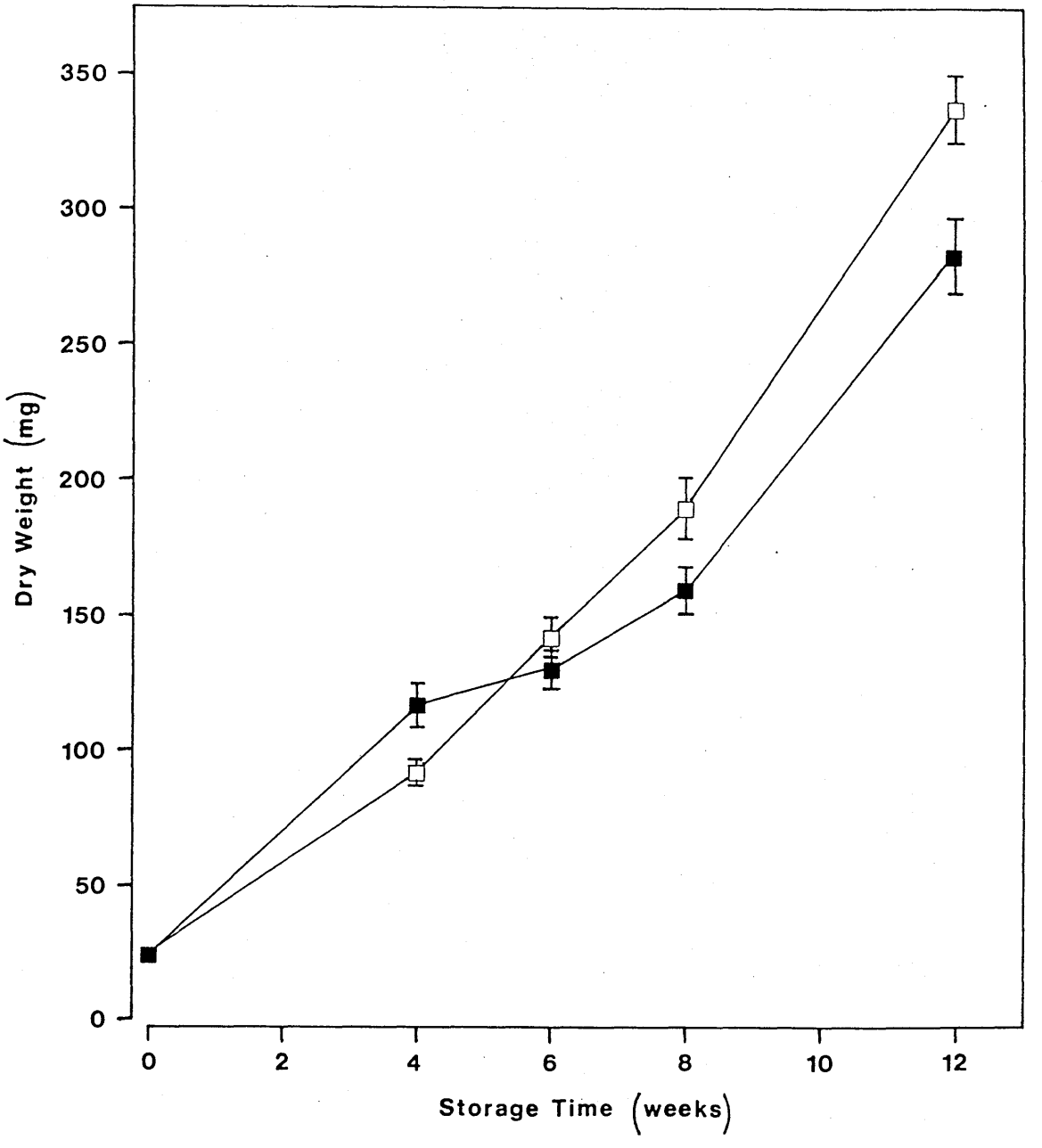

350
300
250
200
150
100
50
0
Dry Weight (mg)
0
2
4
6
8
10
12
Storage Time (weeks)

TABLE 4 Shoot length, measured at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

STORAGE PERIOD (weeks)	AVERAGE LENGTH +/-SE (mm)	
	5°C	17°C
0	9.5 +/-0.52	9.5 +/-0.52
4	22.4 +/-0.76	20.4 +/-0.54
6	23.2 +/-0.79	25.0 +/-0.63
8	25.0 +/-0.94	28.9 +/-0.71
12	34.9 +/-0.95	38.3 +/-0.73

TABLE 5 Relative growth rate (RGR) of the shoot at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

STORAGE PERIOD (weeks)	RGR +/-SE (g g^{-1} day^{-1})	
	5°C	17°C
0 - 4	0.058 +/-0.004	0.049 +/-0.004
4 - 6	0.008 +/-0.005	0.032 +/-0.006
6 - 8	0.015 +/-0.004	0.021 +/-0.006
8 - 12	0.020 +/-0.003	0.021 +/-0.003

Basal plate

The dry weight of the basal plate was not measured separately in the initial sample and so there was no 0 week value, but the dry weight increased between 4 and 12 weeks at both 5°C and 17°C (Table 6, Figure 2). The dry weights were relatively similar at both temperatures with the standard errors overlapping. The RGR was also similar except during the last stage when it was higher at 17°C (Table 7).

TABLE 6 Dry weight (DW) of the basal plate at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

STORAGE PERIOD (weeks)	AVERAGE DW +/-SE (g)	
	5°C	17°C
4	0.21 +/-0.02	0.21 +/-0.01
6	0.25 +/-0.02	0.23 +/-0.01
8	0.28 +/-0.02	0.26 +/-0.02
12	0.30 +/-0.02	0.34 +/-0.02

TABLE 7 Relative growth rate (RGR) of the basal plate at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

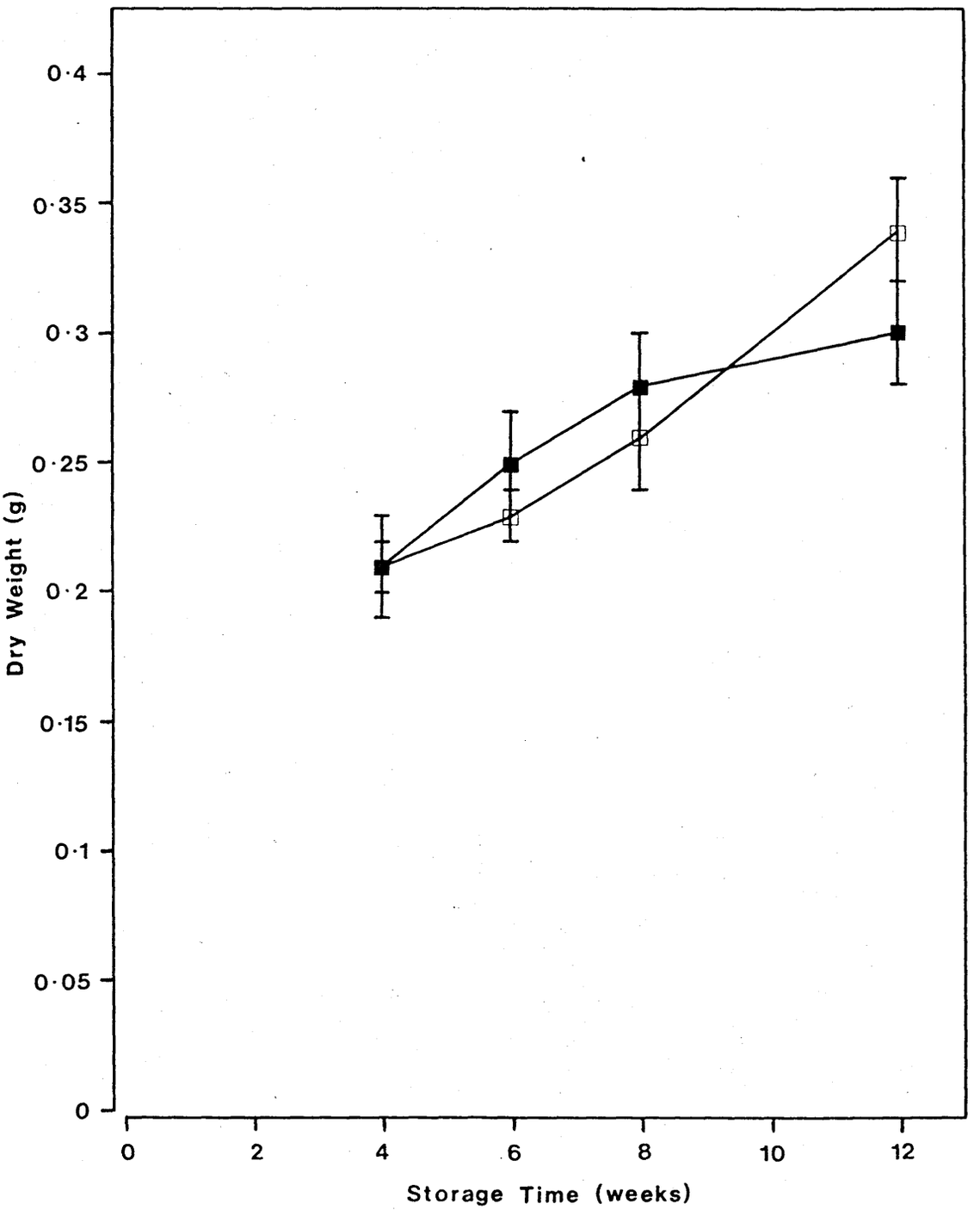
STORAGE PERIOD (weeks)	RGR +/-SE ($g\ g^{-1}\ day^{-1}$)	
	5°C	17°C
4 - 6	0.013 +/-0.010	0.008 +/-0.005
6 - 8	0.008 +/-0.007	0.006 +/-0.005
8 - 12	0.002 +/-0.003	0.010 +/-0.003

FIGURE 2

Change in the dry weight of the basal plate during 12 weeks storage at 5°C and 17°C.

■= 5°C storage

□= 17°C storage


0·4
0·35
0·3
0·25
0·2
0·15
0·1
0·05
0
Dry Weight (g)
0
2
4
6
8
10
12
Storage Time (weeks)

Scales

The scales make up the bulk of the bulb and they appeared to lose dry weight during storage but, due to the scales and basal plate being weighed together, there was no accurate 0 week value for the loss to be measured against. Between 4 and 12 weeks the basal plate gained weight steadily (Table 6) and so it seemed likely that if any change occurred in the first 4 weeks of storage it would be a gain rather than a loss in weight. Subtracting the 4 week dry weight of the basal plate from the 0 week scale plus basal plate value gave an approximate, and probably underestimated, value for the scale alone. Using the 5°C average value of 0.214 g gave a scale dry weight of 15.583 g and this indicated that the dry weight of the scales decreased during storage, particularly at 5°C where the average decrease was almost 2.5 g (Table 8, Figure 3). At 17°C the average dry weight fluctuated but, as in the 5°C samples, the standard errors were high.

The RGR was probably negative during the first four weeks but at both temperatures it later fluctuated between being positive and negative although the standard errors were high (Table 9).

TABLE 8 Dry weight (DW) of the scales at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

* The 0 week value was the combined dry weight of the scales and the basal plate.

STORAGE PERIOD (weeks)	AVERAGE DW +/-SE (g)	
	5°C	17°C
0*	15.80 +/-0.58	15.80 +/-0.58
4	15.22 +/-1.13	13.75 +/-0.73
6	15.24 +/-0.37	14.38 +/-0.75
8	14.50 +/-0.93	13.75 +/-0.52
12	13.13 +/-0.79	14.97 +/-0.69

TABLE 9 Relative growth rate (RGR) of the scales at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

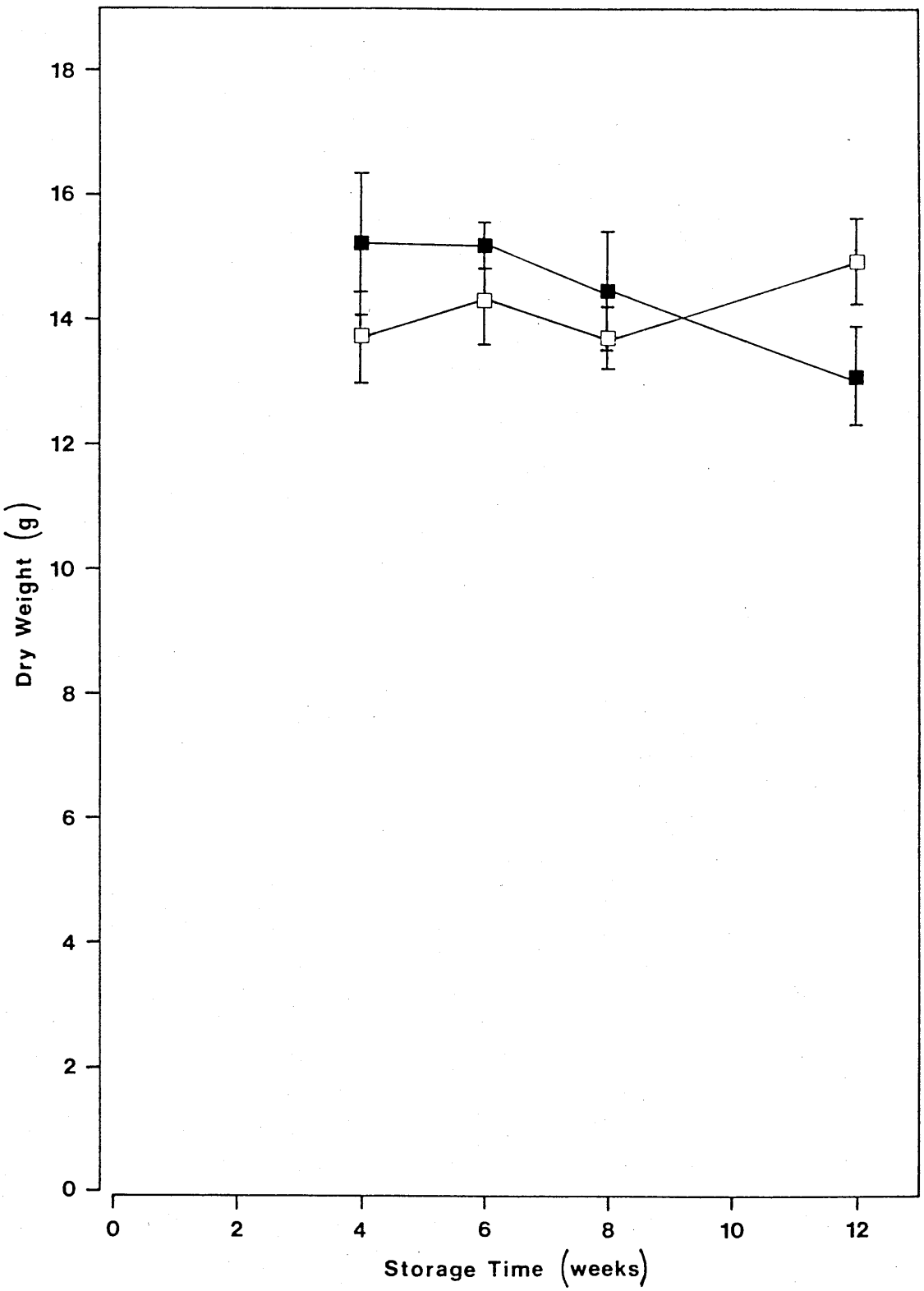
STORAGE PERIOD (weeks)	RGR +/-SE (g g^{-1} day^{-1})	
	5°C	17°C
4 - 6	0.002 +/-0.005	0.003 +/-0.006
6 - 8	-0.004 +/-0.004	-0.003 +/-0.004
8 - 12	-0.004 +/-0.003	0.003 +/-0.002

FIGURE 3

Change in the dry weight of the scales during 12 weeks storage at 5°C and 17°C.

■ = 5°C storage

□ = 17°C storage


18
16
14
12
10
8
6
4
2
0
Dry Weight (g)
0
2
4
6
8
10
12
Storage Time (weeks)

Daughter Bulbs

At both temperatures the combined dry weight of the daughter bulbs increased during storage, but the final dry weight was higher at 17°C (Table 10a, Figure 4). However, within the overall increase there were apparent decreases in the 6 week 5°C and 4 week 17°C samples.

The outer daughter bulb was largest, and in the 4, 6 and 8 week 5°C samples it accounted for 70-71% of the total dry weight, dropping to approximately 54% after 12 weeks. At 17°C the percentage was between 64 and 75% but after 12 weeks it was also 54%. In general the changes in dry weight of the outer daughter bulb followed the pattern observed for the total daughter bulbs (Table 10b, Figure 5).

At 5°C the RGR for the total daughter bulbs was negative in the 4 - 6 week period, but positive during the other stages (Table 11a). At 17°C the initial RGR was negative but thereafter became positive and continued at a high level although, as at 5°C, the standard errors were high. The main difference in RGR between the temperatures was in the 4 - 6 week period. Overall the changes in RGR of the outer daughter bulb were similar to those of the total, and again the standard errors were high (Table 11b).

TABLES 10a and b Dry weight (DW) at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

TABLE 10a Total daughter bulbs

STORAGE PERIOD (weeks)	AVERAGE DW +/-SE (mg)	
	5°C	17°C
0	32 +/-9	32 +/-9
4	36 +/-6	25 +/-3
6	27 +/-4	36 +/-5
8	34 +/-4	54 +/-16
12	48 +/-2	66 +/-4

TABLE 10b Outer daughter bulb

STORAGE PERIOD (weeks)	AVERAGE DW +/-SE (mg)	
	5°C	17°C
4	25.5 +/-5.7	16.2 +/-2.0
6	19.0 +/-2.3	27.0 +/-4.2
8	23.8 +/-3.2	39.3 +/-14.0
12	26.1 +/-3.4	35.8 +/-4.3

FIGURE 4

Change in the dry weight of the total daughter bulbs during 12 weeks storage at 5°C and 17°C.

■ = 5°C storage

□ = 17°C storage

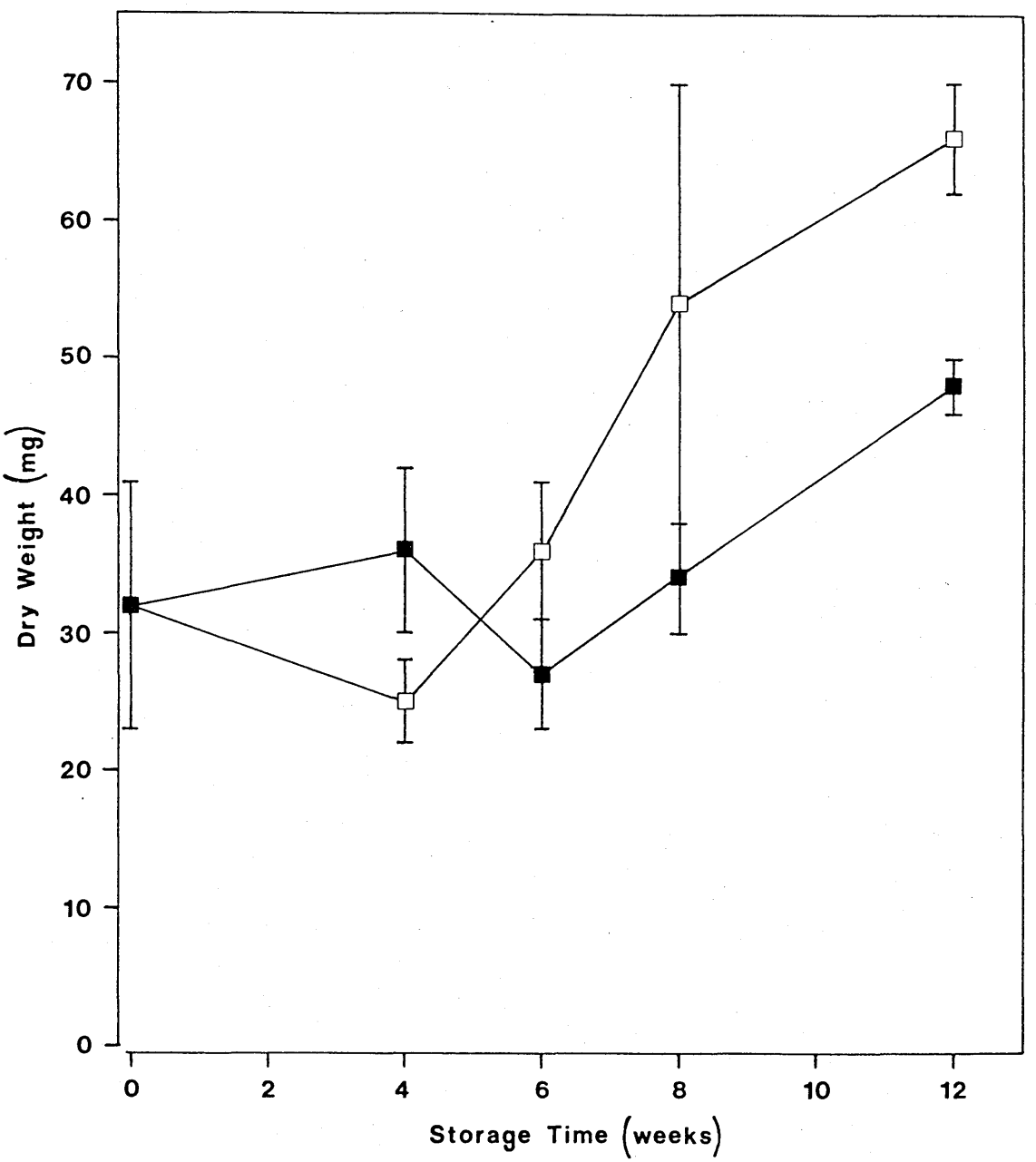
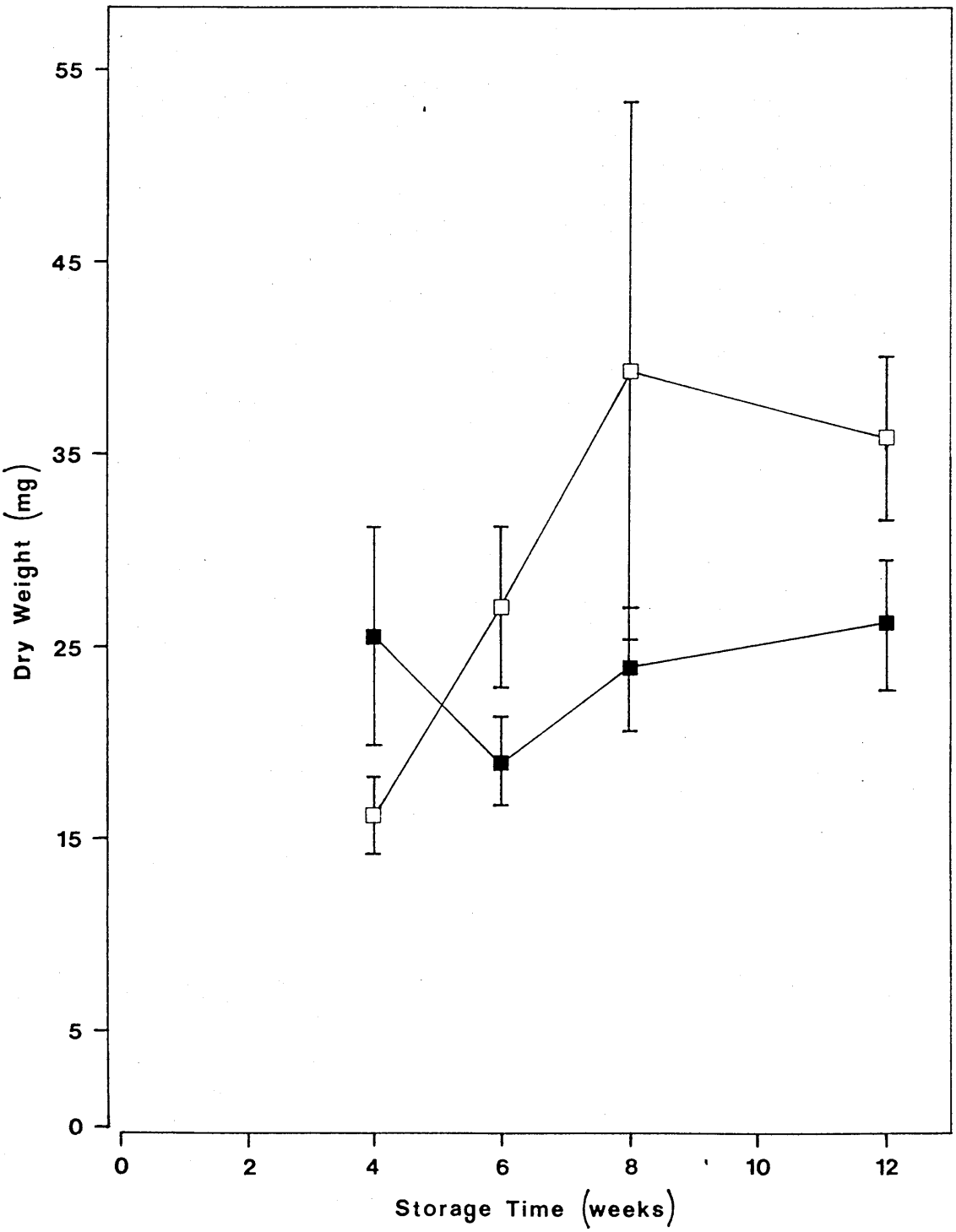

70
60
50
40
30
20
10
0
Dry Weight (mg)
0
2
4
6
8
10
12
Storage Time (weeks)

FIGURE 5

Change in the dry weight of the outer daughter bulb during 12 weeks storage at 5°C and 17°C.

■ = 5°C storage

□ = 17°C storage


55
45
35
25
15
5
0
Dry Weight (mg)
0
2
4
6
8
10
12
Storage Time (weeks)

TABLES 11a and b Relative growth rate (RGR) at 2 and 4 week intervals during 12 weeks storage at 5°C and 17°C.

TABLE 11a Total daughter bulbs

STORAGE PERIOD (weeks)	RGR +/-SE (g g^{-1} day^{-1})	
	5°C	17°C
0 - 4	0.010 +/-0.010	-0.002 +/-0.008
4 - 6	-0.020 +/-0.017	0.022 +/-0.016
6 - 8	0.019 +/-0.015	0.015 +/-0.023
8 - 12	0.014 +/-0.005	0.018 +/-0.008

TABLE 11b Outer daughter bulb

STORAGE PERIOD (weeks)	RGR +/-SE (g g^{-1} day^{-1})	
	5°C	17°C
4 - 6	-0.009 +/-0.018	0.024 +/-0.023
6 - 8	0.007 +/-0.026	0.013 +/-0.031
8 - 12	0.007 +/-0.013	0.010 +/-0.009

Relative Growth Rates

The overall RGRs for each bulb component during the 12 weeks storage are given in Table 12. In each case the RGR was slightly greater at 17°C than 5°C although the differences were small. At both temperatures the shoot had the highest RGR followed by the daughter bulbs, basal plate and scales. The scale RGR (measured from 4 to 12 weeks) was negative at 5°C but positive at 17°C. However, if the RGR was measured using the calculated 0 week dry weight (see scale section), the RGR would be -0.001 at 17°C and -0.002 at 5°C.

TABLE 12 Relative growth rate (RGR) of the individual bulb components from the beginning (0 weeks) until the end of 12 weeks storage at 5°C and 17°C.

* RGR calculated using the 4 and 12 week dry weights.

BULB TISSUE	RGR +/-SE ($g\ g^{-1}\ day^{-1}$)	
	5°C	17°C
Shoot	0.030 +/-0.001	0.032 +/-0.001
Basal plate*	0.006 +/-0.002	0.008 +/-0.002
Scales*	-0.002 +/-0.001	0.002 +/-0.001
Daughter Bulbs	0.008 +/-0.003	0.012 +/-0.002

Discussion

The shoot, basal plate and daughter bulbs increased in dry weight during storage at 5°C and 17°C, but the increase was generally greater at 17°C. For the shoot and the daughter bulbs the 4 week 5°C dry weights were higher, but during the 4 - 6 week period the RGR was greater at 17°C and thereafter the 17°C dry weights were higher. Between 4 and 8 weeks the average dry weight of the basal plate was consistently higher at 5°C, but during the last 4 weeks of storage the RGR at 17°C increased and the final dry weight was higher at 17°C. Therefore, although the final dry weight of these components was generally greater at 17°C than 5°C, this was not necessarily the case throughout storage.

The bulbs are stored dry in the dark, and growth takes place at the expense of the stored material in the scales. As would be expected, the dry weight of the scales appeared to decrease during storage, but the reduction was greatest at 5°C although the other components appeared to gain slightly more weight at 17°C. At 5°C the overall dry weight of the whole bulb also fell, the increase in dry weight of the shoot, basal plate and daughter bulbs not completely compensating for the loss of dry weight of the scales. The final dry weight of the 17°C whole bulbs was similar to the initial dry weight implying that the loss of scale weight was countered by the growth of the other components.

CHAPTER 2

INITIAL RADIOIMMUNOASSAY ANALYSES

Introduction

Prior to radioimmunoassays (RIA), using the GA_1-methyl ester (Me) antiserum raised in Glasgow, these samples were methylated then fractionated using gradient HPLC. Tritiated standards of GA-Mes were used to determine their retention times and to indicate what fractions they would appear in if present in the samples (Table 1). Each sample contained [^{3}H]GA_9-Me used as an internal standard, and this eluted in fractions 55 and 56 of the samples, rather than fraction 52 as expected from the tritiated standard. Therefore the GA_9-Me had been shifted by 3-4 fractions but it is impossible to know whether the effect was the same throughout the HPLC gradient. The relative impurity of the samples in comparison to the radiolabelled standards may have caused problems. More polar GAs, eluting early in the gradient, may have been unaffected with the effect increasing with increasing time and percentage MeOH, so that the less polar GAs were affected to a greater extent. However, taking into consideration the shift in GA_9-Me, a range of predicted fractions where each GA-Me might be expected to elute is given in Table 1, and these adjusted figures have been used on the diagrams to indicate elution times.

Three main peaks were found in all the samples and they are referred to as peaks 1, 2 and 3 respectively, in order of elution.

The competition of a known amount of labelled tracer antigen with an unknown amount of sample antigen, for a limited number of antibody binding sites is the basic principle of immunoassays (Van Vunakis, 1980; Weiler, 1982, 1984). However, problems can occur when substances in the sample interfere with antigen-antibody binding, by competitive or non-specific inhibition, and so the accuracy of the assay must be checked (Crozier *et al.*, 1986; Pengelly, 1986). The method of assay validation used here was to assay a range of sample dilutions. In the absence of interference the resultant dilution curve will be parallel with the standard curve, but non-parallel if sample contaminants are affecting antigen-antibody binding.

TABLE 1 HPLC Fractions where tritiated GA-Me standards eluted during a 40-100% MeOH gradient.

GA-Me	STANDARD FRACTION NO.	PREDICTED FRACTION NOS.
GA_8-Me	11	13-16
GA_1-Me	18	20-23
GA_5-Me	34	36-39
GA_{20}-Me	35-36	37-41
GA_4-Me	46	48-51
GA_9-Me	52	55-56

WHOLE BULB EXTRACTS

0 week 5°C Whole Bulb Extract

A preliminary assay of all the HPLC fractions indicated the presence of four peaks in this sample and these results were confirmed by subsequent assays. The largest peaks occurred in fractions 22-23 (Peak 1) and 29-30 (Peak 2) whilst the other two peaks were somewhat smaller and appeared in fractions 39-40 (Peak 3) and 49-51 (Peak 4) (Table 2, Figure 1).

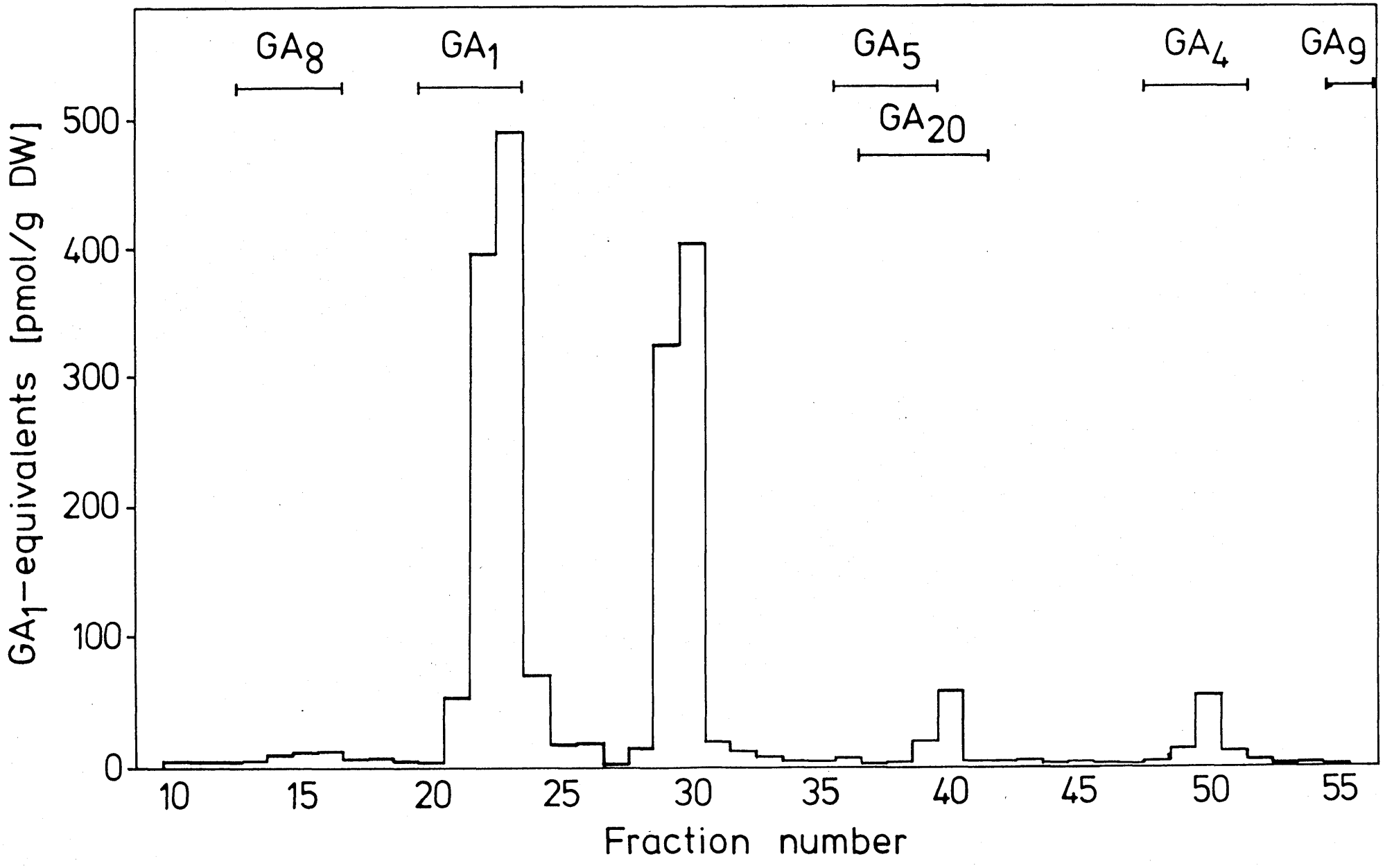
None of the sample peaks corresponded exactly with the standards analysed but, taking into account the shift in GA_9-Me, three of them fell within the predicted range of fractions (Tables 1 and 2, Figure 1). Peaks 1 and 4 corresponded to GA_1-Me and GA_4-Me respectively, whilst peak 3 was in the areas of GA_5-Me and GA_{20}-Me, which overlap, but possibly nearer to GA_{20}-Me. Peak 2 eluted between GA_1-Me and GA_5-Me and thus did not correspond to any of the standards.

TABLE 2 Size of peaks 1-3 detected by RIA in the 0 week 5°C whole bulb extract, using the GA_1-Me antiserum.

PEAK NUMBER	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL	RETENTION PROPERTY
1	22	342.11	832.24	GA_1
	23	490.13		
2	29	324.85	728.24	
	30	403.39		
3	39	19.49	78.94	GA_5/GA_{20}
	40	59.45		
4	49	13.16	79.19	GA_4
	50	54.68		
	51	11.35		

FIGURE 1

Distribution of immunoreactivity in the HPLC fractions of the 0 week 5°C whole bulb extract.


GA8
GA1
GA5
GA20
GA4
GA9
500
400
300
200
100
0
GA1-equivalents [pmol/g DW]
10
15
20
25
30
35
40
45
50
55
Fraction number

Dilution Curves - Dilution curves of fractions 19, 22 and 23 of peak 1 were carried out using 1, 2, 5, 10, 20 and 50 µl of each fraction and the slopes of each are given in Table 3 with the slope of the standard curve. Fraction 19 was not part of peak 1 and the slope of its dilution curve was quite different from that of the standard curve (Figure 2). Peak 1 occurred in fractions 22 and 23 and the slopes of their dilution curves were closer to the slope of the standard curve, particularly that of fraction 23 (Figures 2 and 3). The fraction 23 dilution curve was repeated and the slope was again very similar to that of the standard curve with a difference of 0.074, exactly the same as the difference found in the first set of curves (Table 3, Figure 4).

TABLE 3 Slopes of the RIA standard curves and of the extract dilution curves of HPLC fractions 19, 22 and 23 from the 0 week 5°C whole bulb extract.

SAMPLE	SLOPE
Standards	-2.079
Fraction 19	-1.573
Fraction 22	-1.818
Fraction 23	-2.005
Standards	-1.891
Fraction 23	-1.817

FIGURE 2

Parallelism of a standard curve, from an RIA using GA_1-Me antiserum, and dilution curves of HPLC fractions 19 and 22 from the 0 week 5°C whole bulb extract.

● = GA_1-Me standard curve

▲ = Fraction 19 dilution curve

△ = Fraction 22 dilution curve

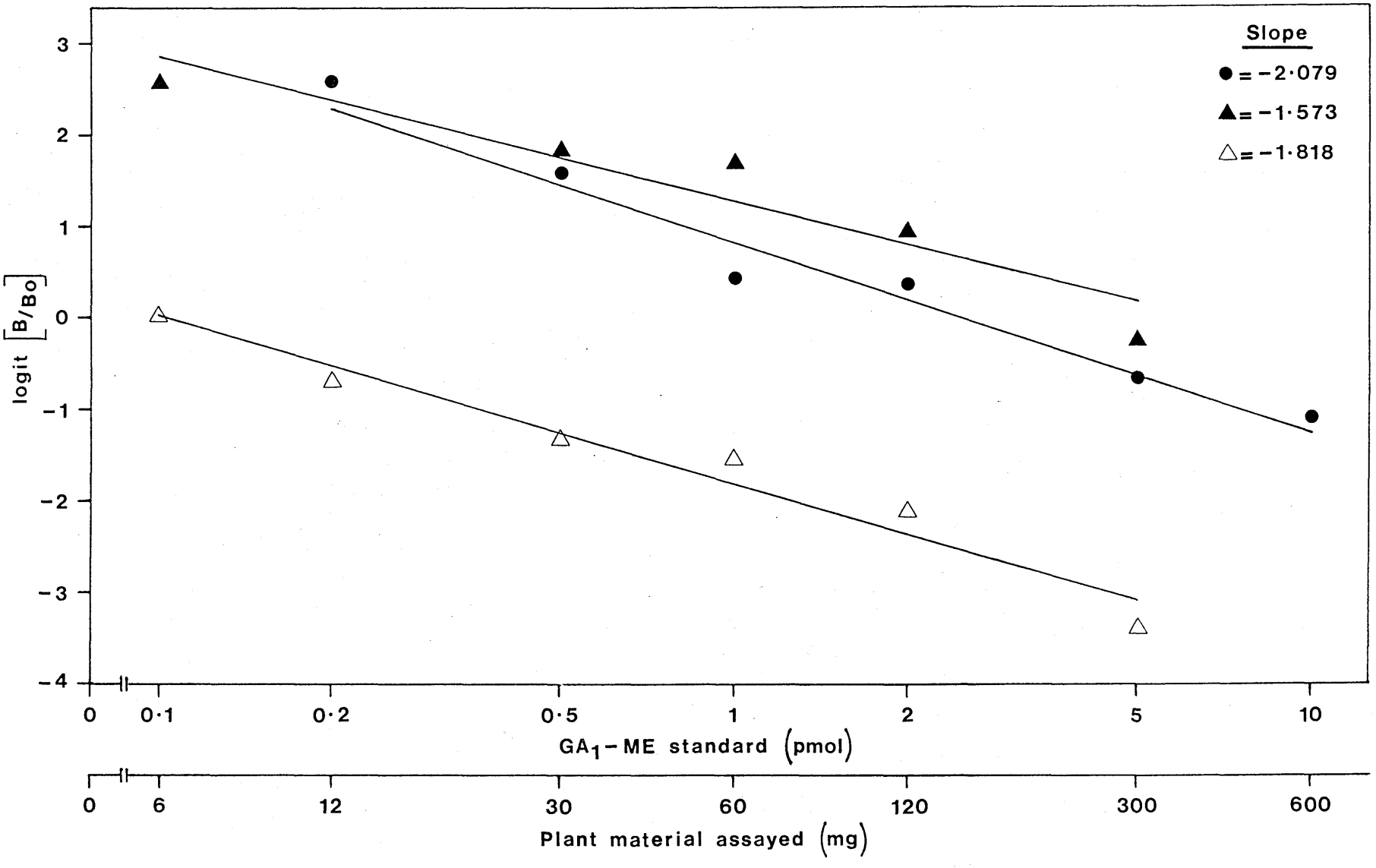

Slope
● = −2·079
▲ = −1·573
△ = −1·818
logit [B/Bo]
3
2
1
0
−1
−2
−3
−4
0
0·1
0·2
0·5
1
2
5
10
GA1 − ME standard (pmol)
0
6
12
30
60
120
300
600
Plant material assayed (mg)

FIGURE 3

Parallelism of a standard curve, from an RIA using GA_1-Me antiserum, and a dilution curve of HPLC fraction 23 from the 0 week 5°C whole bulb extract.

● = GA_1-Me standard curve

○ = Fraction 23 dilution curve

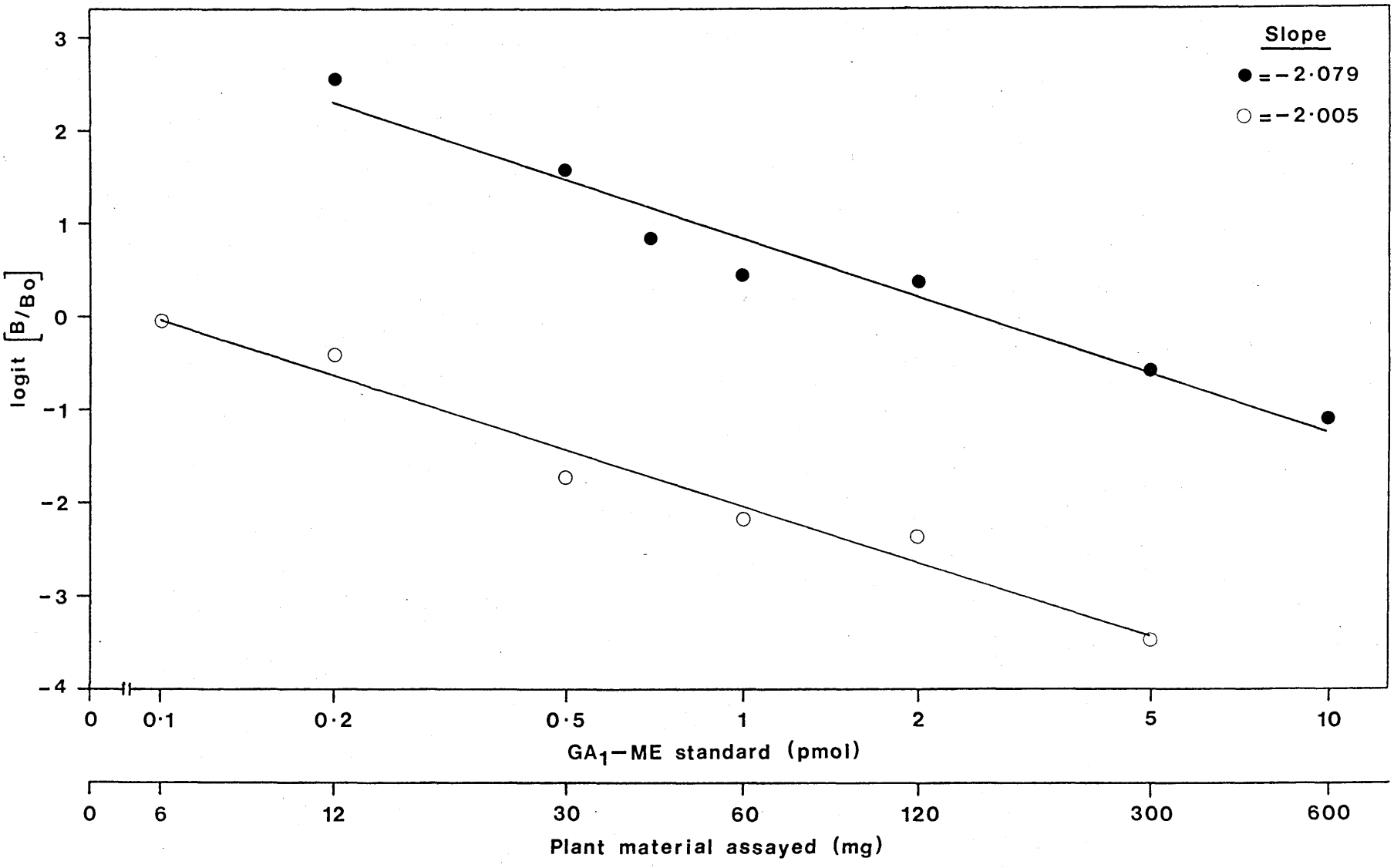
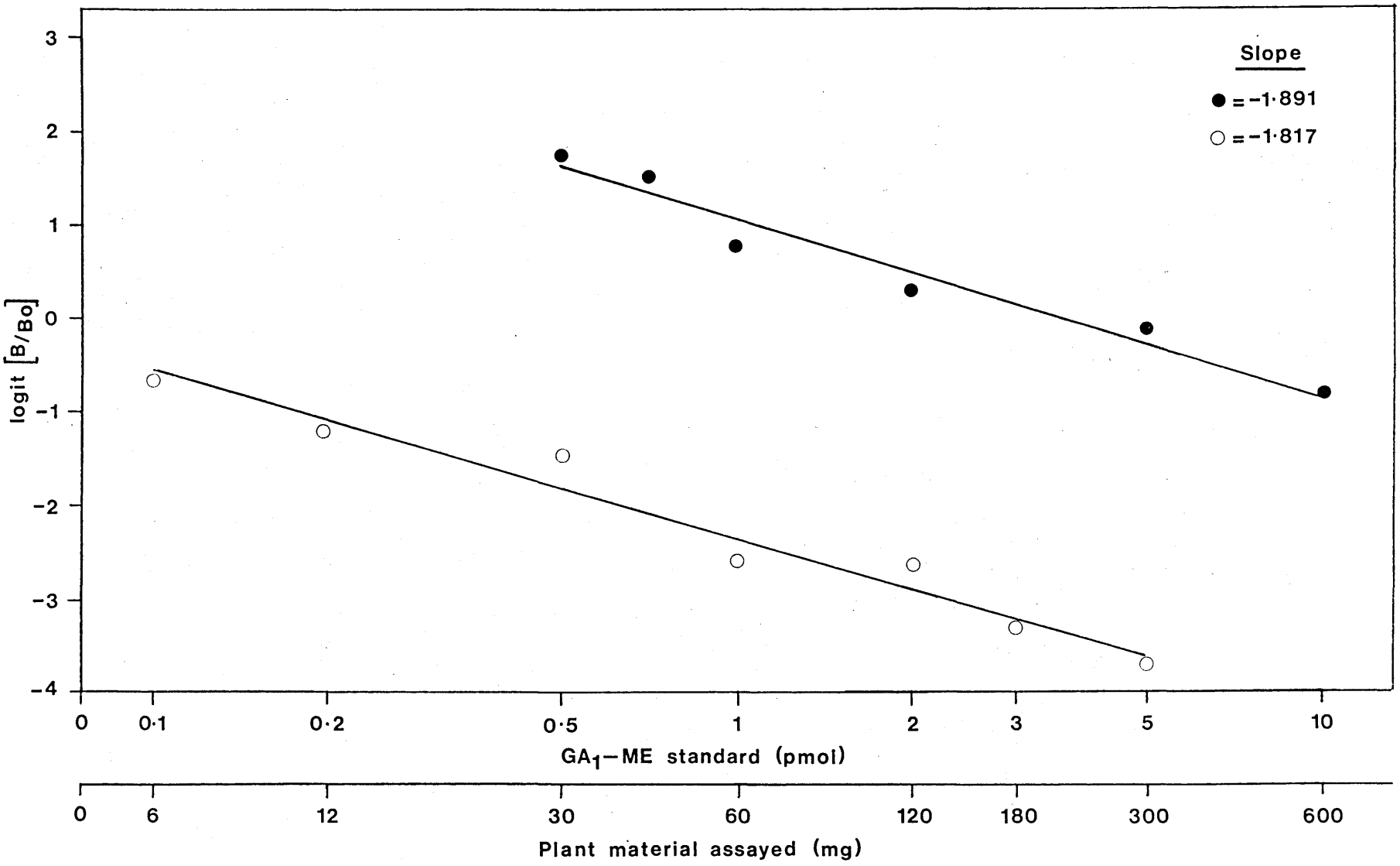

Slope
● = −2·079
○ = −2·005
logit [B/Bo]
3
2
1
0
−1
−2
−3
−4
0
0·1
0·2
0·5
1
2
5
10
GA1−ME standard (pmol)
0
6
12
30
60
120
300
600
Plant material assayed (mg)

FIGURE 4

Parallelism of a standard curve, from an RIA using GA_1-Me antiserum, and a dilution curve of HPLC fraction 23 from the 0 week 5°C whole bulb extract.

● = GA_1-Me standard curve

○ = Fraction 23 dilution curve


Slope
● = -1·891
○ = -1·817
logit [B/Bo]
3
2
1
0
-1
-2
-3
-4
0
0·1
0·2
0·5
1
2
3
5
10
GA1–ME standard (pmol)
0
6
12
30
60
120
180
300
600
Plant material assayed (mg)

Isocratic HPLC - The differing chromatography of the sample and standards made it difficult to make tentative identifications of the peaks but it was considered that peak 1 could be GA_1-Me. This peak occurred in fractions 22 and 23 but in case there was any overlap into surrounding fractions, fractions 21-26 were used for the next step. Twenty percent of each fraction was taken and the resultant combined sample was split in half. [^{3}H]GA_8-Me and [^{3}H]GA_1-Me were added to one half which was then fractionated by isocratic HPLC at 40% MeOH. The [^{3}H]GA_1-Me eluted in fractions 14-15. The remaining half of the sample was also fractionated as previously described but without the tritiated GAs. The resultant fractions were assayed and a peak was found in fractions 13-16 with the maximum part in fraction 16 (Table 4). It is possible that this could have been GA_1-Me although the peak was spread over four fractions and the main part was in fraction 16 rather than 14 or 15. The only other GA with a retention time similar to GA_1 and detected by this antiserum, was GA_3, but it would elute earlier rather than later than GA_1. The estimated value for this peak was 773 pmol GA_1-equivalents g^{-1} DW (Table 4) and so was similar to the amount estimated before as 832 pmol GA_1-equivalents g^{-1} DW (Table 2).

TABLE 4 Re-analysis by RIA of Peak 1 from the 0 week 5°C whole bulb extract, after fractionation by isocratic HPLC at 40% MeOH.

FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
13	95.94	
14	123.35	773.01
15	168.58	
16	385.14	

6 weeks 5°C Whole Bulb Extract

Three peaks were found in this sample, in fractions 18-22, 27-29 and 38-39 (Table 5, Figure 5). Peak 1, the largest peak, overlapped the predicted elution range of GA_1-Me but eluted over a large number of fractions, beginning earlier than this range. Peak 2 eluted between GA_1-Me and GA_5-Me and therefore did not correspond to any of the GA-Me standards whilst Peak 3, the smallest peak, occurred in the GA_5-Me/GA_{20}-Me area.

Dilution Curves - Dilution curves were carried out on fractions 19 and 22, where fraction 19 was the main part of Peak 1 with fraction 20, whilst fraction 22 was on the edge of the peak. The amount of each fraction used was 1.25, 2.5, 6.25, 12.5, 25 and 62.5 µl. The similarity between the slopes of the standard curve and the fraction 19 dilution curve (Table 6, Figure 6) was less than for the fraction 22 dilution curve (Figure 7) which was an unexpected result. The fraction 19 dilution curve was repeated using 1.25, 2.5, 6.25, 12.5, 25, 37.5 and 50 µl. In this case the difference between the sample and standard slopes was only 0.08, approximately half the difference found before (Table 6, Figure 8). The R^2 value for this curve was also better than the first, indicating that the second result was more accurate.

FIGURE 5

Distribution of immunoreactivity in the HPLC fractions of the 6 weeks 5°C whole bulb extract.

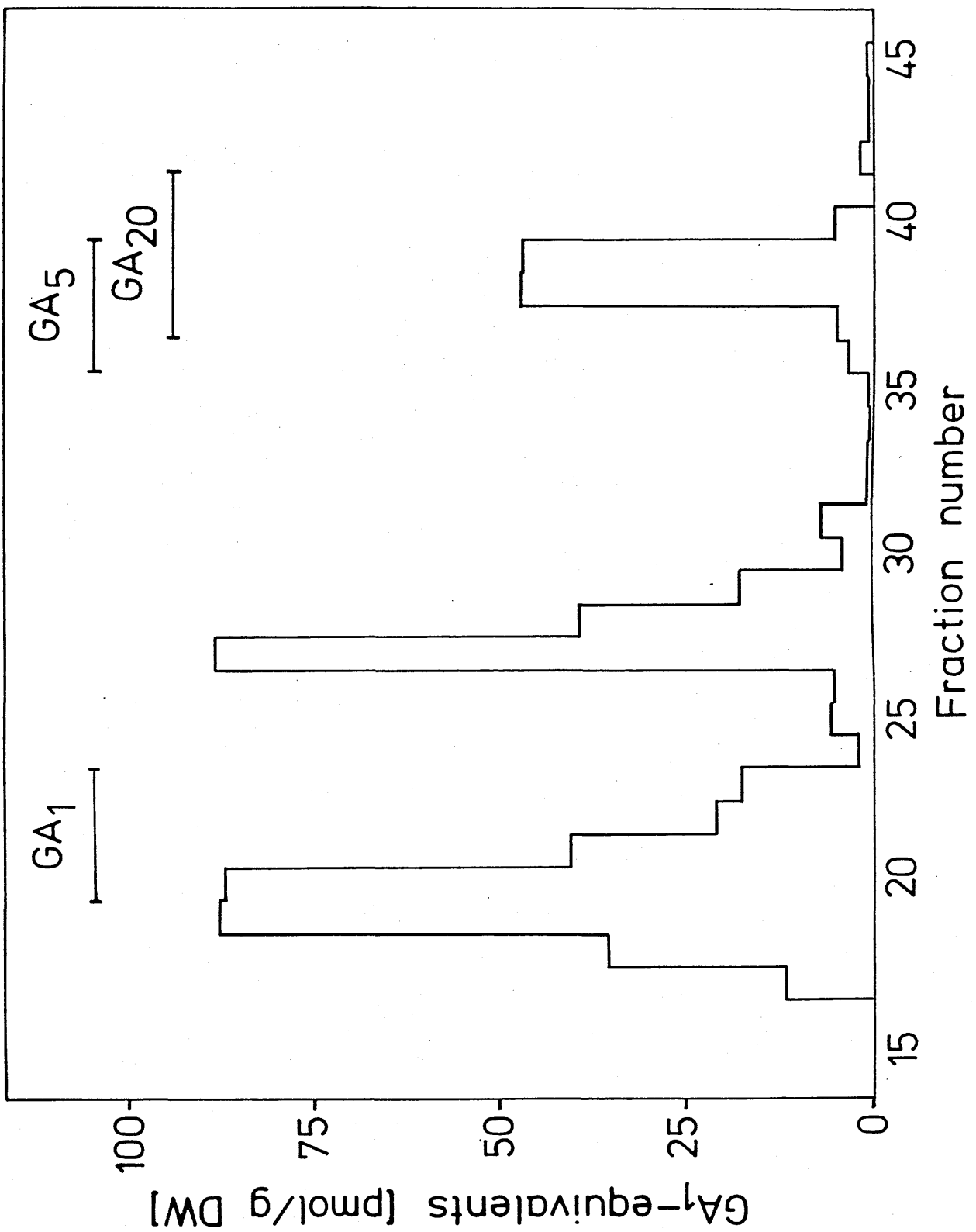

GA1
GA5
GA20
GA1-equivalents [pmol/g DW]
0
25
50
75
100
15
20
25
30
35
40
45
Fraction number

TABLE 5 Size of peaks 1-3 detected by RIA in the 6 weeks 5°C whole bulb extract, using the GA_1-Me antiserum.

PEAK NUMBER	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL	RETENTION PROPERTY
1	18	35.27	271.02	GA_1
	19	87.51		
	20	86.86		
	21	40.49		
	22	20.89		
2	27	88.09	144.98	
	28	39.04		
	29	17.55		
3	38	47.18	94.20	GA_5/GA_{20}
	39	47.02		

TABLE 6 Slopes of the RIA standard curves and of the extract dilution curves of HPLC fractions 19 and 22 from the 6 week 5°C whole bulb extract.

SAMPLE	SLOPE
Standards	-1.900
Fraction 19	-2.062
Fraction 22	-1.931
Standards	-1.891
Fraction 19	-1.971

FIGURE 6

Parallelism of a standard curve, from an RIA using GA_1-Me antiserum, and a dilution curve of HPLC fraction 19 from the 6 weeks 5°C whole bulb extract.

● = GA_1-Me standard curve

○ = Fraction 19 dilution curve

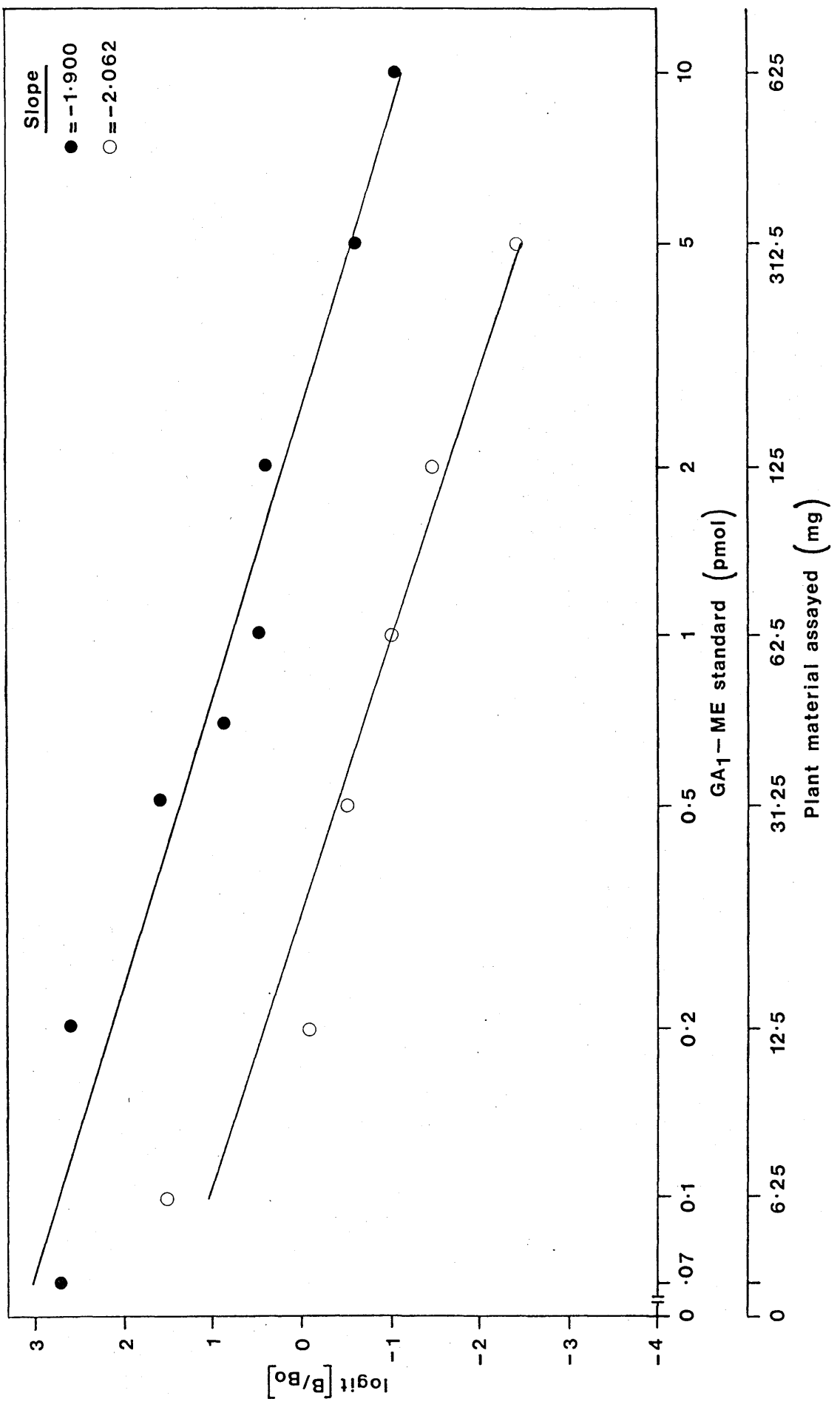

Slope
● = -1·900
○ = -2·062
logit [B/Bo]
3
2
1
0
-1
-2
-3
-4
0
·07
0·1
0·2
0·5
1
2
5
10
GA1 – ME standard (pmol)
0
6·25
12·5
31·25
62·5
125
312·5
625
Plant material assayed (mg)

FIGURE 7

Dilution curve of HPLC fraction 22 from the 6 weeks 5°C whole bulb extract.

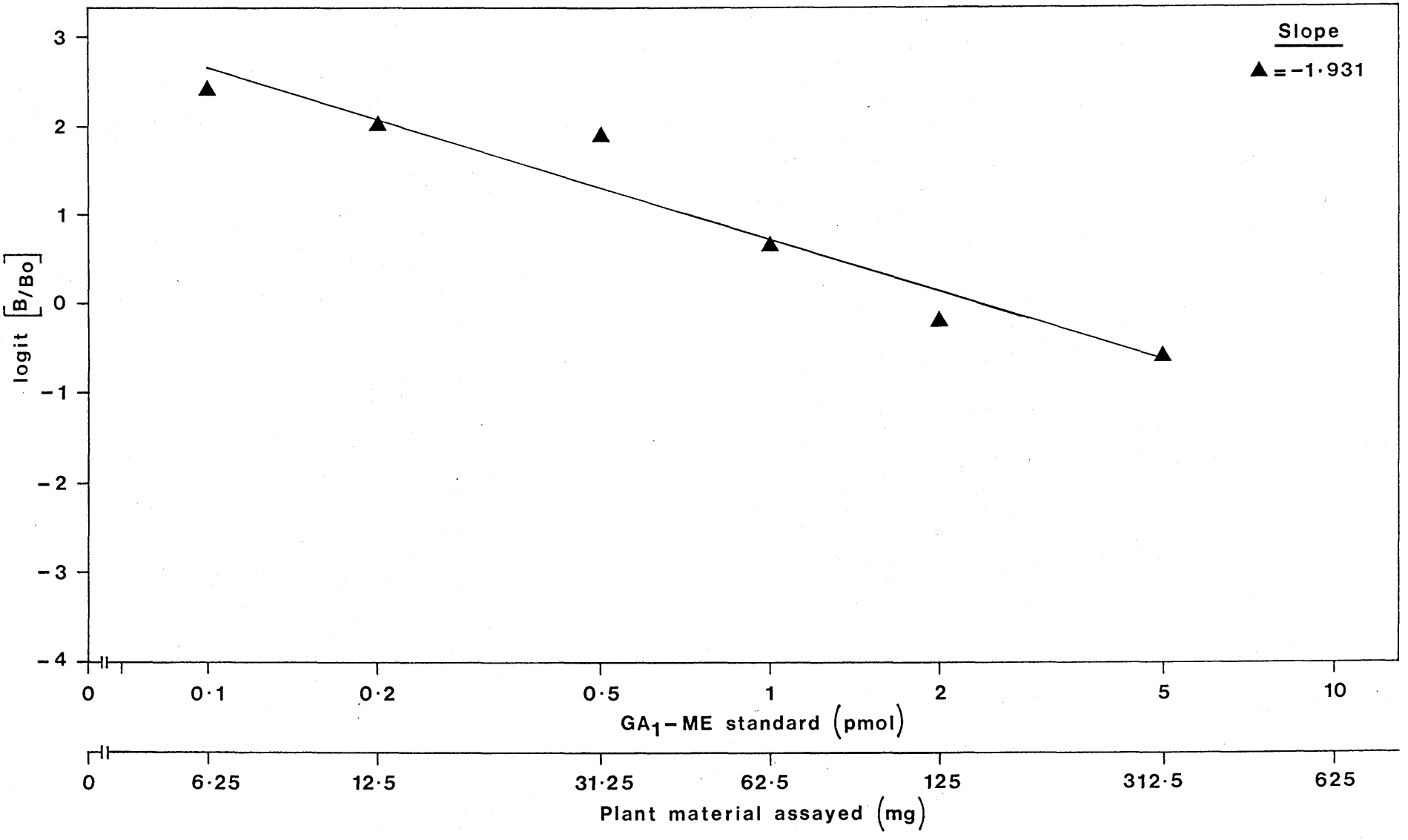
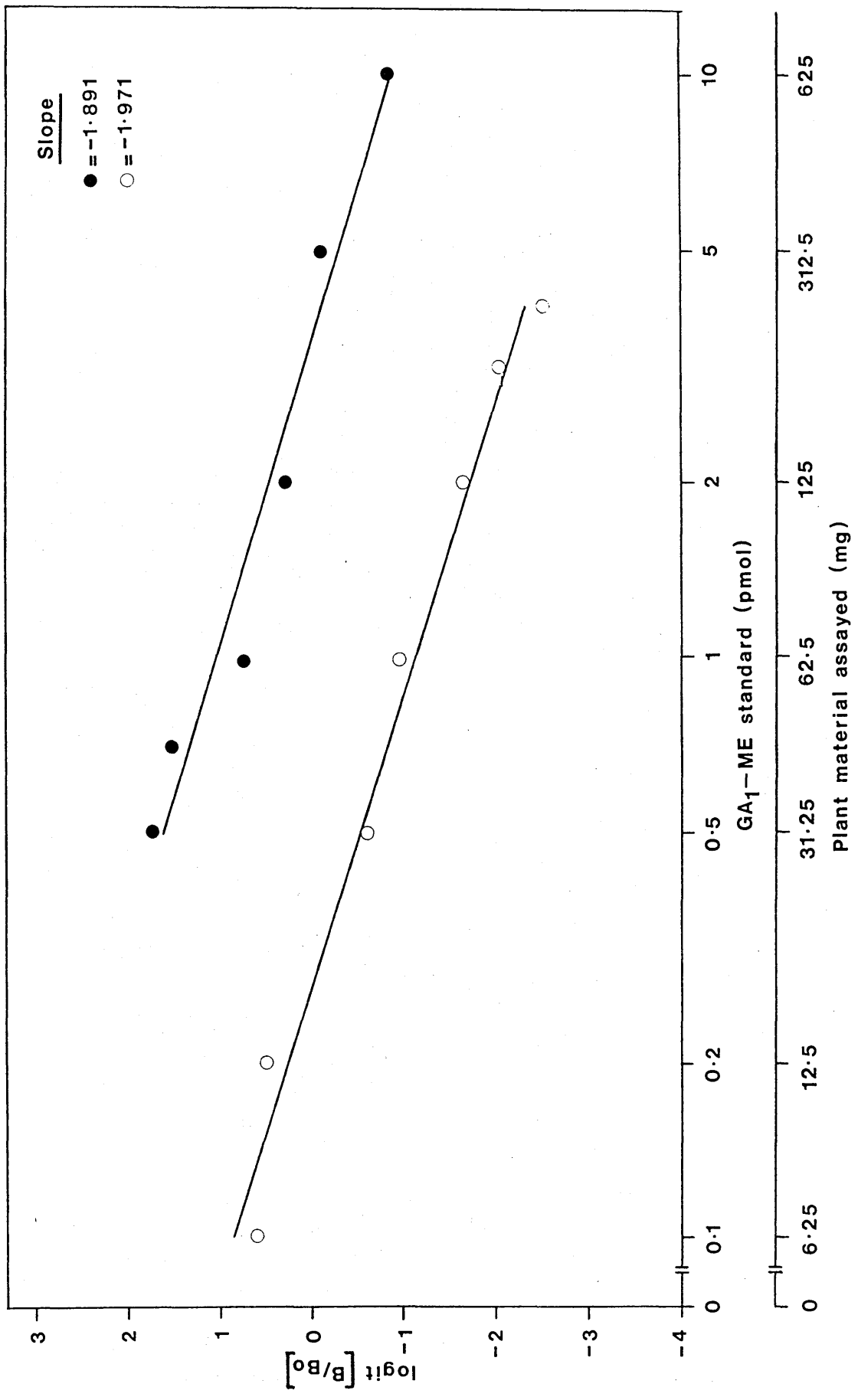

Slope
▲ = −1·931
logit [B/Bo]
3
2
1
0
−1
−2
−3
−4
0
0·1
0·2
0·5
1
2
5
10
GA1 − ME standard (pmol)
0
6·25
12·5
31·25
62·5
125
312·5
625
Plant material assayed (mg)

FIGURE 8

Parallelism of a standard curve, from an RIA using GA_1-Me antiserum, and a dilution curve of HPLC fraction 19 from the 6 weeks 5°C whole bulb extract.

● = GA_1-Me standard curve

○ = Fraction 19 dilution curve


Slope
● = -1·891
○ = -1·971
logit [B/Bo]
3
2
1
0
-1
-2
-3
-4
0
0·1
0·2
0·5
1
2
5
10
GA1—ME standard (pmol)
0
6·25
12·5
31·25
62·5
125
312·5
625
Plant material assayed (mg)

12 weeks 5°C Whole Bulb Extract

Peaks 1-3 were detected in fractions 18-22, 27-29 and 38-39 (Table 7, Figure 9). The largest was peak 1, which eluted partly in the range of GA_1-Me, although the highest part of the peak, fraction 19, was out-with the predicted fractions. Peaks 2 and 3 were similar in size, with peak 2 eluting between GA_1-Me and GA_5-Me and peak 3 close to GA_5-Me/GA_{20}-Me.

Dilution Curves - Dilution curves of fractions 19 and 22 were carried out, using 1.25, 2.5, 6.25, 12.5, 25 and 62.5 µl of each, where fraction 19 was the highest part of peak 1 and fraction 22 was at the edge. The slopes of both dilution curves were similar to that of the standard curve, but fraction 19 was closest with a difference of only 0.057 (Table 8, Figure 10). Another dilution curve was carried out for fraction 19 using 1.25, 2.5, 6.25, 12.5, 25, 37.5 and 50 µl. This time the difference between the slopes of the standard and dilution curve was greater, at 0.149 compared to 0.057 before (Table 8). However this result seemed less reliable than the first, as the points on this dilution curve were more variable (Figure 11) and it had a poor R^2 value indicating its inaccuracy.

FIGURE 9

Distribution of immunoreactivity in the HPLC fractions of the 12 weeks 5°C whole bulb extract.

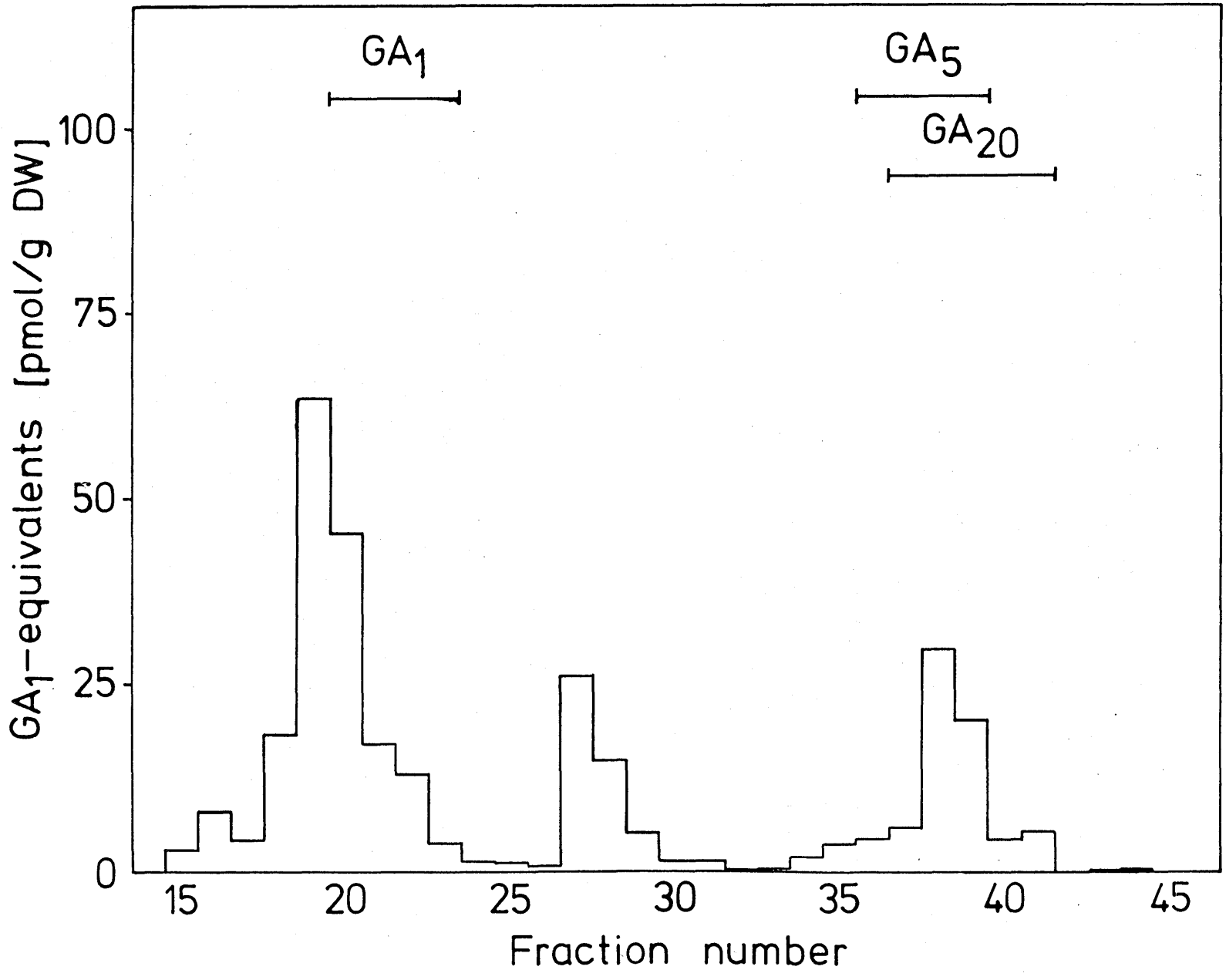

GA1
GA5
GA20
100
75
50
25
0
GA1-equivalents [pmol/g DW]
15
20
25
30
35
40
45
Fraction number

TABLE 7 Position and size of peaks detected by RIA, using the GA_1-Me antiserum, in the HPLC fractions of the 12 week 5°C whole bulb extract.

PEAK NUMBER	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL	RETENTION PROPERTY
1	18	18.36	157.82	GA_1
	19	63.55		
	20	45.47		
	21	17.20		
	22	13.24		
2	27	26.22	46.07	
	28	14.89		
	29	4.96		
3	38	29.63	49.71	GA_5/GA_{20}
	39	20.08		

TABLE 8 Slopes of the RIA standard curves and of the extract dilution curves of HPLC fractions 19 and 22 from the 12 week 5°C whole bulb extract.

SAMPLE	SLOPE
Standards	-1.900
Fraction 19	-1.957
Fraction 22	-1.991
Standards	-1.891
Fraction 19	-2.040

FIGURE 10

Parallelism of a standard curve, from an RIA using GA_1-Me antiserum, and dilution curves of HPLC fractions 19 and 22 from the 12 weeks 5°C whole bulb extract.

● = GA_1-Me standard curve

▲= Fraction 19 dilution curve

△= Fraction 22 dilution curve

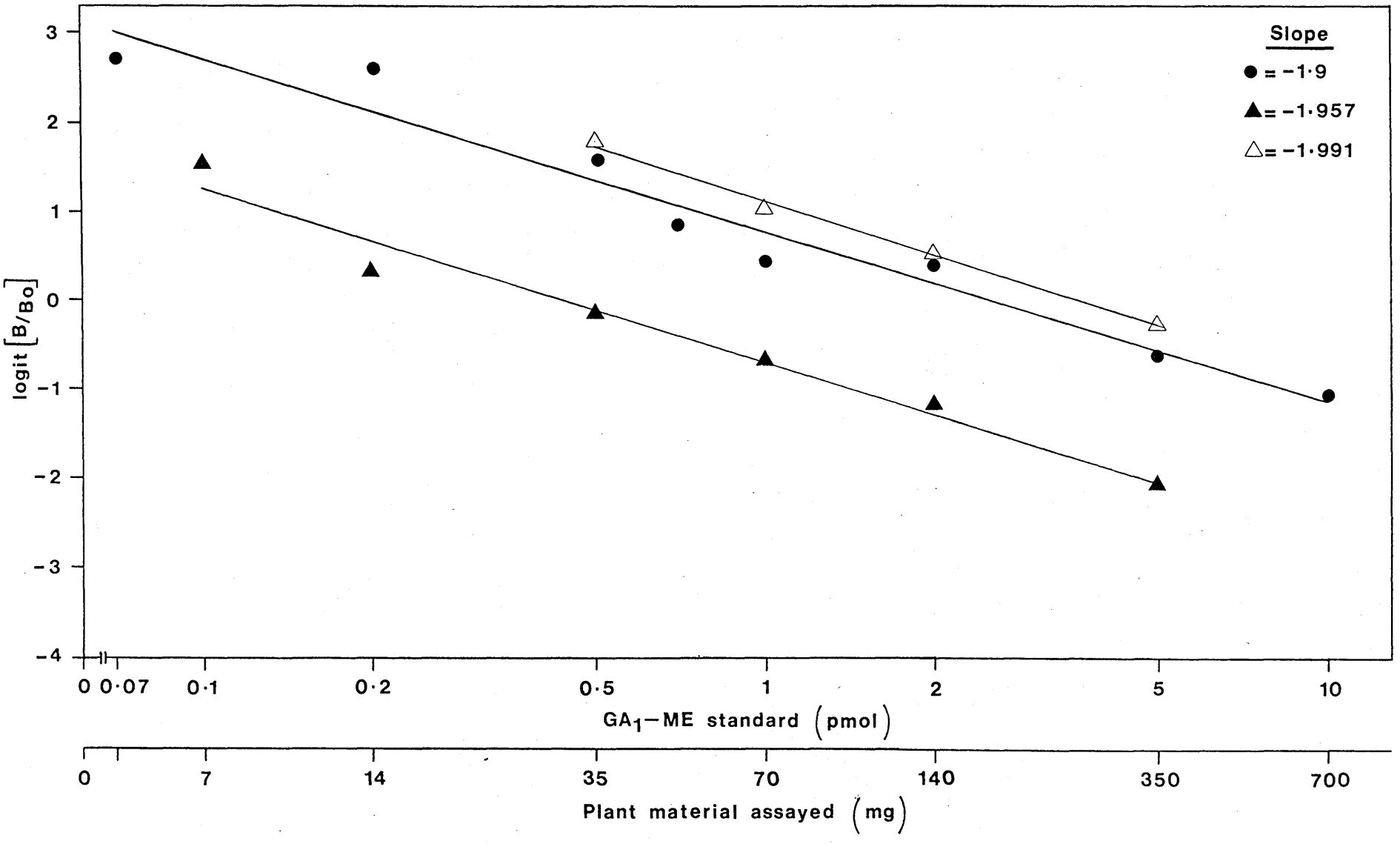
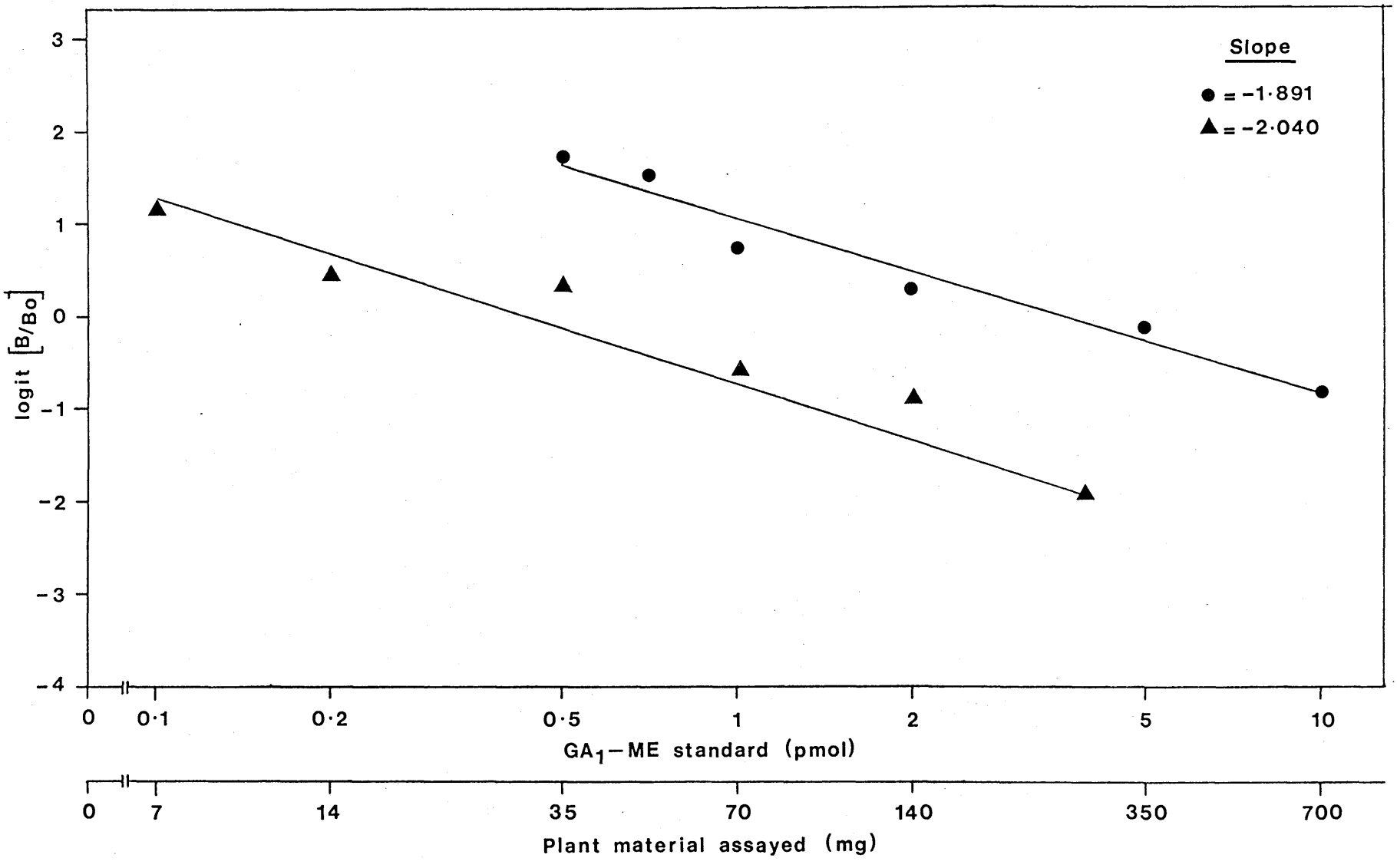

Slope
● = −1·9
▲ = −1·957
△ = −1·991
logit [B/Bo]
3
2
1
0
−1
−2
−3
−4
0 0·07 0·1 0·2 0·5 1 2 5 10
GA1−ME standard (pmol)
0 7 14 35 70 140 350 700
Plant material assayed (mg)

FIGURE 11

Parallelism of a standard curve, from an RIA using GA_1-Me antiserum, and a dilution curve of HPLC fraction 19 from the 12 weeks 5°C whole bulb extract.

● = GA_1-Me standard curve

▲ = Fraction 19 dilution curve


Slope
● = -1·891
▲ = -2·040
logit [B/Bo]
3
2
1
0
-1
-2
-3
-4
0
0·1
0·2
0·5
1
2
5
10
GA1—ME standard (pmol)
0
7
14
35
70
140
350
700
Plant material assayed (mg)

12 weeks 5°C Scale Extract

This sample appeared to contain peaks 1-3 plus a very small peak in fraction 16 (Table 9, Figure 12). This early peak was very small and may just have been background. However, it has been included because this is the area where GA_8-Me elutes and a very large peak was found in fractions 15-17 of the 12 weeks 5°C basal plate sample. Peak 1 may have been GA_1-Me - it spread over a large number of fractions, overlapping the predicted range of GA_1-Me. None of the GA-Me standards corresponded to Peak 2 which eluted between GA_1-Me and GA_5-Me but Peak 3 occurred in the area of GA_5-Me and GA_{20}-Me.

12 weeks 5°C Basal Plate Extract

The three peaks detected in this sample occurred in fractions 15-17, 22-24 and 36-38 with the peaks decreasing in size with increasing retention time (Table 10, Figure 13). They did not appear in the same fractions as the peaks in the other samples - each was earlier than the expected peak. The first peak appeared in the predicted range of GA_8-Me which was fractions 13-16, whilst the next peak was in the range of fractions 20-23 of GA_1-Me although the GA_1-like peaks detected in other samples generally appeared in fractions 18-22. The last peak was probably correspondent to peak 3 and GA_5-Me/GA_{20}-Me as it eluted only slightly earlier than peak 3 in the other samples.

FIGURE 12

Distribution of immunoreactivity in the HPLC fractions of the 12 weeks 5°C scale extract.

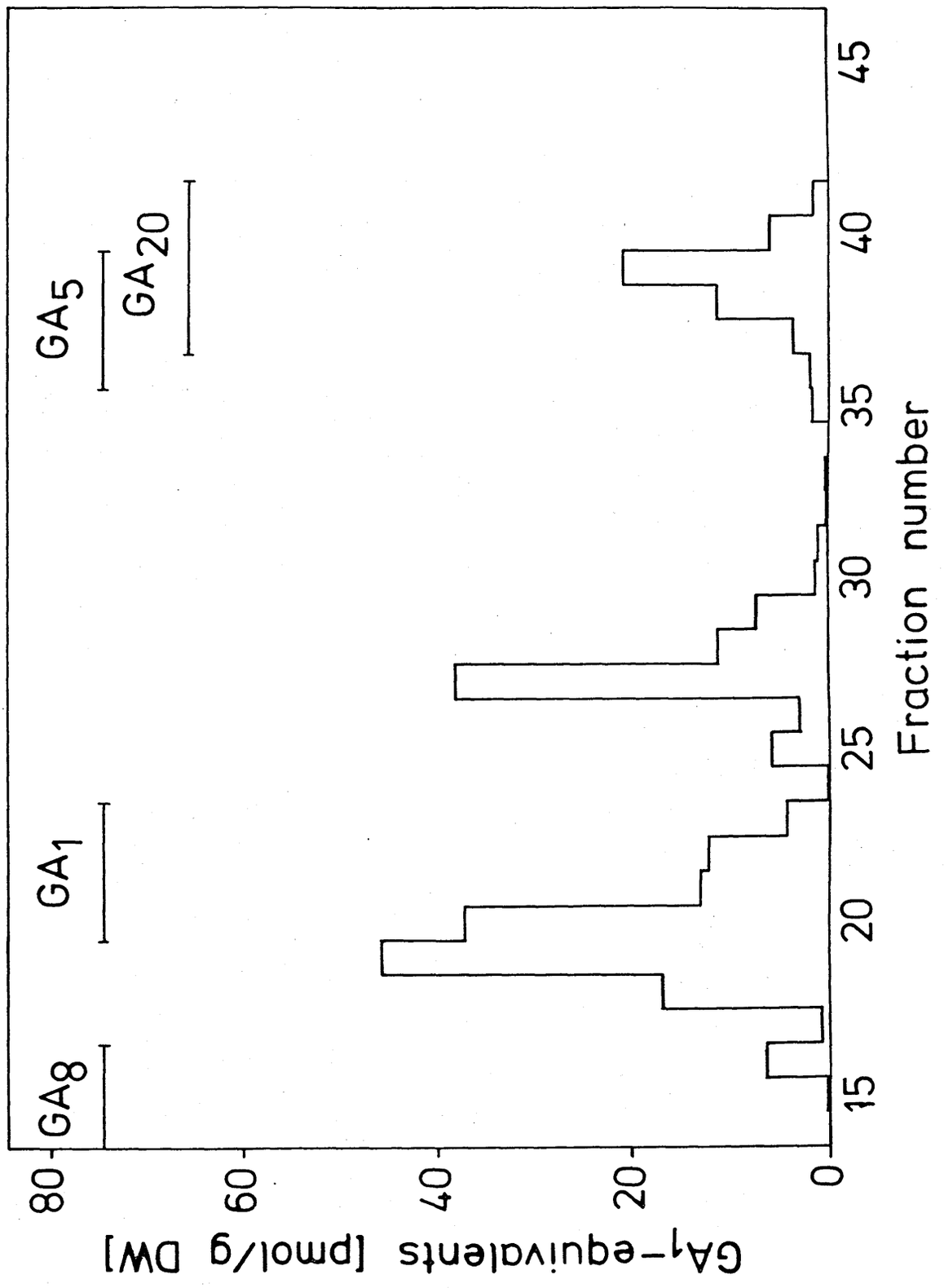

GA8
GA1
GA5
GA20
0
20
40
60
80
GA1-equivalents [pmol/g DW]
15
20
25
30
35
40
45
Fraction number

TABLE 9 Position and size of the peaks detected by RIA, using the GA_1-Me antiserum, in the HPLC fractions of the 12 weeks 5°C scale extract.

PEAK NUMBER	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL	RETENTION PROPERTY
1	18	16.97	125.44	GA_1
	19	45.85		
	20	37.36		
	21	12.99		
	22	12.27		
2	27	38.45	49.64	
	28	11.19		
3	38	11.37	32.31	GA_5/GA_{20}
	39	20.94		

FIGURE 13

Distribution of immunoreactivity in the HPLC fractions of the 12 weeks 5°C basal plate extract.

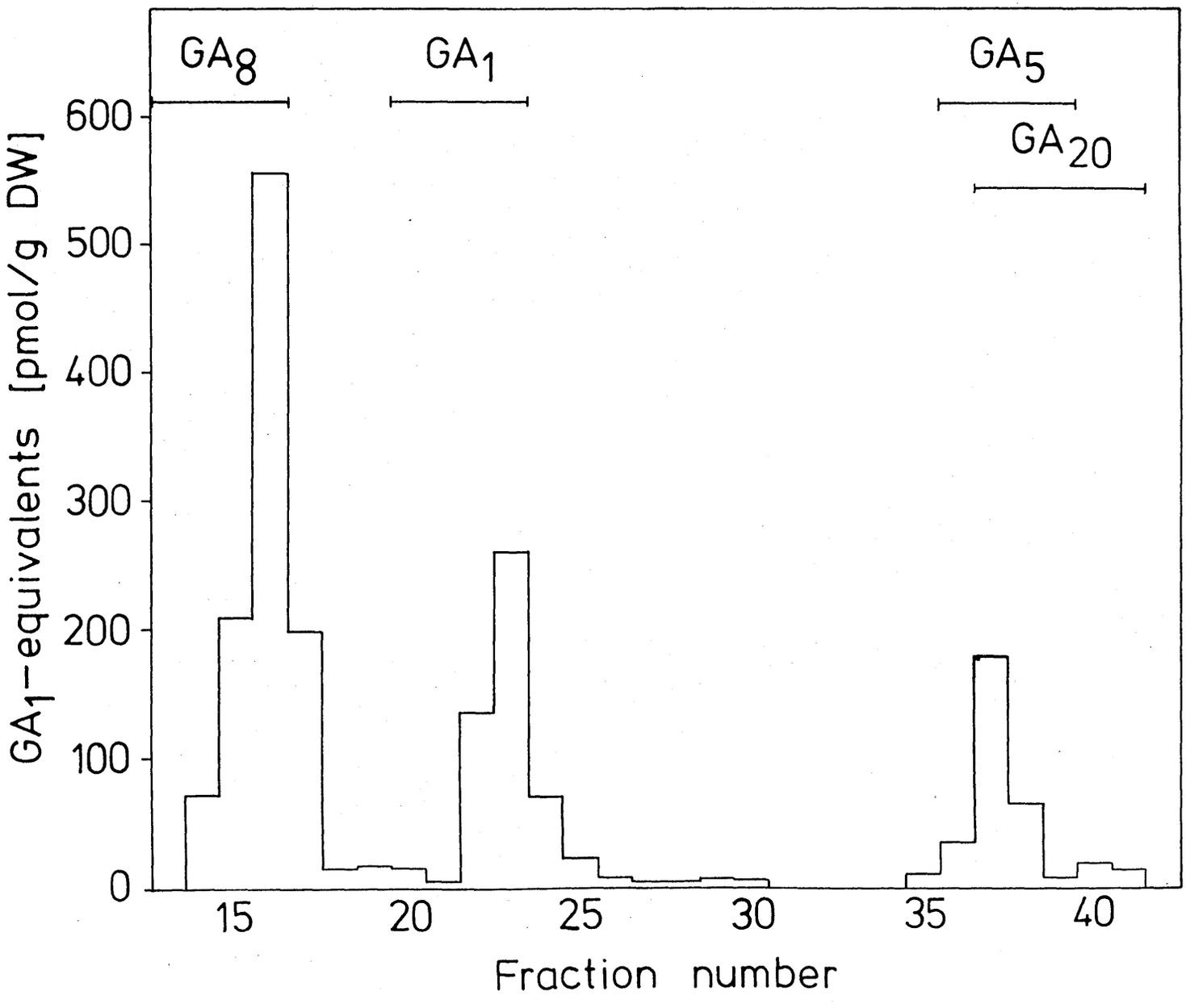

GA8
GA1
GA5
GA20
600
500
400
300
200
100
0
GA1-equivalents [pmol/g DW]
15
20
25
30
35
40
Fraction number

TABLE 10 Position and size of the peaks detected by RIA, using the GA_1-Me antiserum, in the HPLC fractions of the 12 week 5°C basal plate extract.

PEAK NUMBER	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL	RETENTION PROPERTY
1	15	209.48	962.60	GA_1
	16	556.11		
	17	197.01		
2	22	135.66	464.83	
	23	259.35		
	24	69.82		
3	36	33.42	277.80	GA_5/GA_{20}
	37	178.55		
	38	65.83		

DISCUSSION

Whole Bulb Samples - The three whole bulb samples each appeared to contain identical substances, peaks 1, 2 and 3, whilst in the 0 week sample another later peak was detected.

The results were expressed in pmol GA_1-equivalents g^{-1} DW, where GA_1-Me had a cross reactivity of 100% with the antiserum. Other GA-Mes had lower cross reactivities than GA_1-Me, therefore results adjusted to take into account the identity of the peak could be somewhat different and alter the relative proportions of each peak. However, using the results in GA_1-equivalents, peak 1 was the largest in each sample followed by peaks 2 and 3. Peaks 1 and 2 had both decreased by a similar large amount in the 6 week sample and a further smaller decrease had occurred in the 12 week sample (Table 11). The initial level of Peak 3 was considerably lower and thus the changes were not so dramatic. There was a relatively small increase in the 6 week sample followed by a fall to below the initial value. Peak 4 was only detected in the 0 week sample. The trend was therefore one of decreasing size of each peak with increasing storage time, and the combined total of the 12 week sample was less than one sixth of the initial combined total (Table 11).

TABLE 11 Size of peaks 1-4 in the 0, 6 and 12 week 5°C whole bulb extracts and the combined total for each extract. Results are expressed in pmol GA_1-equivalents g^{-1} DW.

PEAK NUMBER	0W5°C	% OF TOTAL	6W5°C	% OF TOTAL	12W5°C	% OF TOTAL
1	832.24	48.42	271.02	53.12	157.82	62.23
2	728.24	42.37	144.98	28.42	46.07	18.17
3	78.94	4.59	94.20	18.46	49.71	19.60
4	79.19	4.61	N.D.		N.D.	
COMBINED TOTAL	1718.61		510.20		253.60	

TABLE 12 Size of peaks 1-3 in the 12 week 5°C scale and basal plate extracts and the combined total for each extract. The results are expressed in pmol GA_1-equivalents g^{-1} DW.

PEAK NUMBER	12W5°C SCALE	% OF TOTAL	12W5°C BASAL PLATE	% OF TOTAL
1	125.44	60.48	962.60	56.45
2	49.64	23.93	464.83	27.26
3	32.31	15.58	277.80	16.29
COMBINED TOTAL	207.39		1705.23	

Although peak 1 reduced in size with time its percentage of the combined total increased (Table 11). The percentage of peak 3 also increased, particularly between 0 and 6 weeks, but the percentage of peak 2 decreased from a level only slightly lower than that of peak 1, to a level similar to that of peak 3.

12 weeks 5°C Scale and Basal Plate Samples - The scale extract contained peaks 1-3 which had been detected in the whole bulb extracts (Table 12). Three peaks were also detected in the basal plate sample but each occurred earlier than, although still in the same region as, peaks 1-3 (Table 12). If the peaks detected in these two samples are taken to be equivalent to peaks 1-3, then they correspond to the pattern found in the whole bulb samples where peak 1 was the highest followed by peak 2 then peak 3. The peaks found in the basal plate sample were much larger than those found in the scale sample but when considered as a percentage of the total the individual peaks had a similar value in both extracts (Table 12).

Possible Identities of the Peaks Detected - Due to the apparent difference in the chromatography of the tritiated standards and the samples, the exact position of GAs in the HPLC fractions was unknown.

Three of the five samples contained peaks around fractions 18-22 (Peak 1) with the apex of the peak in fractions 19 or 19-20. Tritiated GA_1-Me eluted in

fraction 18 whilst the predicted elution range was fractions 20-23 and it seemed possible that peak 1 was GA_1-Me. The exceptions were the 0 week 5°C whole bulb sample and the 12 weeks 5°C basal plate sample. In the whole bulb sample the first peak was detected later, in fraction 22-23, but the other peaks detected also appeared later than the equivalent peaks in the other samples - in fractions 29-30 and 39-40 rather than 27-29 and 38-39. However, the [^{3}H]GA_9-Me in the 0 week sample was found in fraction 56 as in the other samples, but perhaps the chromatography early in the gradient was affected by extract impurities to a greater extent than in the other samples. Subsequent isocratic HPLC of this peak followed by further analysis by RIA, appeared to confirm the assumption that this peak probably was GA_1-Me. Another possibility was that peak 1 could be GA_9-Me rather than GA_1-Me as it also had a high cross reactivity, but considering the result for the isocratic analysis of the 0 week sample it seemed unlikely.

The basal plate sample was more difficult to interpret as the peaks appeared in fractions 15-17, 22-24 and 36-38 rather than 18-22, 27-29 and 38-39 in three of the other samples. It seems possible that the peaks were equivalent but that each one eluted earlier than expected, although peak 2 seemed particularly early. The first peak also appeared in the predicted range of GA_8-Me but although this antiserum did detect GA_8-Me the cross reactivity was only 7% and so it seems unlikely that such a large peak

could be GA_6-Me. Therefore despite the apparent differences it seemed likely that these peaks were 1-3 as in the other samples.

Peak 2, which occurred around fractions 27-30 in four of the extracts (22-24 in the 12 weeks 5°C basal plate extract), eluted after GA_1-Me and before GA_5-Me and thus did not correspond with any of the tritiated standards. The only other GA detected by this antiserum was GA_7-Me but this GA would elute near GA_4, much later than this peak. It seems likely that it was a substance with a high cross reactivity with the antiserum because of its large size in some of the samples.

Peak 3, which was detected in each extract between fractions 36-40, was probably GA_5 and/or GA_{20}, both of which were detected by this antiserum. On the HPLC gradient used GA_5-Me and GA_{20}-Me were not completely separated, and so without further analysis using isocratic HPLC it was difficult to tell whether this peak was GA_5 or GA_{20}. The cross reactivity of GA_{20}-Me was considerably higher than that of GA_5-Me at 43% in comparison to 12%.

Peak 4, only detected in the 0 week whole bulb sample, was probably GA_4-Me as the predicted elution range was fractions 48-51 and the peak eluted in fractions 49-51. The antiserum also detected GA_7-Me which would have a retention time similar to that of GA_4-Me but it would elute before GA_4-Me and so it is unlikely that this peak was GA_7-Me.

At this stage research was hampered because of shortage of antiserum and an attempt to raise more took time and was ultimately unsuccessful. Further antiserum was obtained eventually from outside sources but not until sometime later. Each peak should have been subjected to further analysis, with continuation of the isocratic HPLC and subsequent RIA carried out for peak 1 of the 0 week whole bulb sample. Dilution curves indicated that there was no interference with peak 1, but verification of the accuracy of peaks 2 and 3 should also have been carried out, and further verification of peak 1 using internal standardisation in addition to dilution curves (Crozier *et al.*, 1986; Pengelly, 1986).

CHAPTER 3

WHOLE BULB SAMPLES - *Radioimmunoassays using the MAC 136 and 398 Pool antisera.*

Introduction

The whole bulb extracts had originally been assayed with the GA_1-Me antiserum raised in Glasgow. Attempts to raise more of these antibodies were unsuccessful and so two other antisera were obtained and used - first the MAC 136 antiserum (free GAs, Knox *et al.*, 1987) and later the 398 Pool antiserum (methylated GAs).

In addition to analysing extracts it had not previously been possible to analyse (i.e. 6 weeks 17°C and 4 and 8 weeks 5°C and 17°C whole bulb extracts plus the individual component extracts), it was decided to re-assay the 0, 6 and 12 week 5°C whole bulb samples using the new antisera. Newly extracted non-methylated samples were fractionated by a 30 min HPLC gradient of 30-100% MeOH prior to RIA with the MAC 136 antiserum. The fractions where radiolabelled standards of free GAs eluted on this gradient are given in Table 1. The [^{3}H]GA_9 internal standard was generally found in fraction 51 of the extracts. The details for the assay with the 398 Pool antiserum are given later with the appropriate results.

For the assays using the MAC 136 antiserum GA_9 standards were used for the standard curve. However, GA_9 and GA_1 both had cross reactivities of 100% and so the results were expressed in pmol GA_1 so that they were expressed in the same manner as the other results.

Standards of GA_4-Me were used for the assay with the 398 Pool antiserum but the results have been adjusted to pmol GA_1 - GA_1-Me and GA_4-Me had cross reactivities of 100% and 109% respectively.

0 week 5°C Whole Bulb Extract

Fractions 18-26 were assayed initially to look for a possible GA_1 peak as GA_1 was expected around fractions 23-25 (Table 1). Considering the results previously obtained a large peak was expected in this area but little was evident apart from a very slight lowering of the radioactivity in fractions 18 and 21.

TABLE 1 HPLC fractions where tritiated GA standards plus [^{14}C]GA_3 eluted when separated by a 30 min 30-100% MeOH HPLC gradient.

GA	FRACTION NUMBER
GA_8	12-13
GA_3	20-21
GA_1	23-25
GA_5	36
GA_{20}	37
GA_4	47
GA_9	51-52

During this assay the standard curve had appeared normal and the assay appeared to be working properly but the result was so surprising it was thought that something was perhaps interfering with the the assay. The assay method was tested - the method advised for this antiserum used horse serum in the incubation mixture (Knox *et al.*, 1987) rather than γ-globulin which had been used for the GA_1-Me antiserum assays. Standard curves were run using γ-globulin rather than horse serum and different rinsing methods after precipitation of the protein pellet. As expected, the incubation mixture using horse serum was best although the method was modified slightly by precipitating and then rinsing with saturated ammonium sulphate. The original method did not include a rinse but the UB values were rather high when no rinse was carried out.

Therefore the assay method appeared to be suitable and there was no outward sign that the assay was not functioning properly. Each sample fraction was dissolved in a small volume of MeOH (usually 10 µl) before dilution with PBS buffer and MeOH can interfere with the antibody-antigen binding. Assays were carried out using the same concentration of GA_3 standard with increasing concentrations of MeOH in the incubation mixture. A high concentration of MeOH did appear to affect the binding resulting in lowered radioactivity but only to a limited extent and not at the concentrations which would have been present in the sample assays. In any case the symptoms of

this type of interference (lowered or variable radioactivity) were not apparent in the sample assays.

Fractions 18-34 were assayed, using twice the quantity in the first assay, in case a peak had eluted later than expected. Although this indicated that there may have been very small peaks in fractions 18-19, 20-21, 27 and 32-33 (Table 2), there were certainly no large peaks and nothing in the GA_1 area of fractions 23-25.

A possible GA_5/GA_{20} peak had also been found when the first 0 week 5°C whole bulb sample was assayed and so fractions 35-39, around where these GAs were expected, were assayed but no peak was found.

TABLE 2 Possible peaks found in HPLC fractions 18-34 of the 0 weeks 5°C whole bulb sample using the MAC 136 antiserum.

FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
18	6.69	10.75
19	4.06	
20	5.51	11.72
21	6.21	
27	4.21	4.21
32	5.49	8.90
33	3.41	

TABLE 3 Peak found in HPLC fractions 23-25 of the original 0 week 5°C whole bulb sample using the MAC 136 antiserum.

FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
23	143.87	
24	83.53	243.30
25	15.90	

Original 0 week 5°C Extract Only half of the original 0W5°C whole bulb sample, in which a large GA_1-like peak had been found, had been used for immunoassays with the GA_1-Me antiserum. The remaining non-methylated half was fractionated by gradient HPLC and assayed with the MAC 136 antiserum to see whether it detected the peak previously found with the GA_1-Me antiserum. Fractions 23-26 were assayed using approximately the same amount as had been used for the assay of the above sample. A large peak was found in fractions 23-25 where GA_1 was expected (Table 3) but earlier fractions should have been assayed as the peak may have begun earlier. However, this at least seemed to confirm that the assay itself was functioning properly and that a large peak had been found in the GA_1 area of the 0 week 5°C whole bulb sample.

0 weeks 5°C Repeat Extract At this stage another sample of the 0 week tissue was extracted in exactly the same manner as the original sample using 30 g DW, the only

difference being that [^{3}H]GA_1 was used as an internal standard in addition to [^{3}H]GA_9. Half of the resultant extract was fractionated and the [^{3}H]GA_1 eluted in fractions 22-24. An assay of fractions 23-26 with the MAC 136 antiserum detected nothing.

The other half of the repeat sample was methylated and fractionated using a 40-100% MeOH HPLC gradient over 30 min. The fractions where radiolabelled GA-Mes eluted are given in Table 4. The [^{3}H]GA_1-Me in the extract eluted in fractions 21 and 22 and fractions 20-23 were assayed for GA_1 with the 398 Pool antiserum. Unlike the previous assay using the MAC 136 antiserum, a peak was detected in fractions 21 and 22 (Table 5).

After this positive result fractions 11-50 were assayed and a peak was again detected in fractions 21 and 22 but the only other peak was in fraction 37 around where GA_5-Me and GA_{20}-Me were expected (Table 5, Figure 1).

Therefore a possible GA_1 peak was detected in this repeat sample by the 398 Pool antiserum, but it was very small compared to the peak detected in the original sample. A very small peak was also evident in fraction 37 of this sample possibly equivalent to a considerably larger peak which had been detected in the original sample in fractions 39-40. There was no sign of the large peak which had been detected in fractions 29-30 of the original extract.

TABLE 4 HPLC fractions where tritiated standards of GA-Mes plus [^{14}C]GA_3-Me eluted when separated by a 30 min 40-100% MeOH gradient.

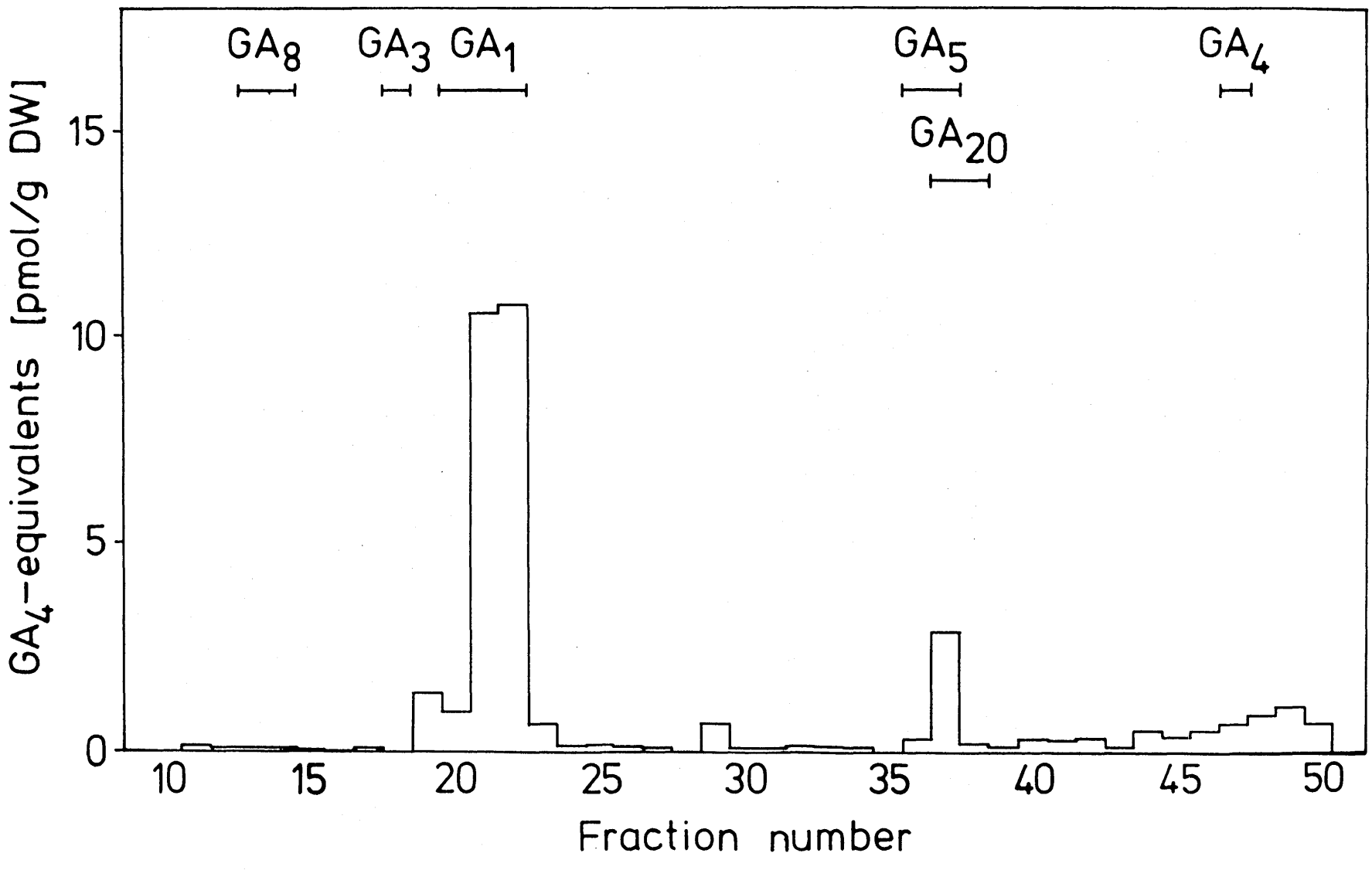
GA-Me	FRACTION NUMBER
GA_8-Me	13-14
GA_3-Me	18
GA_1-Me	20-21
GA_5-Me	36-37
GA_{20}-Me	37-38
GA_4-Me	47
GA_9-Me	53-54

TABLE 5 Peaks detected in the methylated HPLC fractions of the 0 week 5°C whole bulb sample by the 398 Pool antiserum in two separate assays. The results of the assay of fractions 20-23 are shown first.

FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
21	11.26	22.96
22	11.70	
21	9.58	19.35
22	9.77	
37	2.56	2.56

FIGURE 1

Distribution of immunoreactivity in the HPLC fractions of the 0 week 5°C whole bulb repeat extract, detected by the 398 Pool antiserum.


GA8
GA3
GA1
GA5
GA20
GA4
GA4-equivalents [pmol/g DW]
0
5
10
15
10
15
20
25
30
35
40
45
50
Fraction number

6 weeks 5°C Whole Bulb Extract

This sample was fractionated using the gradient for non-methylated samples, then fractions 22-25 were assayed for a possible GA_1 peak with the MAC 136 antiserum. A very small peak was found in fractions 23 and 24 (Table 6) but nothing as large as was expected from the original results. However, these results corresponded with the 0W5°C results which had also been far lower than expected.

6 weeks 5°C Repeat Extract For this extract 30 g DW of the bulb tissue was used - the same amount as had been used in the original extract. Tritiated GA_1 and GA_8 were used as internal standards and after fractionation of half the sample the [^{3}H]GA_1 was found in fractions 24 and 25. Fractions 23-26 were assayed with the MAC 136 antiserum but the only evidence of a peak was a very slight lowering of the radioactivity in fractions 24-26.

12 weeks 5°C Whole Bulb Extract

Due to the unusual results of the 0 and 6 week samples another extract was done of 30 g DW of the 12 week tissue with [^{3}H]GA_8 and [^{3}H]GA_1 as internal standards. The [^{3}H]GA_1 eluted in fractions 23-25 but an assay of fractions 22-26 with the MAC 136 antiserum detected nothing. Fractions 11-38 were then assayed using twice as much as in the previous assay but this only detected a tiny peak in fraction 34 (Table 6).

The GA_1 areas of the 4 week 5°C and 17°C, 6 week 17°C and 8 week 5°C and 17°C whole bulb samples were also assayed with the MAC 136 antiserum but no peaks were found in any of the samples.

TABLE 6 Peaks detected in the HPLC fractions of the 6 and 12 week 5°C whole bulb samples using the MAC 136 antiserum.

SAMPLE	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
6W5°C	23	4.36	8.54
	24	4.18	
12W5°C	34	5.51	5.51

Discussion

Considering the results obtained from earlier whole bulb samples, using the GA_1-Me antiserum raised in Glasgow, several peaks were expected in these samples, but particularly a possible GA_1 peak. Therefore these assays concentrated on the GA_1 area but either nothing or only very small peaks were found. At this stage the original 0W5°C sample was assayed with the MAC 136 antiserum which also detected a large peak and confirmed that the assay was working and that this substance was detected by two different antisera.

Due to the fact that the original extracts of 30 g DW had required high dilution to get the peaks on scale, only 5 g DW had been extracted this time. When these samples gave negative results repeat extracts were carried out, again using 30 g DW. When assayed with the MAC 136 antiserum the results for these repeat samples were no better but an assay of the 0 week sample with the 398 Pool antiserum detected a small peak. This peak was still very much smaller than the peak which had been detected in the original sample.

It is possible that the original results were due to contamination of the samples during extraction and purification. If so, each of the 0, 6 and 12 week samples must have been contaminated with several substances as they each appeared to contain similar peaks which generally decreased from the 0 week sample through 6 weeks to the 12W5°C sample. The most obvious way in which

contamination could take place is from contaminated glassware. All dirty glassware was washed, and dried in a hot oven and if it had been used for radioactive substances it was steeped in detergent for at least 24 hours prior to normal washing. The glassware used was in a large laboratory used by a number of people so the glassware was not only for personal use. It is therefore impossible to say that these cleaning procedures were always rigorously adhered to but if this procedure had been followed it seems unlikely that any contamination could have taken place. Also, if contamination were to take place in this way a whole batch of glassware must have been contaminated for each of these samples to have been affected. GA analysis by other researchers at this time did not yield unusual results and so contamination seems unlikely.

The blender goblet used to macerate the tissue could have been contaminated when the first of these extracts was carried out. However, it was always cleaned thoroughly between extracts which would seem to discount the contamination of subsequent extracts in this manner. The goblet was not being used by anybody else at this time and so contamination could not have taken place from another sample.

Each sample was subjected to rotary evaporation at various stages during the extraction and purification procedure. Some samples have a tendency to froth and bubble during rotary evaporation thus contaminating the

equipment and so if it had not been cleaned properly this could have caused contamination of subsequent samples.

The bulb tissue had been frozen in liquid N_2, freeze-dried and stored frozen and it was assumed that in this state any GAs present would be stable indefinitely. It is possible that breakdown took place between the original and the later extractions which would account for the vast difference in the results. This seems the most likely explanation although it could not have been predicted beforehand.

CHAPTER 4

ANALYSIS OF THE INDIVIDUAL BULB COMPONENTS BY RIA

Introduction

The individual tissue samples were fractionated by gradient HPLC prior to RIA with the MAC 136 antiserum for non-methylated samples (Knox *et al.*, 1987), or the 398 Pool antiserum for methylated extracts. Table 1 gives the fractions where free GAs or GA-Mes eluted during their respective HPLC gradients.

For the assays with the MAC 136 antiserum standards of GA_3 were used rather than GA_1. The results are expressed in pmol GA_1 because both GA_1 and GA_3 had a cross reactivity of 100% with this antiserum and so the results are equivalent whether expressed in pmol GA_1 or pmol GA_3. GA_4-Me standards, with a cross reactivity of 109%, were used for the assays with the 398 Pool antiserum. GA_7-Me had a cross reactivity of 100% and so the results were adjusted so that they were expressed in pmol GA_1.

Scale Extracts

All the scale extracts were assayed with the MAC 136 antiserum except the 4W17°C sample. Fractions 22-26 of the 5°C samples were assayed for a possible GA_1 peak but the only substance detected was a very small peak in the 12 week sample (Table 2). Of the 17°C samples assayed with the MAC 136 antiserum a small peak was found in fractions 21-23 of the 12 week sample when fractions 18-26 were assayed. The 4 week sample was assayed with the 398

TABLE 1 HPLC fractions where radiolabelled free GAs and GA-Mes eluted during 30 min gradients of 30-100% MeOH or 40-100% MeOH respectively.

GA	FRACTION NUMBER	GA-Me	FRACTION NUMBER
GA_8	12-13	GA_8-Me	13-14
GA_3	20-21	GA_3-Me	18
GA_1	23-25	GA_1-Me	20-21
GA_5	36	GA_5-Me	36-37
GA_{20}	37	GA_{20}-Me	37-38
GA_4	47	GA_4-Me	47
GA_9	51-52	GA_9-Me	53-54

TABLE 2 Peaks detected in the HPLC fractions of scale extracts, from bulbs stored at 5°C and 17°C, using the MAC 136 antiserum.

SAMPLE	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
0W5°C		N.D.	N.D.
4W5°C		N.D.	N.D.
6W5°C		N.D.	N.D.
8W5°C		N.D.	N.D.
12W5°C	22	1.71	3.92
	23	2.21	
6W17°C		N.D.	N.D.
8W17°C		N.D.	N.D.
12W17°C	21	1.45	8.48
	22	4.92	
	23	2.11	

Pool antiserum and a peak was found in fractions 20-22 around where GA_1 was expected in fractions 21 and 22 (Table 2).

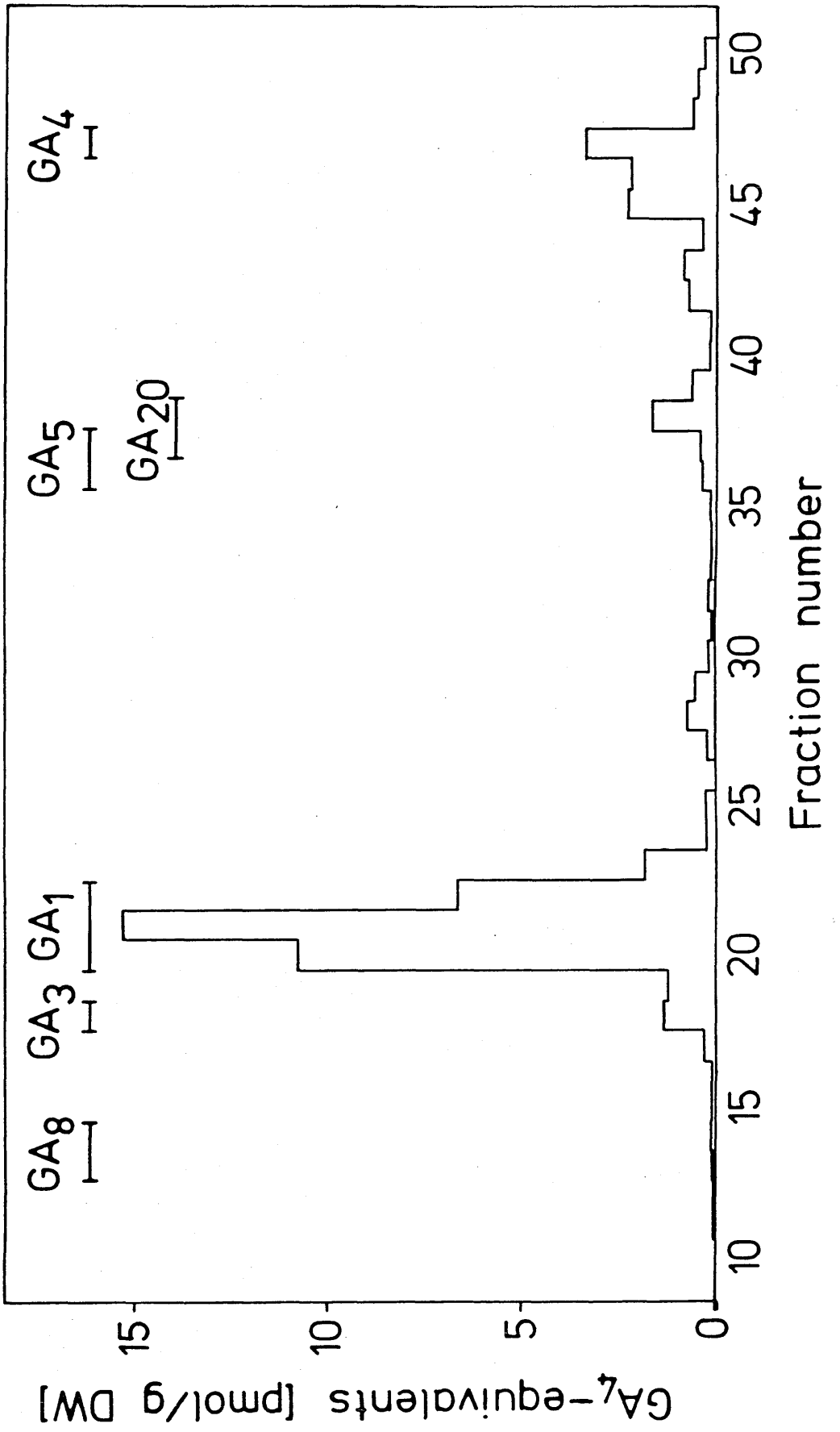
Fractions 11-50 of the methylated 4 week 17°C sample were assayed with the 398 Pool antiserum and three peaks were detected (Table 3, Figure 1). The first appeared in fractions 20-22, in the area where GA_1-Me was expected (Table 1), and was large in relation to the possible GA_1 peaks detected in the other samples (Tables 2 and 3). The other two peaks were smaller and occurred near the areas where GA_5-Me/GA_{20}-Me and GA_4-Me standards had eluted.

TABLE 3 Peaks detected in the HPLC fractions of the 4 weeks 17°C scale sample using the 398 Pool antiserum on methylated fractions.

FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
20	9.73	
21	13.89	29.67
22	6.05	
38	1.50	2.06
39	0.56	
45	2.07	
46	1.96	7.08
47	3.05	

FIGURE 1

Distribution of immunoreactivity in the HPLC fractions of the 4 weeks 17°C Scale extract, detected by the 398 Pool antiserum.


GA4
GA5
GA20
GA8
GA3
GA1
0
5
10
15
10
15
20
25
30
35
40
45
50
Fraction number
GA4-equivalents [pmol/g DW]

Basal Plate Extracts

The GA_1 areas of the basal plate samples were assayed with the MAC 136 antiserum and the peaks detected are shown in Tables 4 and 5. The main parts of the peaks detected in the 5°C samples were in fractions 22-24 although the main part of the 12 week peak was earlier, in fraction 21 (Table 4). It is possible that the substance in the 12 week sample was GA_3 rather than GA_1 as [^{14}C]GA_3 eluted in fractions 20-21. However, assuming that the substances in the other samples were the same, the level fell between 0 and 8 weeks with the greatest decrease between 4 and 6 weeks so that the level in the 6 and 8 week samples was very low. Whether the peak in the 12 week sample was different or not, it was considerably larger than the GA_1-like peaks in the 6 and 8 week samples but smaller than those in the 0 and 4 week samples.

The 12 week peak was detected when fractions 11-50 were assayed with the MAC 136 antiserum, and four other peaks were also found, in fractions 31-34, 38-40, 44-46 and 48-50 (Table 5, Figure 2). The first of these four peaks was the largest with the others decreasing in size in order of elution, but this does not take into account the differing cross reactivities of each substance. None of the peaks corresponded exactly with the standards analysed. The first peak eluted between GA_1 and GA_5 whilst the second peak eluted slightly later than the range of GA_5 and GA_{20}. The third and fourth peaks eluted just before and after GA_4, respectively.

TABLE 4 Peaks detected in the HPLC fractions of the basal plate extracts, from bulbs stored at 5°C, using the MAC 136 antiserum.

SAMPLE	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
0W5°C	21	14.15	85.77
	22	26.55	
	23	26.80	
	24	18.27	
4W5°C	21	7.08	71.30
	22	14.77	
	23	25.98	
	24	15.05	
	25	8.42	
6W5°C	22	8.99	14.61
	23	5.62	
8W5°C	22	4.63	8.90
	23	4.27	
12W5°C	21	23.85	46.11
	22	13.50	
	23	4.50	
	24	4.26	

Apart from the 12 week extract the peaks found in the 17°C samples were smaller than those found in the 5°C samples (Table 6). The 8 week sample appeared to contain two small peaks, in fractions 20 and 24-25 - perhaps GA_3 and GA_1. The peak in the 12 week sample was so large that

the height of the peak in fraction 21 was off scale and the total value was therefore inaccurate. However this peak was considerably larger than any found in the other basal plate samples and seemed more likely to be GA_3 than GA_1 because it eluted around fraction 21 where [^{14}C]GA_3 had eluted. The 12 week 5°C peak also appeared to be closer to GA_3 than GA_1.

TABLE 5 Peaks detected when HPLC fractions 11-50 of the 12 week 5°C basal plate extract were scanned with the MAC 136 antiserum.

FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
31	9.46	
32	23.41	80.94
33	38.37	
34	9.70	
38	20.19	
39	24.33	62.33
40	17.81	
44	21.59	
45	24.92	59.96
46	13.45	
48	11.14	
49	12.91	33.31
50	9.26	

FIGURE 2

Distribution of immunoreactivity in the HPLC fractions of the 12 weeks 5°C Basal Plate extract, detected by the MAC 136 antiserum.

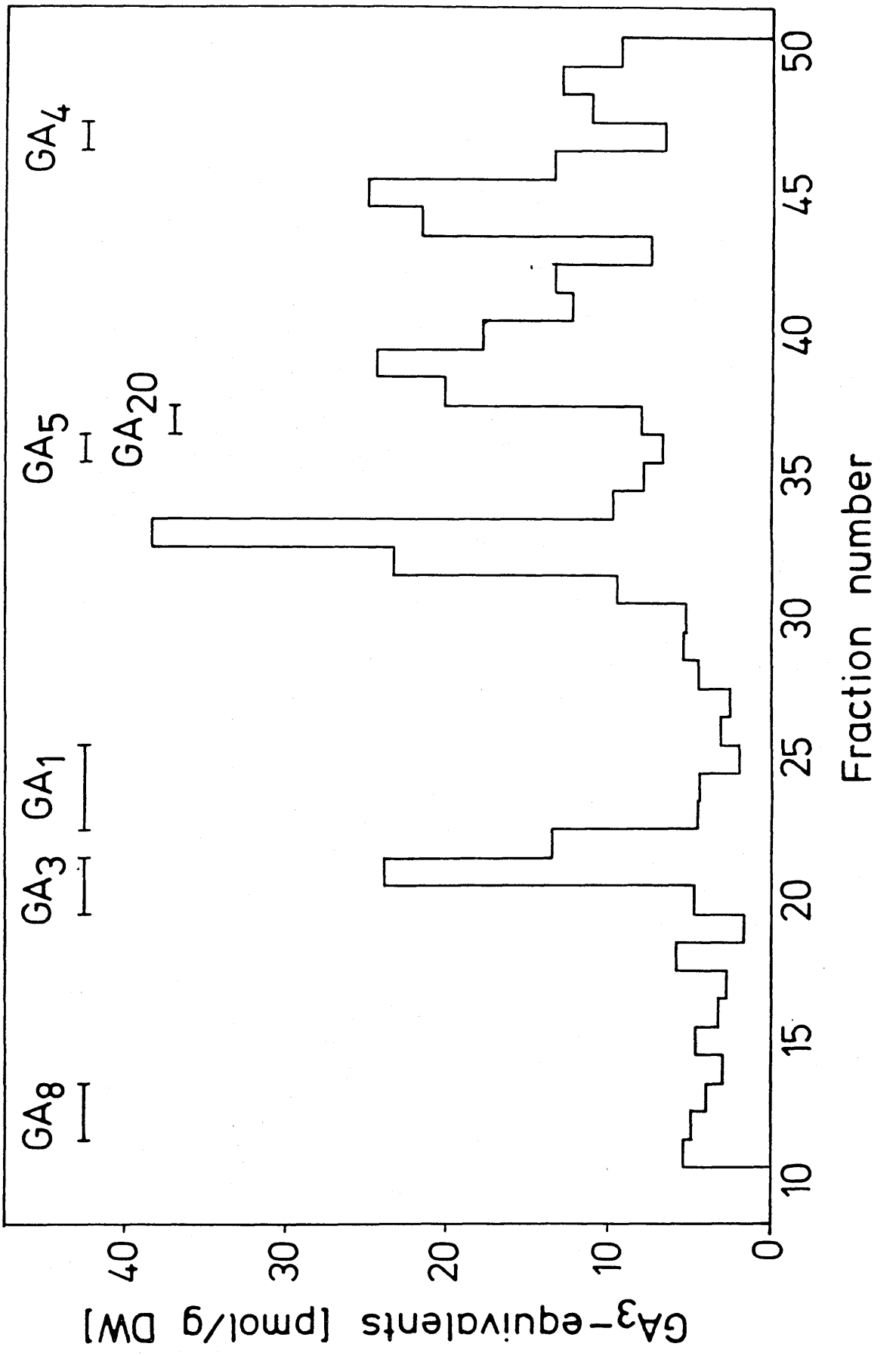

GA3-equivalents [pmol/g DW]
0
10
20
30
40
10
15
20
25
30
35
40
45
50
Fraction number
GA8
GA3
GA1
GA5
GA20
GA4

TABLE 6 Peaks detected in the HPLC fractions of the basal plate extracts, from bulbs stored at 17°C, using the MAC 136 antiserum.

SAMPLE	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
4W17°C	20	4.70	28.94
	21	8.05	
	22	8.91	
	23	7.28	
6W17°C	22	2.11	9.43
	23	3.95	
	24	3.37	
8W17°C	20	9.62	9.62
8W17°C	24	4.92	9.09
	25	4.17	
12W17°C	19	9.39	152.97 plus
	20	88.30	
	21	Off scale	
	22	45.69	
	23	9.59	

Daughter Bulb Extracts

The GA_1 area of each daughter bulb sample was assayed, either with the MAC 136 antiserum or the 398 Pool antiserum which appeared to be more sensitive. The 0 and 6 week samples were methylated and so GA_1 was expected around fractions 20-21 and the peaks detected did appear in this area (Table 7). The 12 week sample was not methylated and thus GA_1 was expected around fractions 23-25 but the peak detected appeared in fractions 21 and 22, nearer to GA_3 which was expected in fractions 20-21 on this HPLC gradient. Assuming that the substances in the 0 and 6 week extracts were the same, the level fell to almost half the original level after 6 weeks. The substance in the 12 week sample had a lower level than that in the 6 week extract but it is not clear whether they were the same substances.

The 4 and 6 week 17°C samples were assayed with the 398 Pool antiserum and contained peaks of a similar size, in the GA_1 area (Table 7). The 8 week sample was assayed with the MAC 136 antiserum and contained a small peak which appeared in the GA_1 area, later than the peaks in the other non-methylated samples (Table 7). The peak detected in the 12 week sample occurred in fractions 21-23, particularly 21 and 22 and so was nearer the area of GA_3 (20-21) than GA_1 (23-25). This peak was also larger than any of the peaks detected in both the 17°C and the 5°C samples.

TABLE 7 Peaks detected in the daughter bulb extracts from the bulbs stored at 5°C and 17°C. Extracts marked with an asterisk were assayed with the 398 Pool antiserum rather than the MAC 136 antiserum.

SAMPLE	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
0W5°C*	20	26.26	67.75
	21	18.34	
	22	13.20	
	23	9.95	
6W5°C*	20	16.90	34.96
	21	6.89	
	22	7.92	
	23	3.25	
12W5°C	21	18.32	27.31
	22	8.99	
4W17°C*	20	18.26	33.50
	21	6.08	
	22	4.06	
	23	5.10	
6W17°C*	21	19.29	32.22
	22	6.41	
	23	6.52	
8W17°C	23	6.54	12.39
	24	5.85	
12W17°C	21	39.12	91.63
	22	41.56	
	23	10.95	

Fractions 16-26 of the 0 week 5°C extract were initially assayed and a GA_1-like peak was detected (Table 7, Figure 3). Subsequently, fractions 11-15 and 27-50 were also scanned using the MAC 136 antiserum and several peaks were found (Table 8, Figure 3). The area from fractions 37 to 43 has been taken as one peak but it is possible that it was actually two peaks. The value fell in fractions 40 and 42 but this fall was interrupted by a slight increase in fraction 41. GA_5 and GA_{20} standards eluted in fractions 36-38 therefore the early part of this peak overlapped this area. The two peaks which eluted in fractions 46-47 and 48-50, were possibly GA_4 and GA_9 respectively, although their elution times did not correspond exactly to those of the standards. The other peak eluted in fractions 44-45 and so did not correspond to any of the GA standards. All these peaks were of a similar size to or larger than the GA_1-like peak (Tables 7 and 8, Figure 3), with the peaks in fractions 37-43 and 48-50 being the largest.

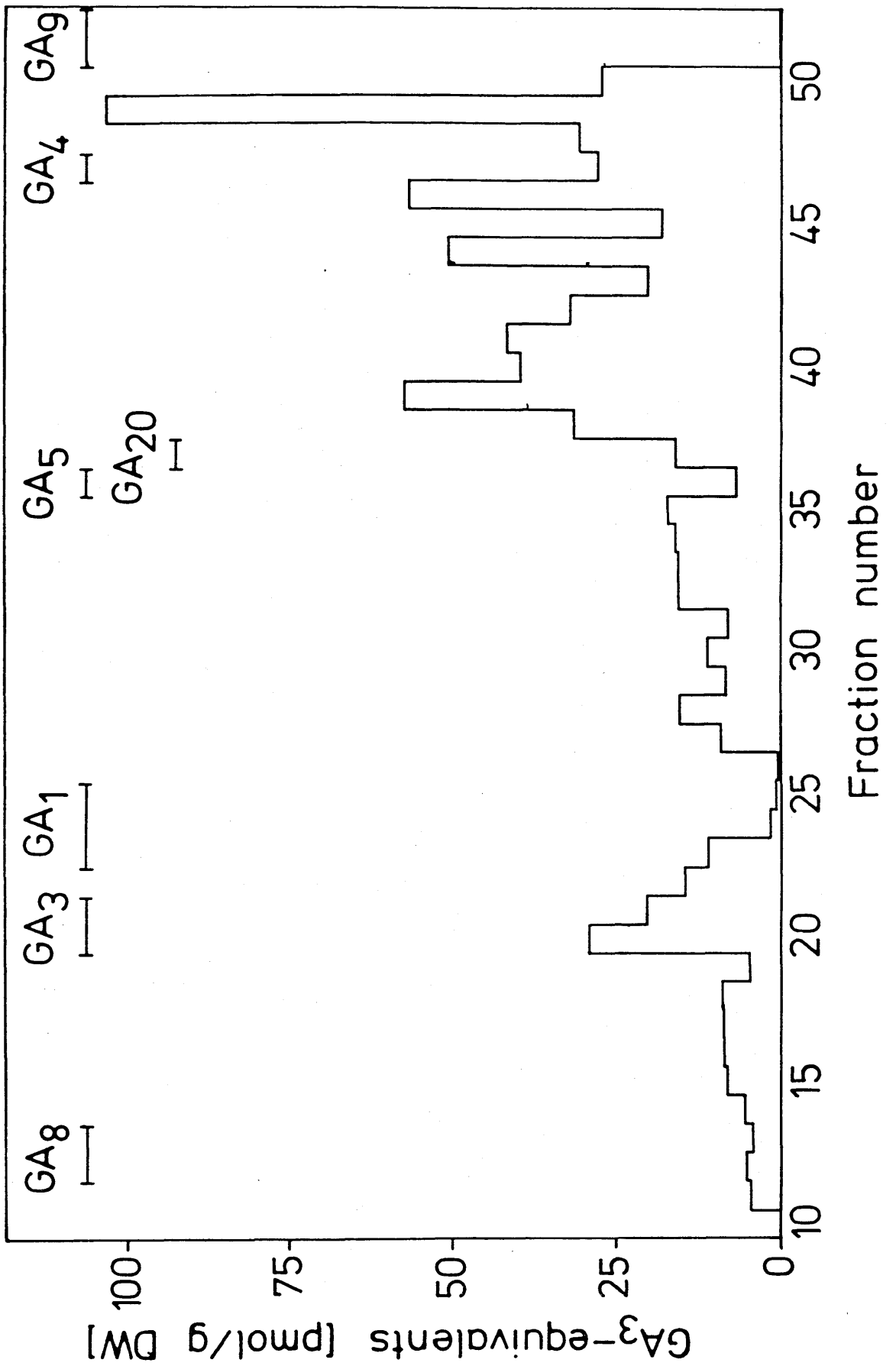
TABLE 8 Peaks detected in the 0 week 5°C daughter bulb extract when fractions 11-15 and 27-50 were assayed with the MAC 136 antiserum.

FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
37	16.03	
38	31.40	
39	57.43	
40	39.87	238.56
41	41.59	
42	32.06	
43	20.18	
44	50.72	68.77
45	18.05	
46	56.91	84.61
47	27.70	
48	30.64	
49	103.03	160.92
50	27.25	

FIGURE 3

Distribution of immunoreactivity in the HPLC fractions of the 0 week 5°C Daughter Bulb extract.

Fractions 11-15 and 27-50 were assayed with the MAC 136 antiserum, whilst fractions 16-26 were assayed with the 398 Pool antiserum.


GA9
GA4
GA5
GA20
GA1
GA3
GA8
100
75
50
25
0
10
15
20
25
30
35
40
45
50
Fraction number
GA3-equivalents [pmol/g DW]

Shoot Extracts

All the samples, apart from the 8 week 5°C sample which was methylated, were assayed with the MAC 136 antiserum (Table 9). Generally, fractions 22-25 were assayed to look for possible GA_1 peaks which would be expected in fractions 23-25. However, peaks were only detected when earlier fractions of the 12 week 5°C and 17°C samples were assayed. They were detected in fractions 19-23 of the 5°C sample and fractions 19-21 of the 17°C sample, with the main part of both being in fraction 20 (Table 9). Due to their position these peaks seemed nearer to GA_3 than GA_1.

The 8 week 5°C shoot sample had been methylated prior to fractionation and was assayed with the 398 Pool antiserum. Fractions 11-50 were scanned but only two peaks were found, in fraction 18-22 and 44-47 (Table 10, Figure 4). On the HPLC gradient for methylated samples GA_1-Me eluted in fractions 20-21 and so the first peak, with its main part in fraction 20, may have been GA_1. The second peak was smaller and eluted just before where GA_4 was expected in fraction 47.

TABLE 9 Peaks detected in the HPLC fractions of the shoot extracts from bulbs stored at 5°C and 17°C. Extracts marked with an asterisk were assayed with the 398 Pool antiserum rather than the MAC 136 antiserum.

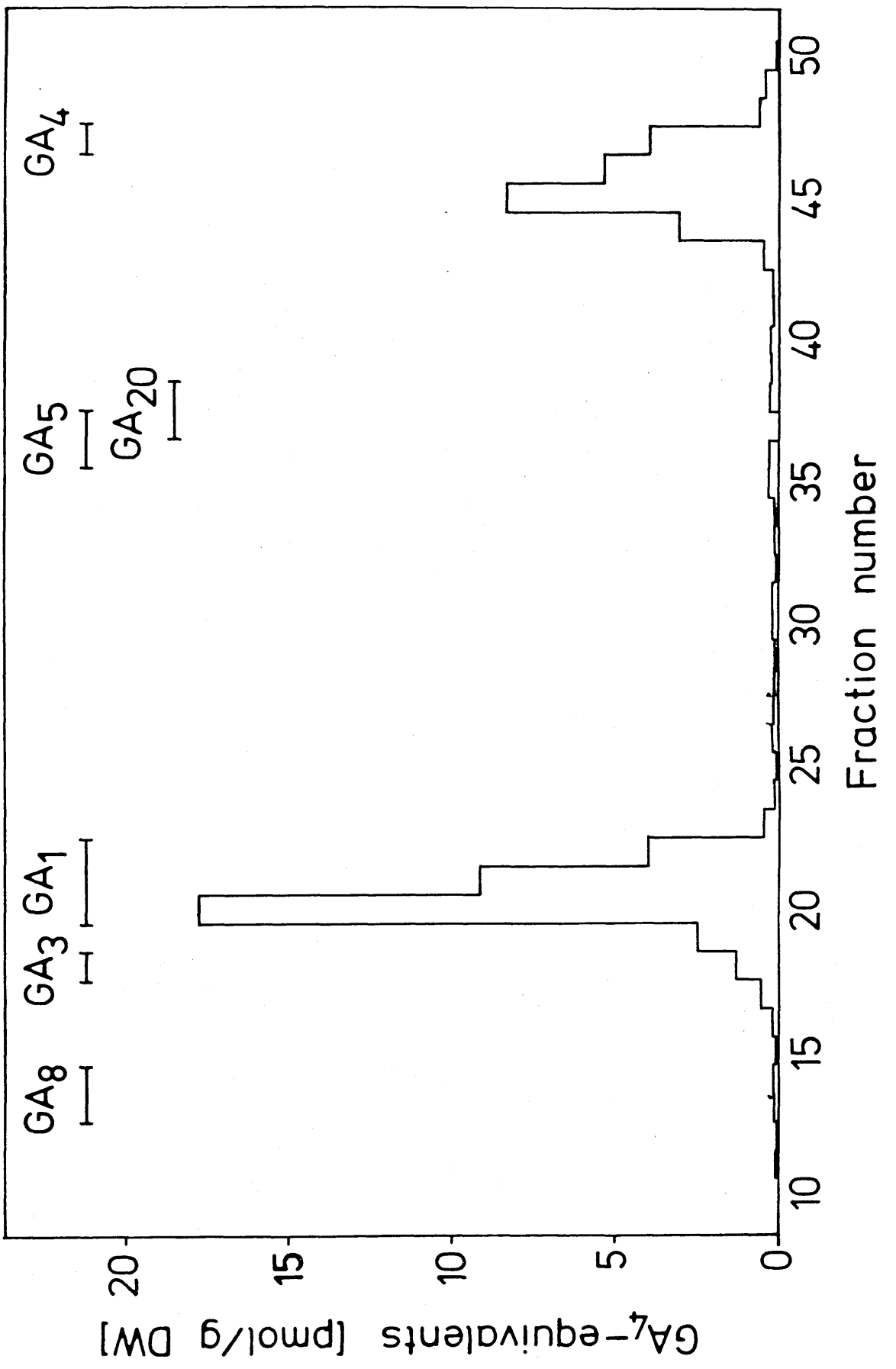
SAMPLE	FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
0W5°C		N.D.	N.D.
6W5°C		N.D.	N.D.
8W5°C*	18	1.17	31.57
	19	2.24	
	20	16.21	
	21	8.32	
	22	3.63	
12W5°C	19	4.49	27.86
	20	10.21	
	21	7.00	
	22	4.03	
	23	2.13	
4W17°C		N.D.	N.D.
6W17°C		N.D.	N.D.
8W17°C		N.D.	N.D.
12W17°C	19	4.25	20.65
	20	9.51	
	21	6.89	

TABLE 10 Peak detected in the 8 week 5°C shoot extract when fractions 11-50 were assayed with the 398 Pool antiserum.

FRACTION NUMBER	pmol GA_1 g^{-1} DW	TOTAL
18	1.17	
19	2.24	
20	16.21	31.57
21	8.32	
22	3.63	
44	2.75	
45	7.62	18.81
46	4.84	
47	3.60	

FIGURE 4

Distribution of immunoreactivity in the HPLC fractions of the 8 weeks 5°C Shoot extract, detected by the 398 Pool antiserum.


GA8
GA3
GA1
GA5
GA20
GA4
0
5
10
15
20
10
15
20
25
30
35
40
45
50
Fraction number
GA4-equivalents [pmol/g DW]

DISCUSSION

Scale Extracts - When the extracts of scale tissue were assayed for GA_1 the only peaks detected were in the 12 weeks 5°C and 17°C, and the 4 weeks 17°C samples. The largest peak was detected in the 4 week sample but this was assayed with the 398 Pool antiserum which appeared to be more sensitive than the MAC 136 antiserum used for the other samples. However, it would seem that GA_1 was not a GA present in large quantities in bulb scale tissue. The main GA-Mes which the 398 Pool antiserum could detect were GA_1-Me, GA_4-Me, GA_3-Me and GA_7-Me and a scan of the 4 week 17°C HPLC fractions revealed two other small peaks. They appeared closest to the GA_{20} and GA_4 standards although the cross reactivity with GA_{20} was <0.4%. Therefore the scale tissue did not appear to be a rich source of GA_1 or the other detectable GAs.

An earlier extract of the 12 week 5°C scale tissue had been assayed with the GA_1-Me antiserum giving a value of 125.44 pmol GA_1 g^{-1} DW compared to the value of 3.92 pmol obtained with the MAC 136 antiserum. A similar situation occurred in the analysis of the whole bulb samples, when assays of new extracts with the MAC 136 antiserum did not detect the large peaks detected by the GA_1-Me antiserum in earlier extracts. In this case it was concluded that the bulb tissue had probably deteriorated in storage resulting in breakdown of any GAs present. This could also have occurred in the individual tissue samples making these

results unreliable.

Basal Plate Extracts - Unlike the scale samples relatively large peaks were detected in the GA_3/GA_1 area of a number of the basal plate extracts. The peaks were not in exactly the same fractions in each sample and the difference was particularly noticeable in the 12 weeks 5°C and 17°C samples where the peaks were earlier, nearer to GA_3 than GA_1. The level fell between 0 and 8 weeks at both 5°C and 17°C with a subsequent increase after 12 weeks which was larger at 17°C than at 5°C. At this stage the activity in the basal plate was greater at 17°C than 5°C with more root growth.

Four other peaks were detected in the 12 week 5°C extract, the first of which eluted between GA_1 and GA_5 and thus did not correspond to any of the radiolabelled standards. However, on reversed phase HPLC both GA_6 and GA_{19} elute after GA_1 and before GA_5 and the MAC 136 antiserum could detect both of them with cross reactivities of 80 and 115% respectively in comparison to 100% for GA_1. Therefore both these GAs had high cross reactivities and although it is unknown precisely where they would elute on the HPLC gradient used it seems possible that this peak was one of these GAs. An earlier extract of this tissue had been assayed with the GA_1-Me antiserum but no such peak was detected although peaks eluting between GA_1 and GA_5 had been found in whole bulb samples.

The second peak appeared just after where the GA_{20} standard eluted. The MAC 136 antiserum had high cross reactivities with GA_5 and GA_{20} (48% and 100% respectively), but the cross reactivity was even higher (167%) with GA_{53} which elutes shortly after GA_{20} on reverse phase HPLC. The earlier extract also appeared to contain a substance in the area of GA_5 and GA_{20} but the value for this peak was considerably higher than that estimated for the later sample. The GA_1-Me antiserum detected GA_5 and GA_{20} with a higher cross reactivity for GA_{20} than GA_5, but it did not detect GA_{53}. It therefore seems most likely that this substance was GA_{20} but the discrepancy in the values can not be accounted for by differing cross reactivities.

The third and fourth peaks eluted just before GA_4 and between GA_4 and GA_9, respectively. The MAC 136 antiserum had cross reactivities of <1% with GA_4 and GA_9 and other GAs which elute close to them on reverse phase HPLC, such as GA_7 and GA_{14}. This antiserum would therefore be unlikely to detect these GAs unless they were very large peaks. Also, an earlier extract had been assayed with the GA_1-Me antiserum, which had a cross reactivity of 38% with GA_4, but nothing was detected in the GA_4 area.

Daughter Bulb Extracts - The problem of whether the peaks detected were likely to be GA_3 or GA_1 was also evident in the daughter bulb assays. However assuming that the peaks were all the same substance the level appeared to fall

during both 5°C and 17°C storage but after 12 weeks 5°C storage the level continued to fall while at 17°C the level had increased to a value higher than that in the initial 0 week sample.

Other peaks were detected in the 0 week daughter bulb sample but the results were very unusual in that there appeared to be a continuous series of five peaks between fractions 37 and 50 where the level never decreased to background between the peaks. They eluted in the area between where GA_5/GA_{20} and GA_9 standards eluted. As explained before the MAC 136 antiserum detected GAs in this area but their cross reactivities were low apart from GA_{53} which had a very high cross reactivity. It may be that interfering substances were present in the sample, giving these strange results (Crozier *et al.*, 1986; Pengelly, 1986).

Shoot Extracts - GA_1 or GA_3-like peaks were only detected in three of the shoot extracts - 8 and 12 week 5°C and 12 week 17°C. The 12 week 5°C and 17°C peaks appeared closer to GA_3 than GA_1, but the 8 week peak, assayed with a different antiserum, eluted where GA_1 was expected. Fractions 11-50 of the 8 week 5°C sample were scanned for other substances but the only other found was in fractions 44-47 just before where GA_4 was expected in fraction 47. The 398 Pool antiserum used had a very high cross reactivity of 109% with GA_4-Me.

Therefore possible GA_1 peaks were detected in some of the extracts of the individual bulb components, but in certain cases they were closer to GA_3 than GA_1. These peaks were more frequently found in the basal plate and daughter bulb extracts than in the scale and shoot extracts, where in many samples no peaks were detected in this area.

For each different type of tissue a scan was done of the HPLC fractions to determine if there were any other substances present. Other peaks were found in each case but, as with the GA_1-like peak, particularly in the basal plate and daughter bulb extracts. In all the extracts the peaks generally appeared around the elution time of GA_5/GA_{20} and later, the exception being in the 12 weeks 5°C basal plate extract where a peak was evident between GA_1 and GA_5.

The comparisons of the 12 weeks 5°C scale and basal plate results with the results from earlier extracts of these tissues were not favourable. The dissimilarity of these results casts doubt on their accuracy and on the results obtained for the other component parts of the bulb. It is possible that the bulb tissue deteriorated during storage resulting in breakdown of GAs and differing results from those obtained earlier.

CHAPTER 5

[^{14}C]GA_3 METABOLISM

Introduction

Due to its commercial availability one of the main GAs used to try and replace the cold treatment of tulips has been GA_3 (Hanks, 1982b; Hanks and Rees, 1980b and references cited therein). One study suggested that radiolabelled GA_3 injected into the bulb was not rapidly metabolised (see Hanks, 1979). In this case bulbs were injected with [^{14}C]GA_3 after 0, 6 or 12 weeks storage at 5°C to determine how exogenous GA_3 was metabolised, and whether metabolism changed with increasing prior cold storage.

Recovery and Distribution of Radioactivity

No radioactivity was found in the aqueous phase of the 0 week 5°C, 1 day sample (Table 1). Radioactivity was detected in the aqueous phase of the 4 day extract and the percentage radioactivity appeared to increase with increasing incubation time, although the accuracy of the 14 day results may be doubtful because the total recovery seemed very high compared to the usual recoveries.

Counts were detected in the 6 week 5°C, 4, 7 and 14 day aqueous fractions with the percentage increasing from the 4 to 7 day samples (Table 2). The percentage in the 14 day sample decreased but the overall recovery was low so this may not be a reliable result.

As before the 12 week 5°C aqueous fractions contained

[^{14}C]GA$_3$ Feeds - Percentage recoveries of applied radioactivity in the EtOAc and aqueous fractions. Data are expressed as percentages of the applied radioactivity.

TABLE 1 0 week 5°C samples

Duration of feed	0 day	1 day	4 day	7 day	14 day
Aqueous	N.D.	N.D.	25.3	33.5	45.6
EtOAc	63.9	59.2	40.4	33.4	41.0
Total	63.9	59.2	65.7	66.9	86.6

TABLE 2 6 week 5°C samples

Duration of feed	0 day	4 day	7 day	14 day
Aqueous	N.D.	15.7	35.0	20.5
EtOAc	60.0	55.5	41.5	32.8
Total	60.0	71.2	76.5	53.3

TABLE 3 12 week 5°C samples

Duration of feed	0 day	4 day	7 day	14 day
Aqueous	N.D.	12.3	15.8	26.9
EtOAc	27.8	75.0	68.1	57.9
Total	27.8	87.3	83.9	84.8

radioactivity, with the percentage increasing with time (Table 3). The EtOAc fractions appeared to have a comparatively high recovery except for the control, part of which had been lost. Chemiluminescence was sometimes a problem when using scintillation counting and so the recoveries in some samples may have been overestimated. The overall mean of the total recoveries was 71.6% (excluding the 12 week, 0 day sample which had been spilt).

From the distribution of the recovered radioactivity it seemed that the percentage found in the aqueous fractions generally increased with time (Table 4). However, in samples with the same incubation time the percentage appeared to decrease with increasing length of prior cold storage.

TABLE 4 [^{14}C]GA$_3$ Metabolism - Distribution of recovered radioactivity between the aqueous and EtOAc fractions. Data are expressed as percentages of the recovered radioactivity.

Prior 5°C Storage	Fraction	0 day	1 day	4 day	7 day	14 day
0W5°C	Aqueous	N.D.	N.D.	38.6	50.1	52.6
	EtOAc	100.0	100.0	61.4	49.9	47.4
6W5°C	Aqueous	N.D.	-	22.0	45.8	38.5
	EtOAc	100.0	-	78.0	54.2	61.5
12W5°C	Aqueous	N.D.	-	14.1	18.9	31.7
	EtOAc	100.0	-	85.9	81.1	68.3

Gradient HPLC

Each EtOAc extract was analysed by reversed-phase HPLC using a gradient of 20-50% MeOH over 20 min. Residual [^{14}C]GA_3 could be identified by comparison with a [^{14}C]GA_3 standard, and from that the other peaks could be numbered. The main peaks were residual [^{14}C]GA_3 and metabolites 1 and 2, which eluted approximately 4.7 and 2.7 min respectively, before [^{14}C]GA_3.

Some metabolism of [^{14}C]GA_3 apparently occurred in the control (0 day feed) samples. The control bulbs were injected with [^{14}C]GA_3 and then frozen in liquid N_2, but as whole bulbs take a relatively long time to freeze it is possible that the bulbs were not completely frozen, leading to metabolism of the applied GA_3.

0 week 5°C Samples

The control sample contained peak 1 but the majority of the radioactivity was still associated with [^{14}C]GA_3 (Table 5, Figure 1). The situation was similar in the 1 day sample with no change in the distribution of radioactivity between peak 1 and [^{14}C]GA_3 (Figures 1 and 2). The percentage of peak 1 increased in the 4, 7 and 14 day samples whilst [^{14}C]GA_3 decreased until they had similar percentages of the radioactivity (Table 5, Figures 2 and 3). Peak 2 started to appear at day 4 and increased in the 7 and 14 day extracts (Figures 2 and 3).

Therefore, the [^{14}C]GA_3 was metabolised after 0 weeks storage at 5°C but a substantial amount remained unmetabolised after 14 days.

TABLE 5 [^{14}C]GA_3 Metabolism - Percentage distribution of radioactivity between peaks 1 and 2 and [^{14}C]GA_3 in the 0 week 5°C samples. N.D. = Not detected.

Incubation Time	% radioactivity Peak 1	Peak 2	[^{14}C]GA_3
0 day	15.0	N.D.	85.0
1 day	15.0	N.D.	85.0
4 day	27.0	2.0	71.0
7 day	37.0	3.0	60.0
14 day	47.0	8.0	45.0

FIGURE 1

HPLC profile of the control (0 day) EtOAc extract from the 0 week 5°C [^{14}C]GA_3 feed.

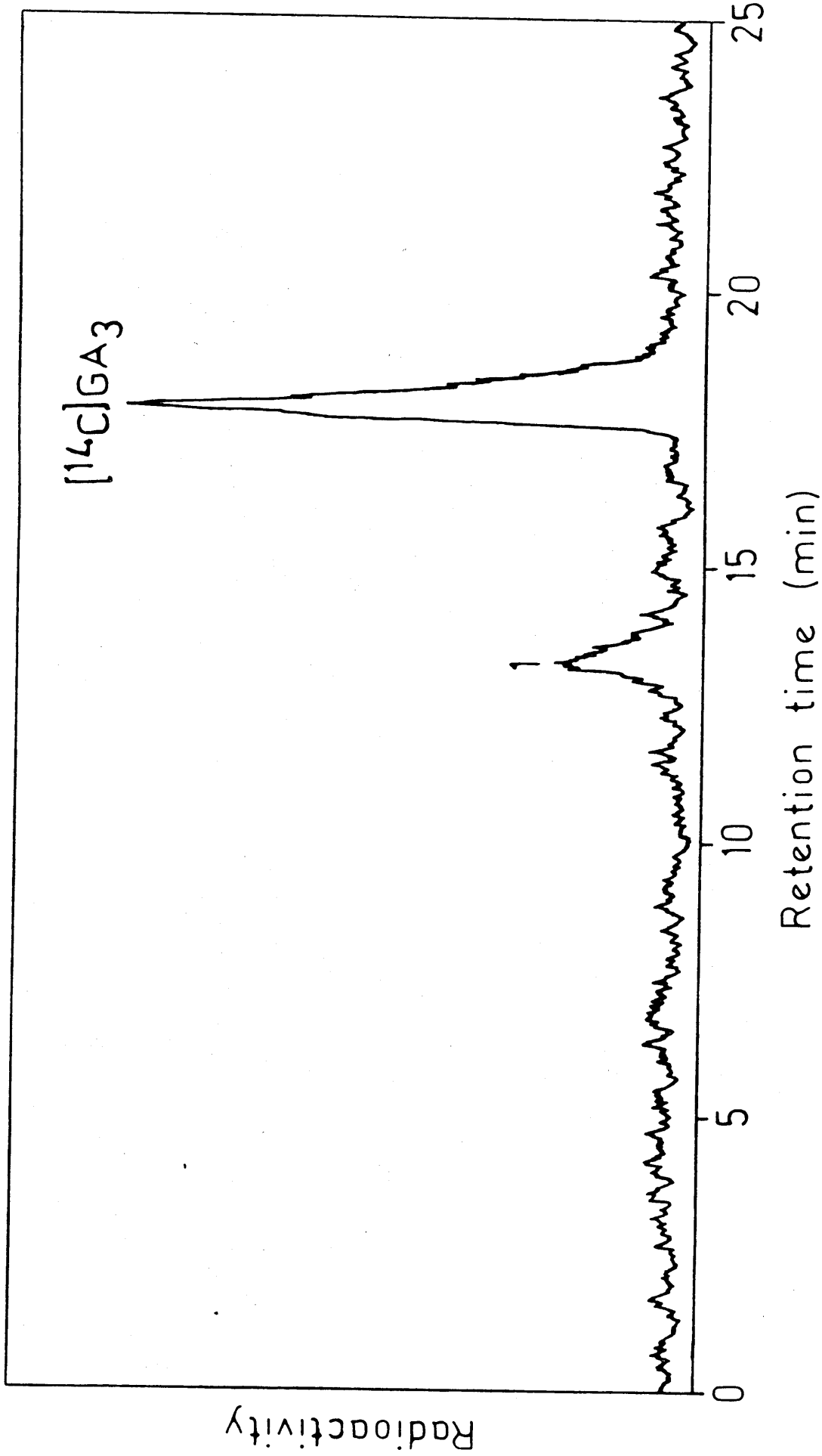

Radioactivity
0
5
10
15
20
25
Retention time (min)
[14C]GA3
1

FIGURE 2

HPLC profiles of the 1 and 4 day EtOAc extracts from the 0 week 5°C [^{14}C]GA_3 feeds.

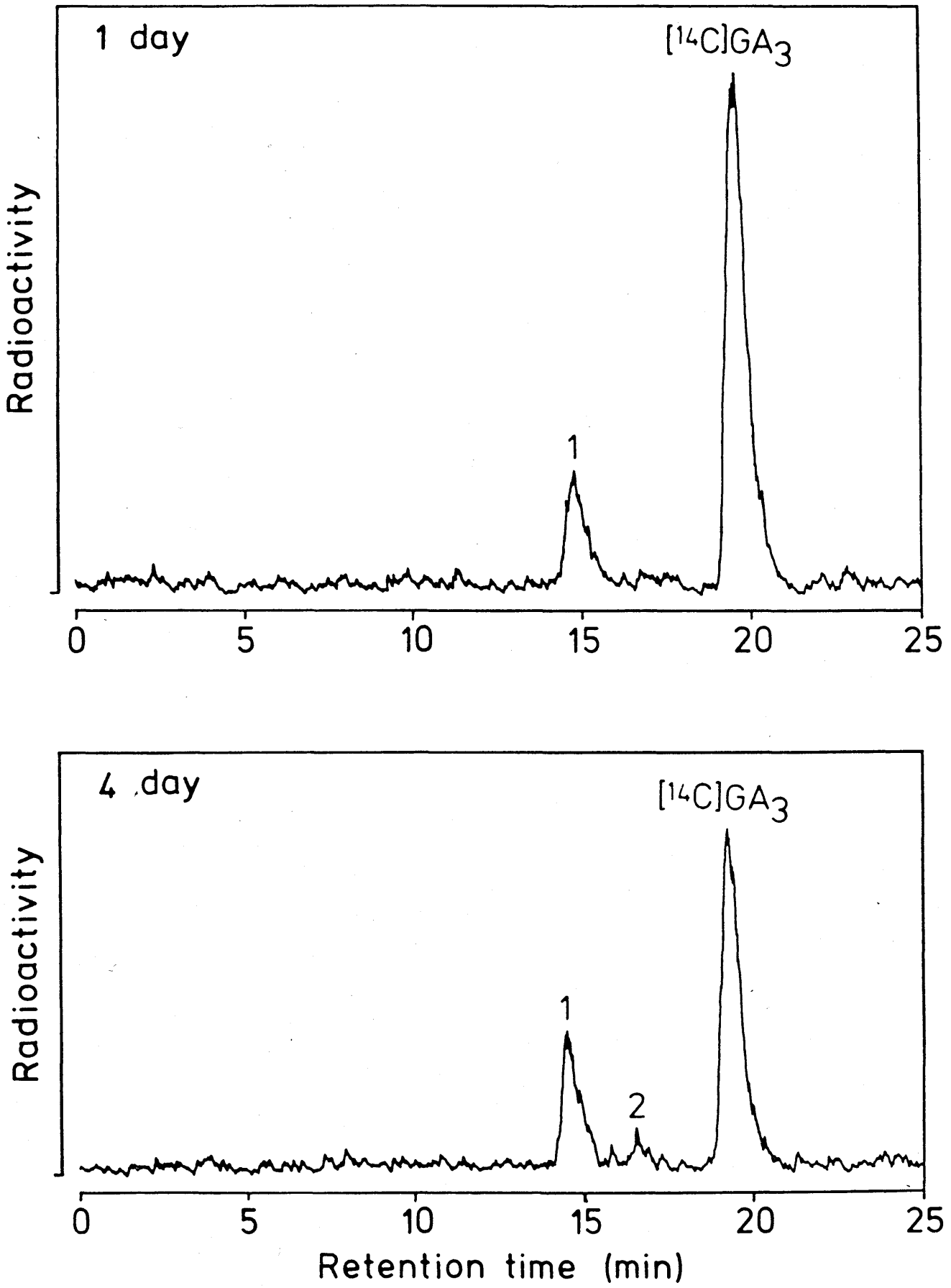
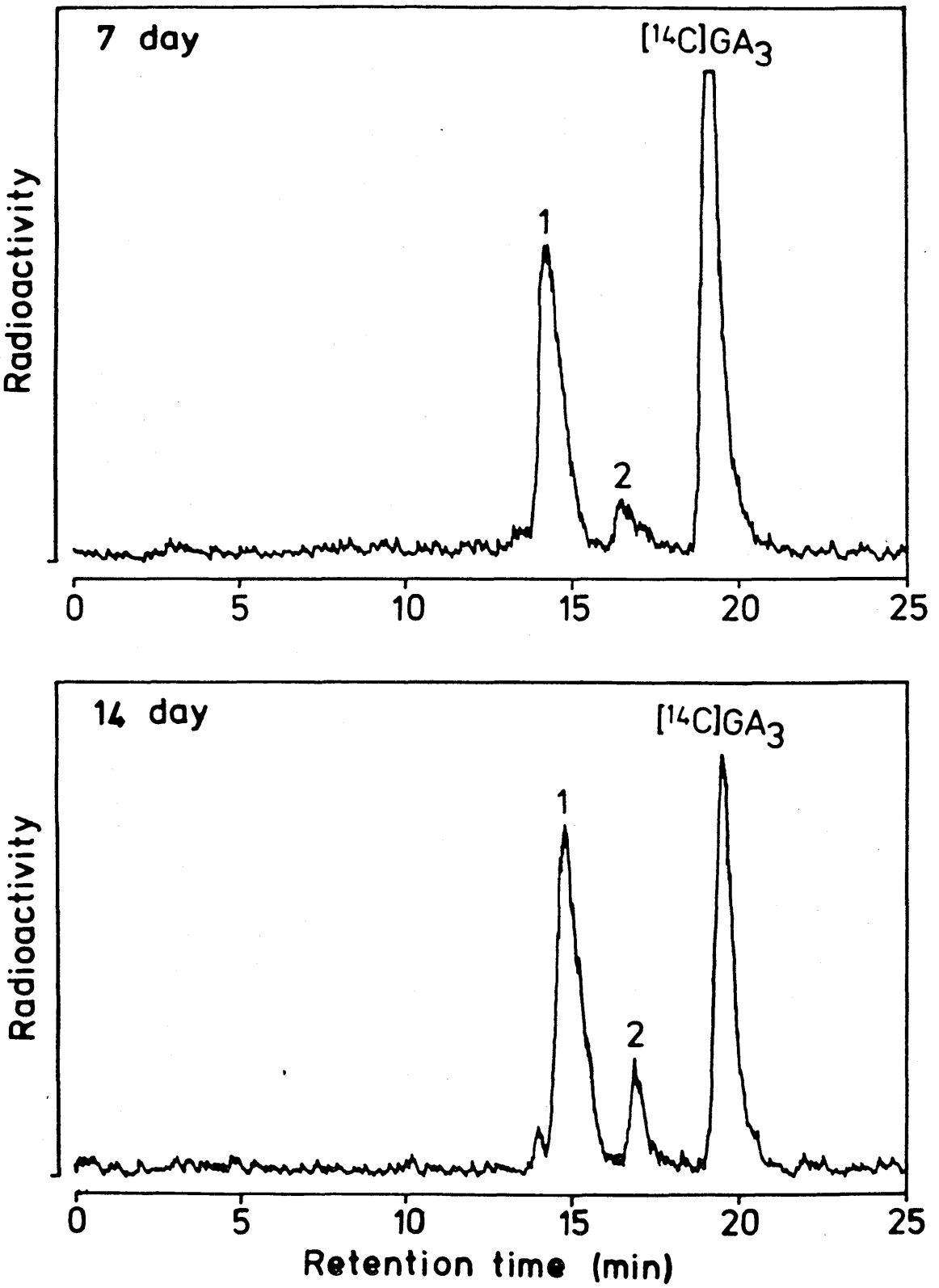

1 day
[14C]GA3
Radioactivity
1
0
5
10
15
20
25
4 day
[14C]GA3
Radioactivity
1
2
0
5
10
15
20
25
Retention time (min)

FIGURE 3

HPLC profiles of the 7 and 14 day EtOAc extracts from the 0 week 5°C [^{14}C]GA_3 feeds.


7 day
[14C]GA3
1
2
Radioactivity
0
5
10
15
20
25
14 day
[14C]GA3
1
2
Radioactivity
0
5
10
15
20
25
Retention time (min)

6 week 5°C Samples

The [^{14}C]GA$_3$ in the control sample had been metabolised to peak 1, which remained the main metabolite in the 4, 7 and 14 day samples (Table 6, Figures 4 and 5). The metabolism of [^{14}C]GA$_3$ increased with time, concomitant with accumulation of peak 1, but after 14 days [^{14}C]GA$_3$ was still the major peak present. Peak 2 was evident in the 14 day sample but only as a minor peak.

Therefore, after 6 weeks storage at 5°C [^{14}C]GA$_3$ was metabolised to one major peak, but the metabolism was slow, and after 14 days [^{14}C]GA$_3$ still accounted for two thirds of the recovered radioactivity.

TABLE 6 [^{14}C]GA$_3$ Metabolism - Percentage distribution of radioactivity between peaks 1 and 2 and [^{14}C]GA$_3$ in the 6 week 5°C samples. N.D. = Not detected.

Incubation Time	% radioactivity		
	Peak 1	Peak 2	[^{14}C]GA$_3$
0 day	14.0	N.D.	86.0
4 day	21.0	N.D.	79.0
7 day	27.0	N.D.	73.0
14 day	30.0	4.0	66.0

FIGURE 4.

HPLC profiles of the 0 and 4 day EtOAc extracts from the 6 weeks 5°C [^{14}C]GA_3 feeds.

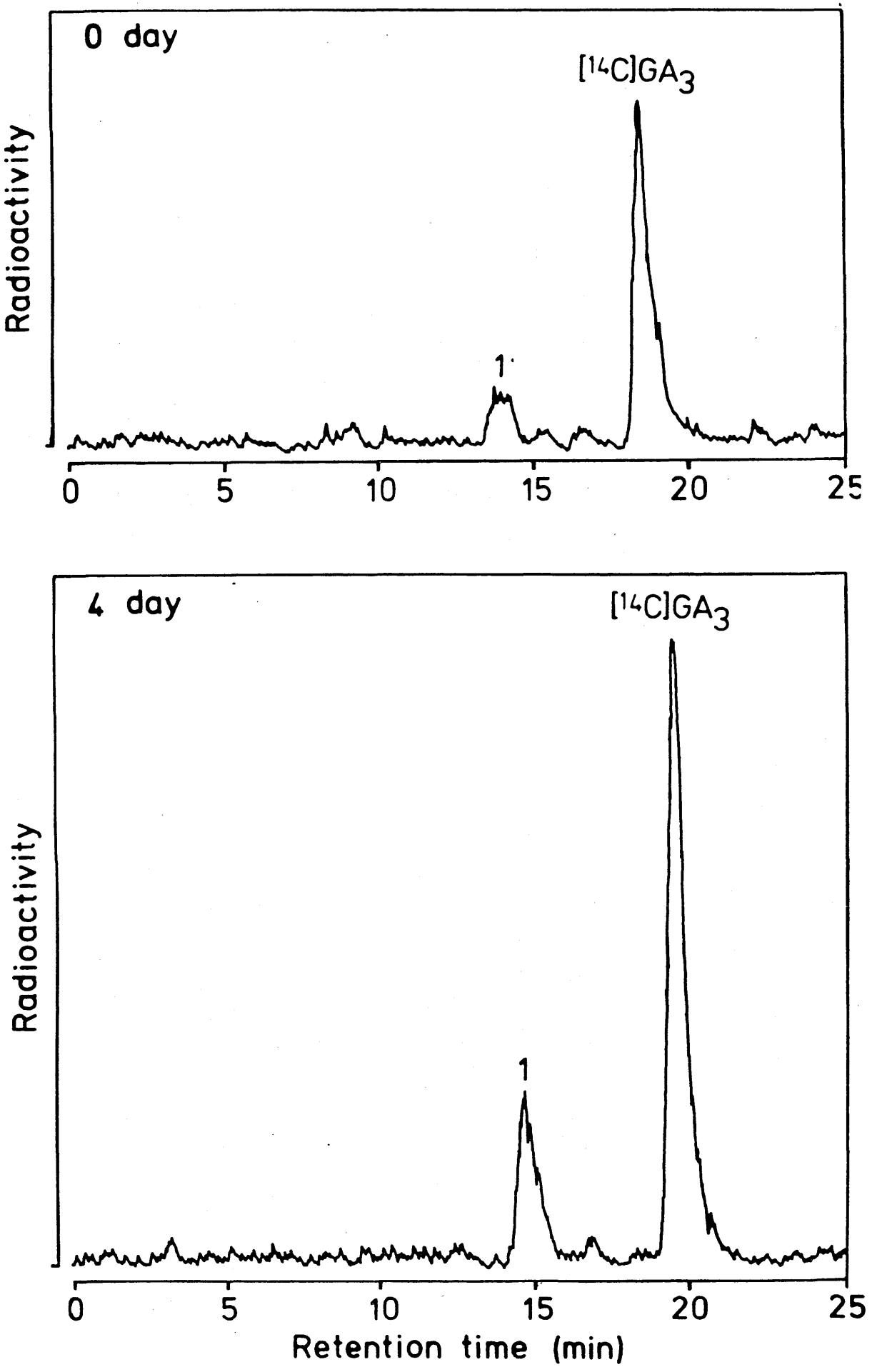
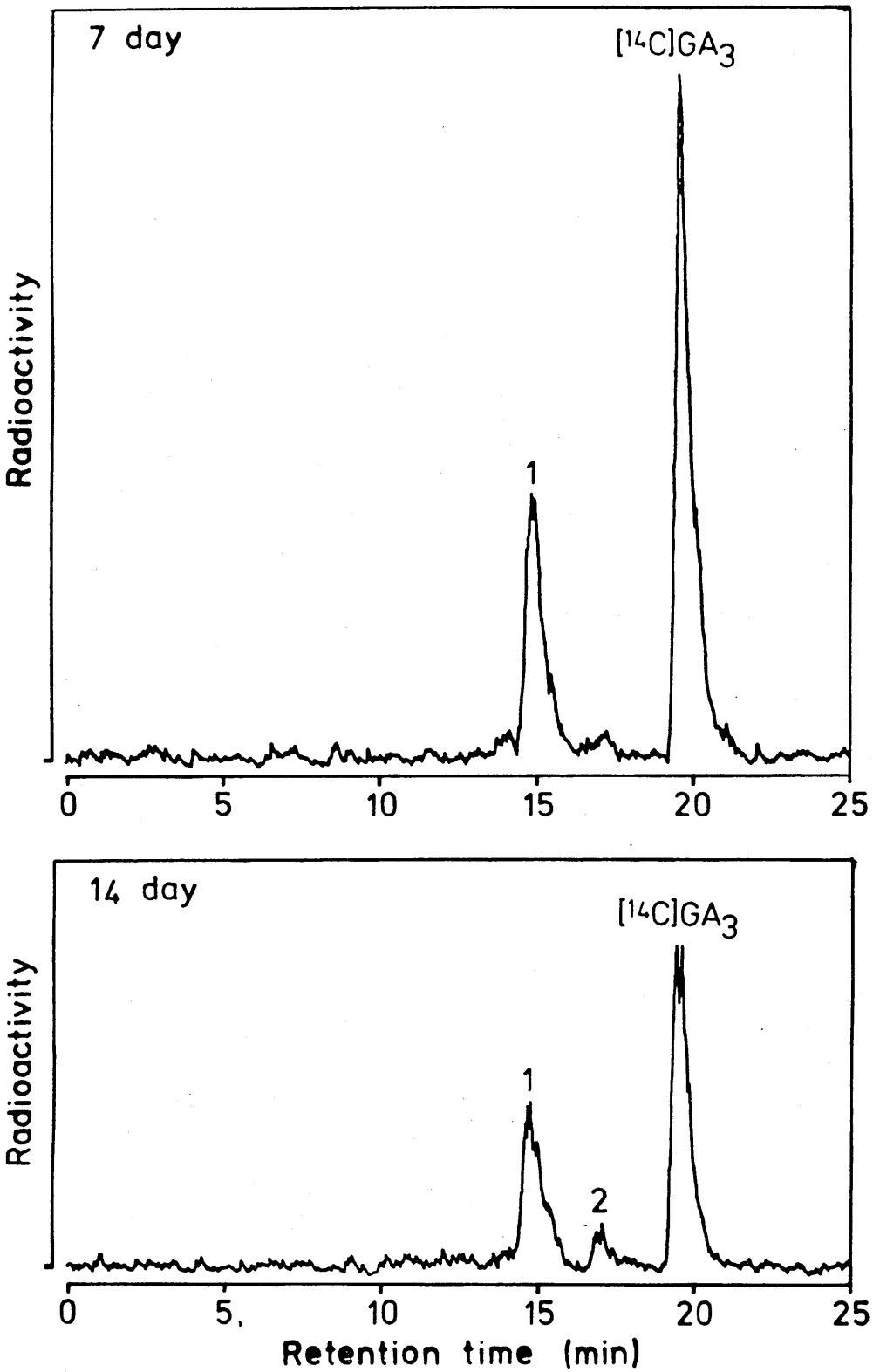

0 day
[14C]GA3
1
Radioactivity
0
5
10
15
20
25
4 day
[14C]GA3
1
Radioactivity
0
5
10
15
20
25
Retention time (min)

FIGURE 5

HPLC profiles of the 7 and 14 day EtOAc extracts from the 6 weeks 5°C [^{14}C]GA_3 feeds.


7 day
[14C]GA3
1
Radioactivity
0
5
10
15
20
25
14 day
[14C]GA3
1
2
Radioactivity
0
5.
10
15
20
25
Retention time (min)

12 week 5°C Samples

The [^{14}C]GA_3 in the control sample had been metabolised to peak 1 as it had been in the 4 day sample, where it had increased to 93.0% of the radioactivity (Table 7, Figure 6). In the 4 day sample two peaks appeared in the region of [^{14}C]GA_3, the first of which was likely to be the residual [^{14}C]GA_3 when compared with the retention time of a standard and the retention time of peak 1.

After 7 and 14 days peak 1 had decreased relative to the [^{14}C]GA_3 peak and very small amounts of peak 2 were evident in the 7 day sample (Table 7, Figure 7).

Therefore, bulbs given [^{14}C]GA_3 after 12 weeks storage at 5°C appeared to have metabolised most of it to peak 1 after 4 days. However, the 7 and 14 day samples were unusual in that peak 1 was still the major metabolite but was smaller than the [^{14}C]GA_3 peak.

TABLE 7 [^{14}C]GA_3 Metabolism - Percentage distribution of radioactivity between peaks 1 and 2 and [^{14}C]GA_3 in the 12 week 5°C samples. N.D. = Not detected.

Incubation Time	% radioactivity		
	Peak 1	Peak 2	[^{14}C]GA_3
0 day	20.0	N.D.	80.0
4 day	93.0	N.D.	7.0
7 day	39.0	2.0	59.0
14 day	27.0	N.D.	73.0

FIGURE 6

HPLC profiles of the 0 and 4 day EtOAc extracts from the 12 weeks 5°C [^{14}C]GA_3 feeds.

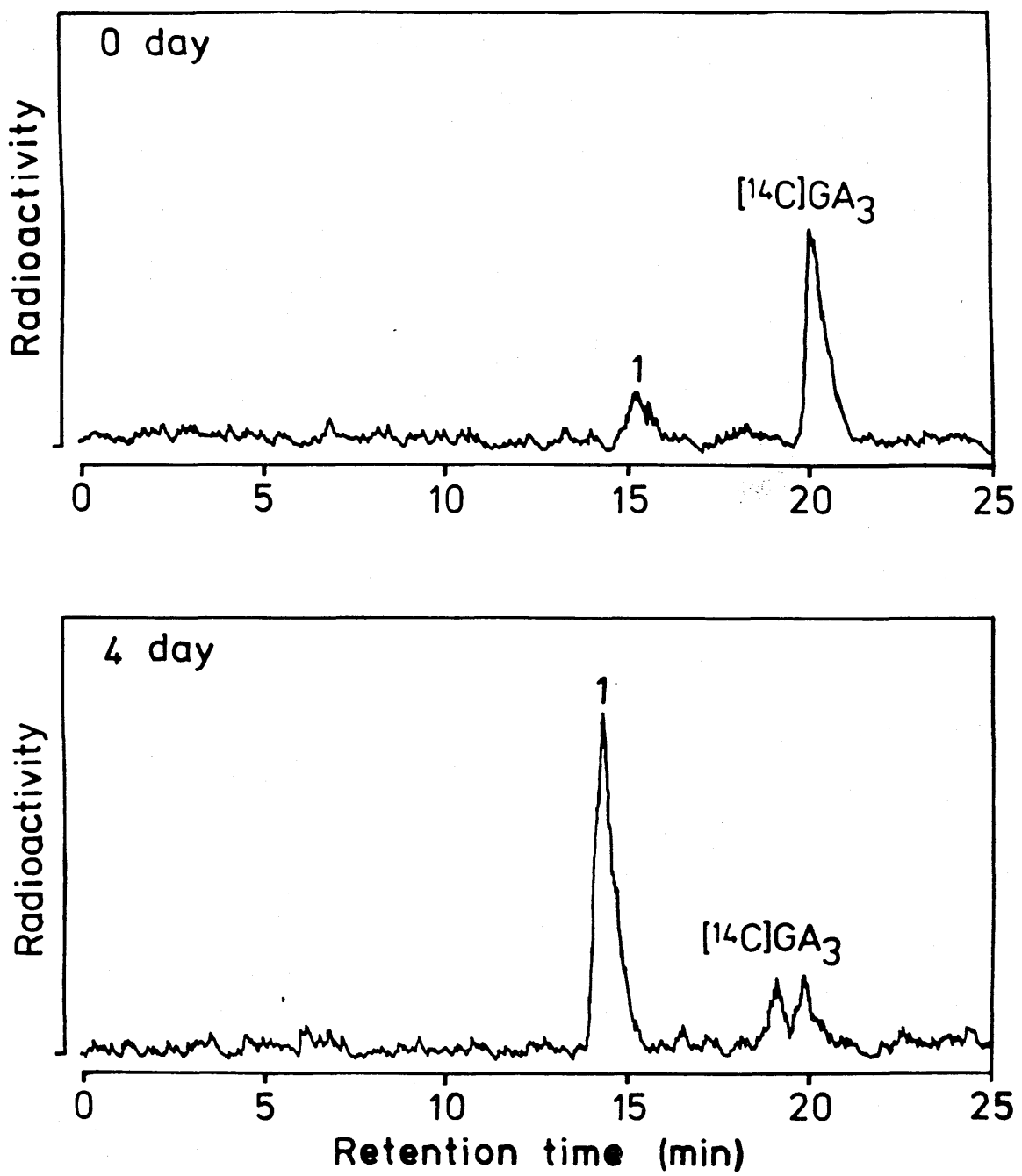
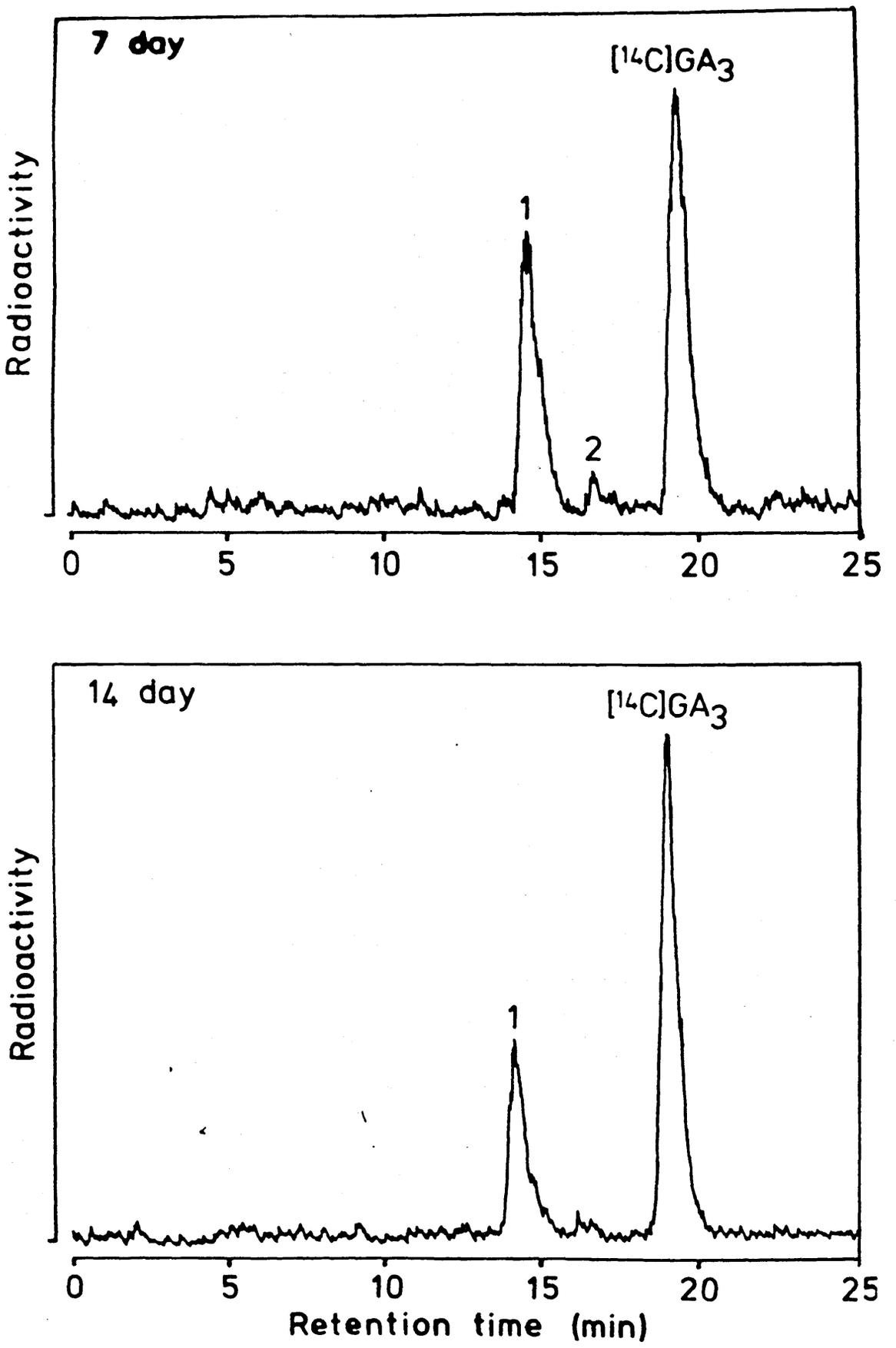

0 day
Radioactivity
[14C]GA3
1
0
5
10
15
20
25
4 day
Radioactivity
1
[14C]GA3
0
5
10
15
20
25
Retention time (min)

FIGURE 7

HPLC profiles of the 7 and 14 day EtOAc extracts from the 12 weeks 5°C [^{14}C]GA_3 feeds.


7 day
[14C]GA3
1
2
Radioactivity
0
5
10
15
20
25
14 day
[14C]GA3
1
Radioactivity
0
5
10
15
20
25
Retention time (min)

Bulk Extracts

Bulk extracts of other 6 and 12 week 5°C samples were carried out. The recovery of radioactivity in the EtOAc fraction of the 6 week 5°C sample was very low (Table 8) although the results indicate that the distribution was similar in both samples (Table 8).

These bulk samples were analysed by gradient HPLC as before and, as expected, peak 1 was the main metabolite with a substantial percentage of the radioactivity still accounted for by [^{14}C]GA_3 (Table 9, Figure 8). The relative percentages of these two peaks were the same in the two bulk samples. There was an indication of trace amounts of peak 2 in the 12 week sample.

TABLE 8 [^{14}C]GA_3 Metabolism - Recovery and distribution of radioactivity in the aqueous and EtOAc fractions of the bulk extracts. Data are expressed as percentages of the applied and recovered radioactivity respectively.

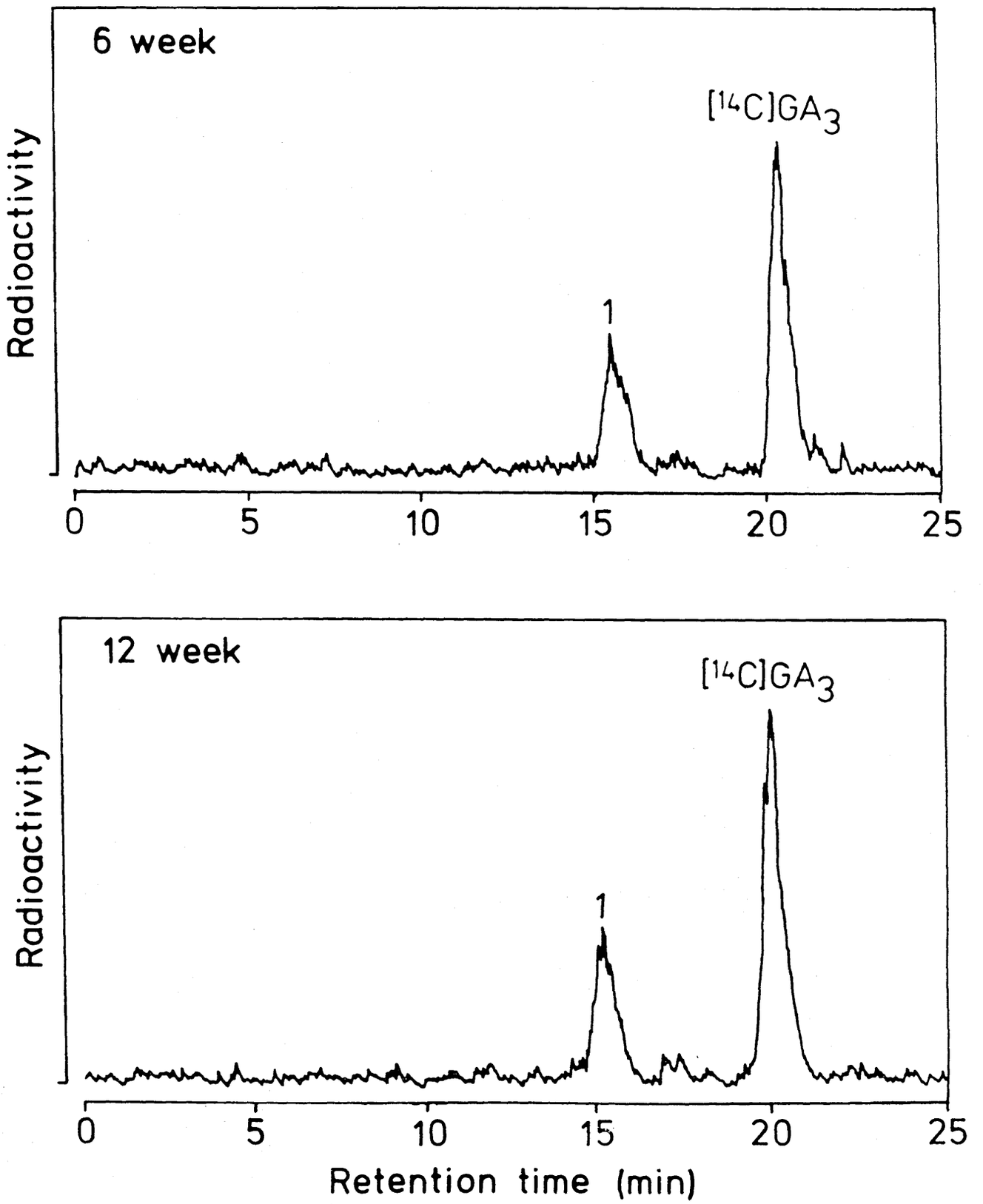
	Sample	Aqueous	EtOAc	Total Recovery
Recovery	6W5°C	13.9	38.2	52.1
	12W5°C	18.5	60.5	79.0
Distribution	6W5°C	26.7	73.3	
	12W5°C	23.4	76.6	

TABLE 9 [^{14}C]GA_3 Metabolism - Percentage distribution of radioactivity between [^{14}C]GA_3 and peak 1 in the 6 and 12 week 5°C bulk extracts.

Extract	% radioactivity	
	Peak 1	[^{14}C]GA_3
6W5°C	29.0	71.0
12W5°C	29.0	71.0

FIGURE 8

HPLC profiles of the 6 and 12 week bulk EtOAc extracts from the [^{14}C]GA_3 feeds.


6 week
[14C]GA3
1
Radioactivity
0
5
10
15
20
25
12 week
[14C]GA3
1
Radioactivity
0
5
10
15
20
25
Retention time (min)

GEL PERMEATION CHROMATOGRAPHY

The major metabolites eluted shortly before [^{14}C]GA_3 suggesting that they may have been other more polar free GAs or GA_3 conjugates. GPC can give an indication of the relative molecular weights of substances and therefore whether they are likely to be free GAs or GA conjugates. All the samples were combined to give one large sample which was then subjected to GPC. Four areas of radioactivity were found in the GPC fractions and the appropriate fractions were combined to give three separate fractions, A-C (Table 10). Fractions A and B eluted earliest in the high molecular weight range and contained putative conjugates. Fraction C eluted later and 83.0% of the recovered radioactivity was associated with it, indicating that it contained free GAs, in particular the residual [^{14}C]GA_3.

TABLE 10 Elution volume and percentage of recovered radioactivity of GPC fractions A-C. Figures in brackets refer to the individual GPC fractions which were combined into one bulk fraction.

Fraction	GPC elution volume (ml)	% of recovered radioactivity
A (4-9)	267-302	6.1
B (15-23)	331-383	10.7
C (24-33)	383-441	83.2

These GPC fractions were analysed by isocratic HPLC and unlabelled standards were used in conjunction with a UV monitor to attempt identification of the metabolites.

Isocratic HPLC

The isocratic HPLC analysis of the GPC fractions could only be superficial due to lack of sample available. Fraction A contained a very polar substance which eluted in the void volume when chromatographed isocratically at 30% and 25% MeOH (Table 11, Figure 9).

Two peaks eluted in fraction C, the second of which had the same retention time as [^{14}C]GA_3 (Table 11, Figure 9). The peaks were similar in size indicating that the earlier eluting peak could be peak 1, the major metabolite, whilst its elution in fraction C indicated that it was a free GA rather than a GA conjugate.

The main peak in fraction B had a similar retention time to GA_3-13-O-glucoside (Table 11, Figure 9). It was expected to be peak 2 but eluted too early in comparison to the peaks in fraction C which were thought to be equivalent to peak 1 and [^{14}C]GA_3.

TABLE 11 Isocratic HPLC - retention times of GPC fractions and GA standards.

Fraction	R_t (min)	Substance	R_t (min)
A	2.4*[1]		
B	6.7[2]	GA_3-13-O-Gluc	6.8[2]
C	8.8[2]	GA_3-3-O-Gluc	8.5[2]
	13.2[2]	[^{14}C]GA_3	13.2[2]

* - Isocratic HPLC conditions. 1 = 25% MeOH

2 = 30% MeOH

GA_3-3-O-Gluc = GA_3-3-O-glucoside

GA_3-13-O-Gluc = GA_3-13-O-glucoside

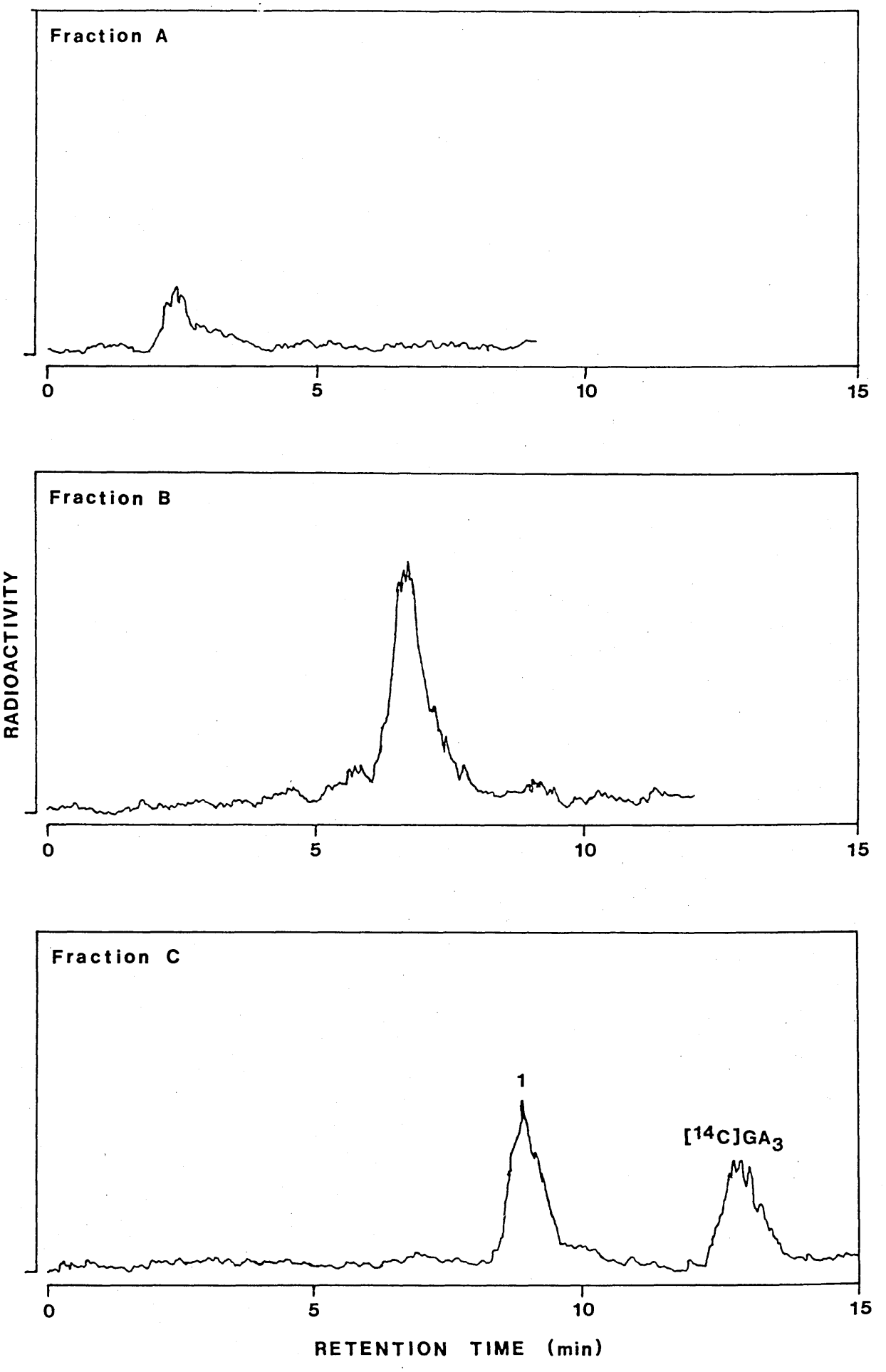
FIGURE 9

HPLC profiles of the GPC fractions A-C from the [^{14}C]GA_3 feeds.

Isocratic HPLC conditions :

Fraction A - 25% MeOH

Fractions B and C - 30% MeOH


Fraction A
0
5
10
15
Fraction B
RADIOACTIVITY
0
5
10
15
Fraction C
1
[14C]GA3
0
5
10
15
RETENTION TIME (min)

Discussion

It has been suggested before that [^{14}C]GA$_3$ is not rapidly metabolised when injected into the bulb (Hanks, 1984), and to an extent these results confirm this. In the 14 day samples after 0, 6 or 12 weeks 5°C storage, a high percentage of [^{14}C]GA$_3$ remained unmetabolised, the amount increasing with increasing length of prior storage. The decreases in [^{14}C]GA$_3$ were accompanied by increases in peak 1, which was the major metabolite in every case, whilst peak 2 was only a very minor product in some of the samples. The 12 week samples were unusual in that they suggested that after 4 days most of the [^{14}C]GA$_3$ had been metabolised to peak 1, then after 7 days the [^{14}C]GA$_3$ peak appeared to have increased again. It is probable that this was due to sample variability or an unusually active bulb, giving an inaccurate result, rather than any true changes occurring in a normal bulb. Neither of the metabolites were identified, but the GPC results indicated that peak 1 was not a conjugate.

The distribution of recovered radioactivity between the acidic EtOAc and aqueous fractions of the 0, 6 and 12 week samples exhibited an increase in the percentage found in the aqueous fraction with time. However, the percentage found in the aqueous phase decreased with longer periods of 5°C storage prior to injection with [^{14}C]GA$_3$. GA conjugates tend to remain in the aqueous fraction when it is partitioned with EtOAc at pH 2.5, as

illustrated by Schneider (1983) where only 4.9% of GA_3-3-O-glucoside was found in the acidic EtOAc fraction. In this experiment only the acidic EtOAc fractions were analysed but in any future experiments analysis of the aqueous fractions would be important. Turnbull *et al* (1986) have described procedures for the analysis of radiolabelled free GAs and GA conjugates, involving various types of chromatography plus derivatisation and enzymic hydrolysis. They were used very successfully to provide a substantial amount of information about, and to identify, the metabolites of [^{3}H]GA_4.

CHAPTER 6

[^{3}H]GA_4 METABOLISM

Introduction

GA_4 is one of the GAs most often used in experiments attempting to partially or wholly replace the cold treatment of tulips (Hanks, 1982b; Hanks and Rees, 1980b and references therein). In these experiments it is usually applied in a mixture with GA_7 as $GA_{4/7}$, and this is considerably more effective than GA_3. When a range of GAs were tested in this type of experiment GA_7 was the most effective followed by GA_4, with GA_3 the least effective (Hanks and Rees, 1980b).

To determine whether cold storage had any effect on the metabolism of GA_4, [^{3}H]GA_4 was injected into bulbs which had received 0, 6 or 12 weeks 5°C storage, after which the bulbs were incubated for 0, 1, 4, 7, 14, 21 or 33 days.

Recovery and Distribution of Radioactivity

The recoveries of the applied radioactivity in the EtOAc and aqueous fractions are given in Tables 1-3. Certain samples appeared to have somewhat higher recoveries than the others - 6 weeks 5°C, 7 and 21 days (Table 2) and 12 weeks 5°C, control and 14 days (Table 3). Inevitably sample losses do vary during extraction and fractionation and these figures may reflect the true recoveries, but it is possible that the counts in the EtOAc fractions were overestimated due to chemi-luminescence. Inaccuracies in the aqueous counts could

[^{3}H]GA$_4$ METABOLISM - Recovery of radioactivity in the aqueous and EtOAc fractions. Data are percentages of the applied radioactivity calculated from dpm.

TABLE 1 0 weeks 5°C storage

Duration of feed	1 day	4 day	7 day	14 day
Aqueous	6.9	13.9	18.4	25.2
EtOAc	56.9	42.5	40.6	40.7
Total	63.8	56.4	59.0	65.9

TABLE 2 6 weeks 5°C storage

Duration of feed	0 day	4 day	7 day	14 day	21 day
Aqueous	13.1	18.8	24.6	24.2	31.3
EtOAc	53.9	49.4	55.9	47.7	50.7
Total	67.0	68.2	80.5	71.9	82.0

TABLE 3 12 weeks 5°C storage

Duration of feed	0 day	4 day	7 day	14 day	33 day
Aqueous	7.6	7.4	22.9	27.7	26.7
EtOAc	78.2	54.4	44.9	62.3	34.6
Total	85.8	61.8	67.8	90.0	61.3

have been caused by quenching. The overall mean of the total recoveries was 70.1%.

The data indicated a general increase, with time, in the percentage radioactivity found in the aqueous fraction. A clearer picture is given in Table 4, where the data are expressed as percentages of the recovered radioactivity. After 7 and 14 days incubation the differences between the aqueous fractions of the 0, 6 and 12 week samples were small, but after 0 and 4 days the percentage was lower in the 12 week samples.

TABLE 4 [^{3}H]GA_4 Metabolism - Distribution of the recovered radioactivity between the EtOAc and aqueous fractions. Data are expressed as percentages of the recovered radioactivity.

Prior 5°C Storage	Fraction	0 day	1 day	4 day	7 day	14 day	21 day	33 day
0 weeks	Aqueous	-	12.5	27.8	31.1	38.2	-	-
	EtOAc	-	87.5	72.2	68.9	61.8	-	-
6 weeks	Aqueous	19.6	-	27.5	30.5	33.6	38.2	-
	EtOAc	80.4	-	72.5	69.5	66.4	61.8	-
12 weeks	Aqueous	8.8	-	12.0	33.9	30.8	-	43.6
	EtOAc	91.2	-	88.0	66.1	69.2	-	56.4

Gradient HPLC Analysis

Aliquots of the EtOAc fractions were analysed by reversed-phase HPLC using a gradient of 40-100% MeOH over 20 min. Tritiated standards of GA_1, GA_4 and GA_8 were run regularly to check on retention times, and sample [^{3}H]GA_4 could be identified by comparison with the GA_4 standard. The average retention times of GA_8, GA_1 and GA_4 were 4.8, 7.8 and 19.6 min respectively. The major peaks are marked as [^{3}H]GA_4 and peaks A and B which eluted, on average, 3.8 and 2.0 min respectively, before [^{3}H]GA_4.

The [^{3}H]GA_4 in the control (0 day feed) samples had been metabolised, probably due to incomplete freezing when the whole bulbs were plunged in liquid N_2 immediately after injection. Considering the control results it is likely that metabolism in the other samples also continued after liquid N_2 treatment, and so the incubation times were probably longer than intended.

0 week 5°C Samples

After a 1 day incubation [^{3}H]GA_4 was still the most abundant substance present, with peaks A and B, especially B, being the major metabolites (Table 5, Figure 1). However, in the 4 day sample the amount of [^{3}H]GA_4 and B had fallen whilst A had increased markedly (Figure 1). In the 7 day sample [^{3}H]GA_4 and peak B had slightly higher percentages with an accompanying lower percentage of peak A which, however, remained the major component (Figure 2). There was also a metabolite peak eluting

immediately prior to A, from which it was not fully resolved. The relative amount of [^{3}H]GA_4 had reduced again after 14 days whilst B had disappeared and A had increased (Figure 2).

Therefore, in the 1 day sample two thirds of the [^{3}H]GA_4 remained unmetabolised and the main metabolite was peak B. With longer incubation times the [^{3}H]GA_4 peak was reduced to a considerably smaller percentage of the total radioactivity with peak A now the main metabolite and B, initially the largest metabolite, finally disappearing.

TABLE 5 [^{3}H]GA_4 Metabolism - Percentage distribution of radioactivity between peaks A and B and [^{3}H]GA_4 in the 0 week 5°C samples. N.D. = not detected.

Incubation Time	% radioactivity Peak A	Peak B	[^{3}H]GA_4
1 day	9.0	23.0	68.0
4 day	86.0	4.0	10.0
7 day	69.0	7.0	24.0
14 day	92.0	N.D.	8.0

FIGURE 1

HPLC profiles of the 1 and 4 day EtOAc extracts from the 0 week 5°C [^{3}H]GA_4 feeds.

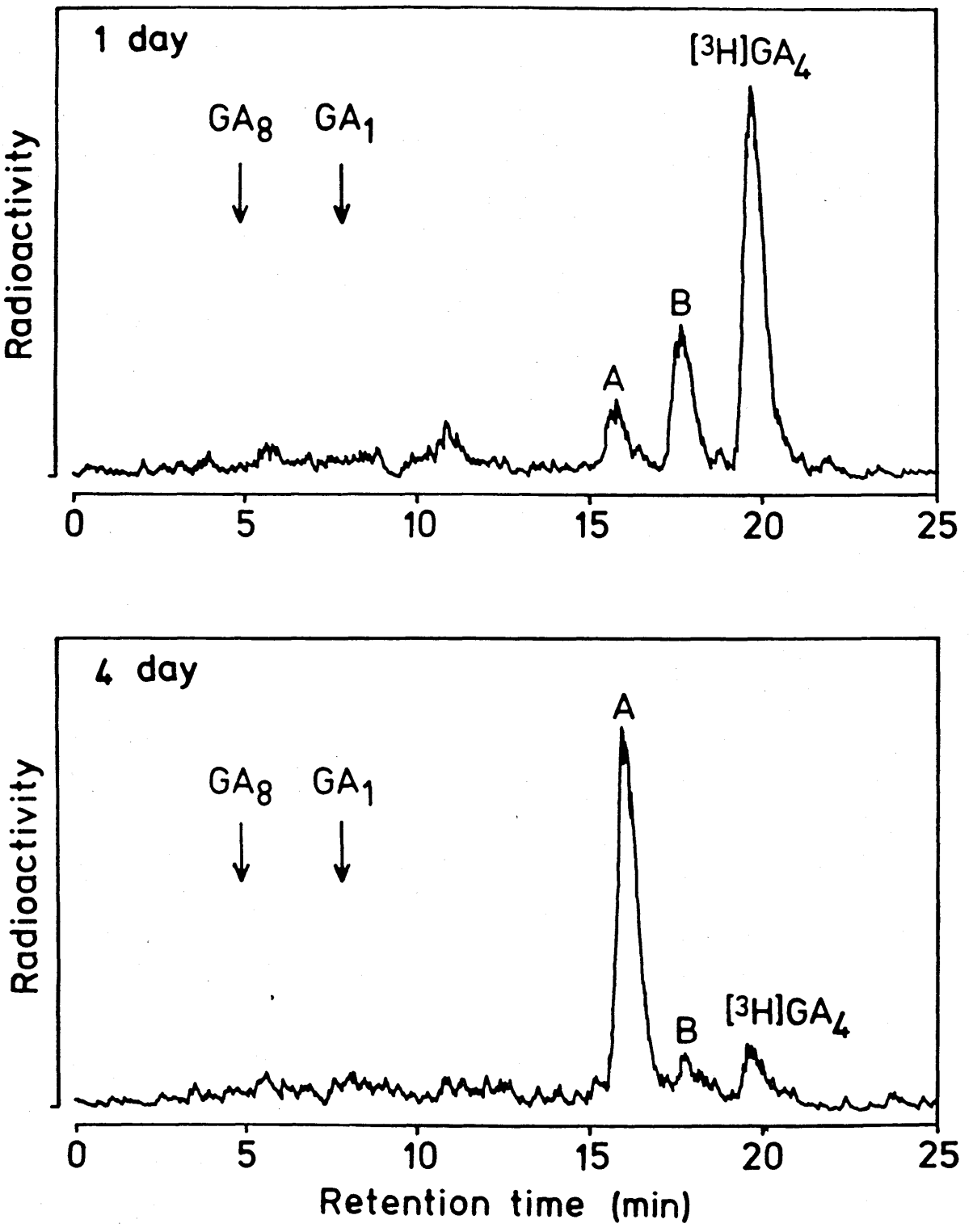
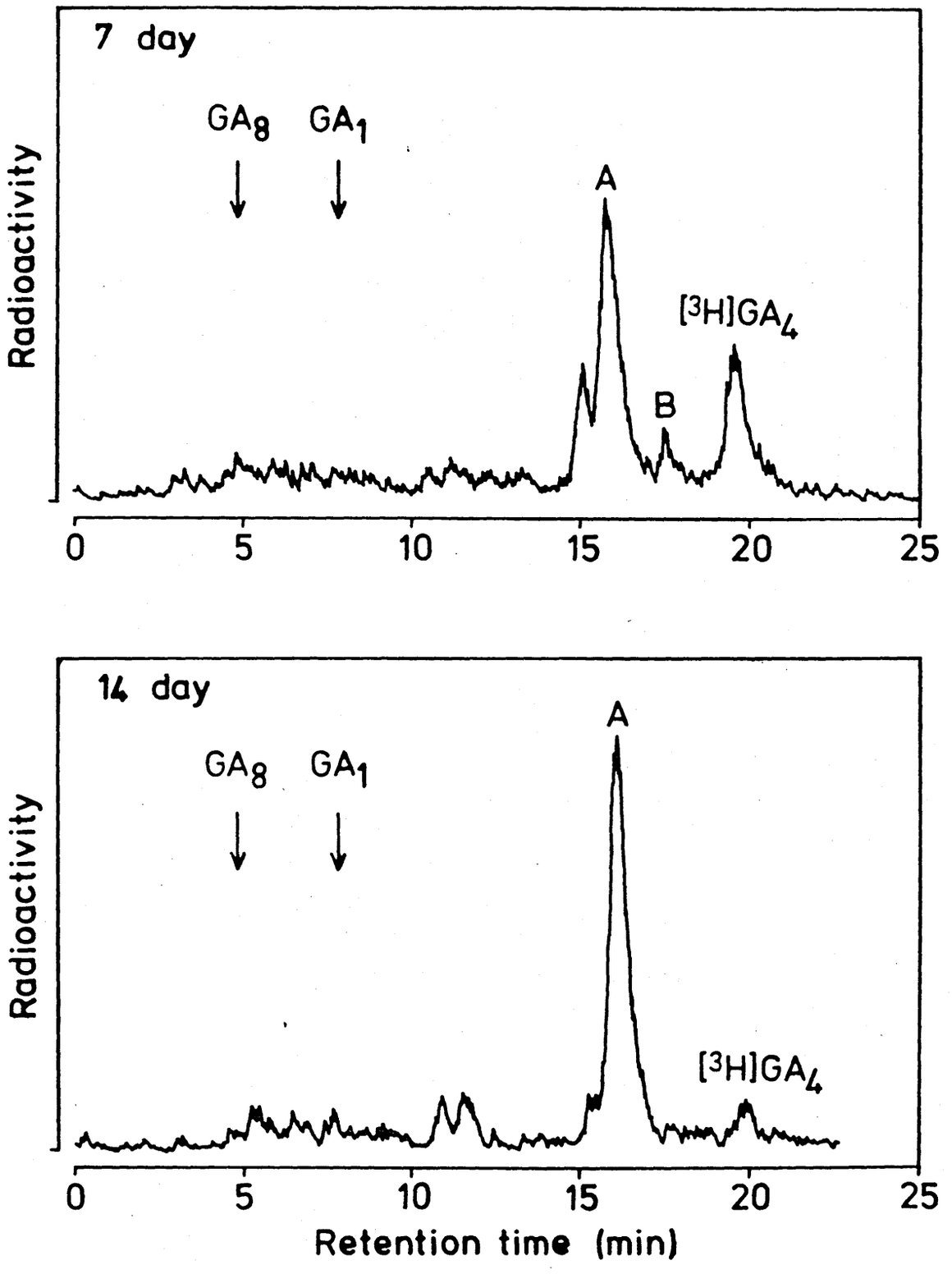

1 day
[3H]GA4
GA8
GA1
B
A
Radioactivity
0
5
10
15
20
25
4 day
A
GA8
GA1
B
[3H]GA4
Radioactivity
0
5
10
15
20
25
Retention time (min)

FIGURE 2

HPLC profiles of the 7 and 14 day EtOAc extracts from the 0 week 5°C [^{3}H]GA_4 feeds.


7 day
GA8
GA1
A
B
[3H]GA4
Radioactivity
0
5
10
15
20
25
14 day
GA8
GA1
A
[3H]GA4
Radioactivity
0
5
10
15
20
25
Retention time (min)

6 week 5°C Samples

The [^{3}H]GA_4 in the control sample had been partly metabolised to peak A (Table 6, Figure 3). Peaks A and B were evident in the 4 day sample as were more polar metabolites (Table 6, Figure 4). The results for the control and 4 day samples would have been clearer if a larger amount of radioactivity had been available for analysis. After 7 days [^{3}H]GA_4 was present in relatively large amounts compared to the 4 day sample and peaks A and B were reduced (Table 6, Figure 4). In the 14 and 21 day samples [^{3}H]GA_4 was again a minor component with peak A rather than peak B being the major metabolite (Figure 5).

Therefore, the main initial metabolite appeared to be peak A but in the 4 and 7 day samples peak B became the main metabolite. This situation was reversed in the 14 and 21 day samples where 80% of the radioactivity was eventually associated with peak A.

TABLE 6 [^{3}H]GA_4 Metabolism - Percentage distribution of radioactivity between peaks A and B and [^{3}H]GA_4 in the 6 week 5°C samples. N.D. = Not detected.

Incubation time	% radioactivity		
	Peak A	Peak B	[^{3}H]GA_4
0 day	16.0	N.D.	84.0
4 day	24.0	51.0	25.0
7 day	8.0	18.0	74.0
14 day	56.0	15.0	29.0
21 day	80.0	5.0	15.0

FIGURE 3

HPLC profile of the control (0 day) EtOAc extract from the 6 weeks 5°C [^{3}H]GA_4 feed.

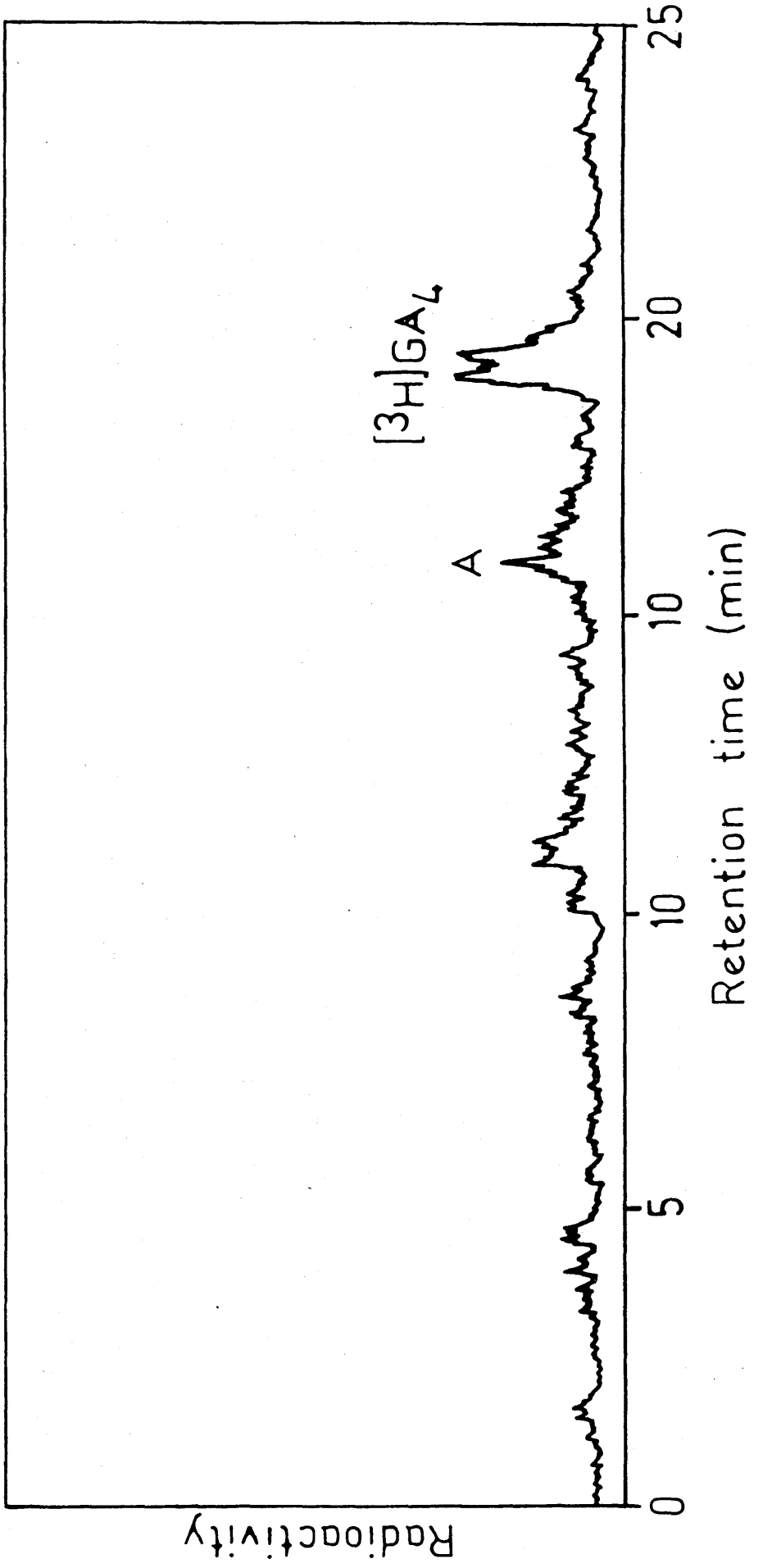

Radioactivity
0
5
10
10
20
25
Retention time (min)
A
[3H]GA4

FIGURE 4

HPLC profiles of the 4 and 7 day EtOAc extracts from the 6 weeks 5°C [^{3}H]GA_4 feeds.

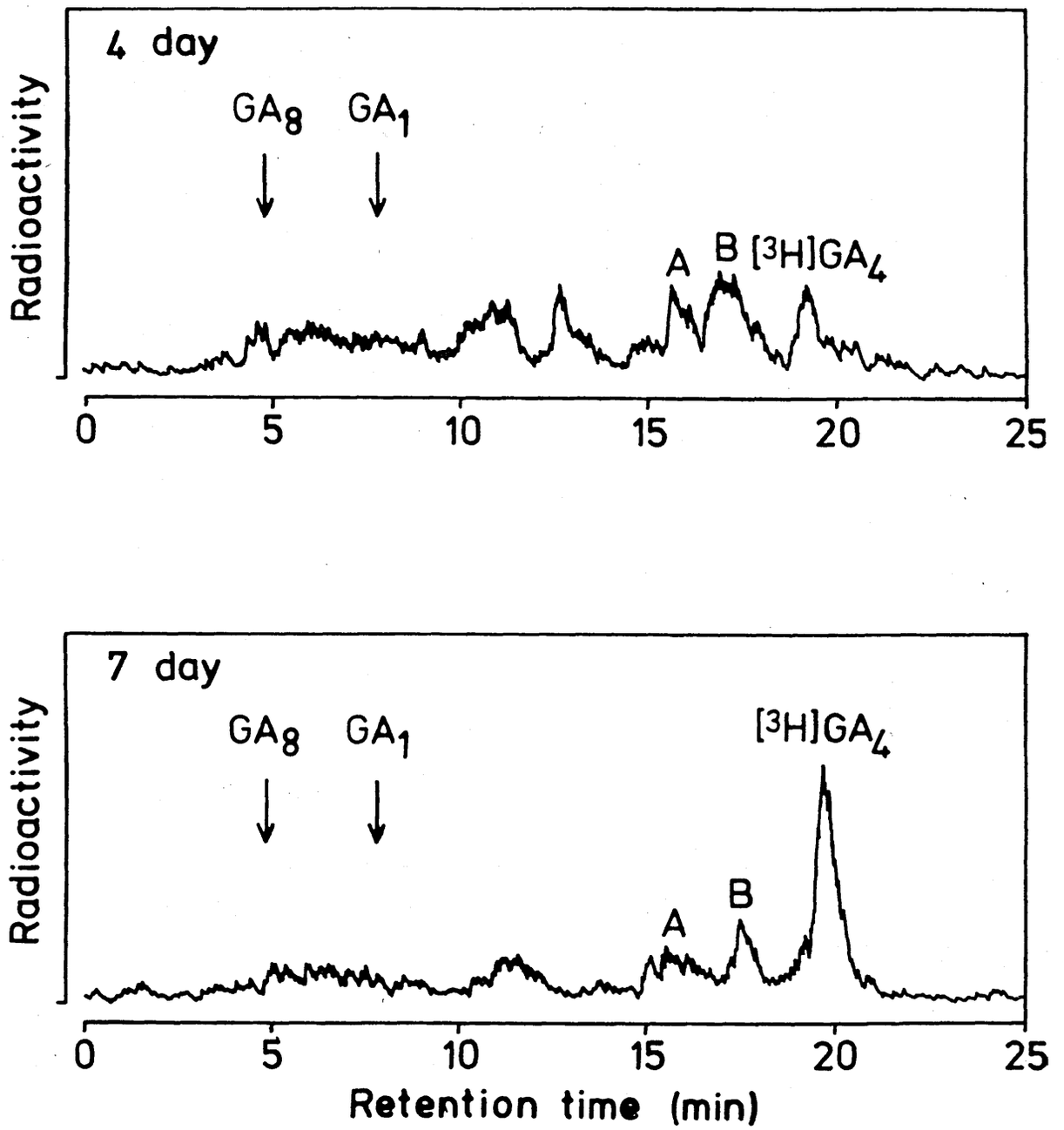
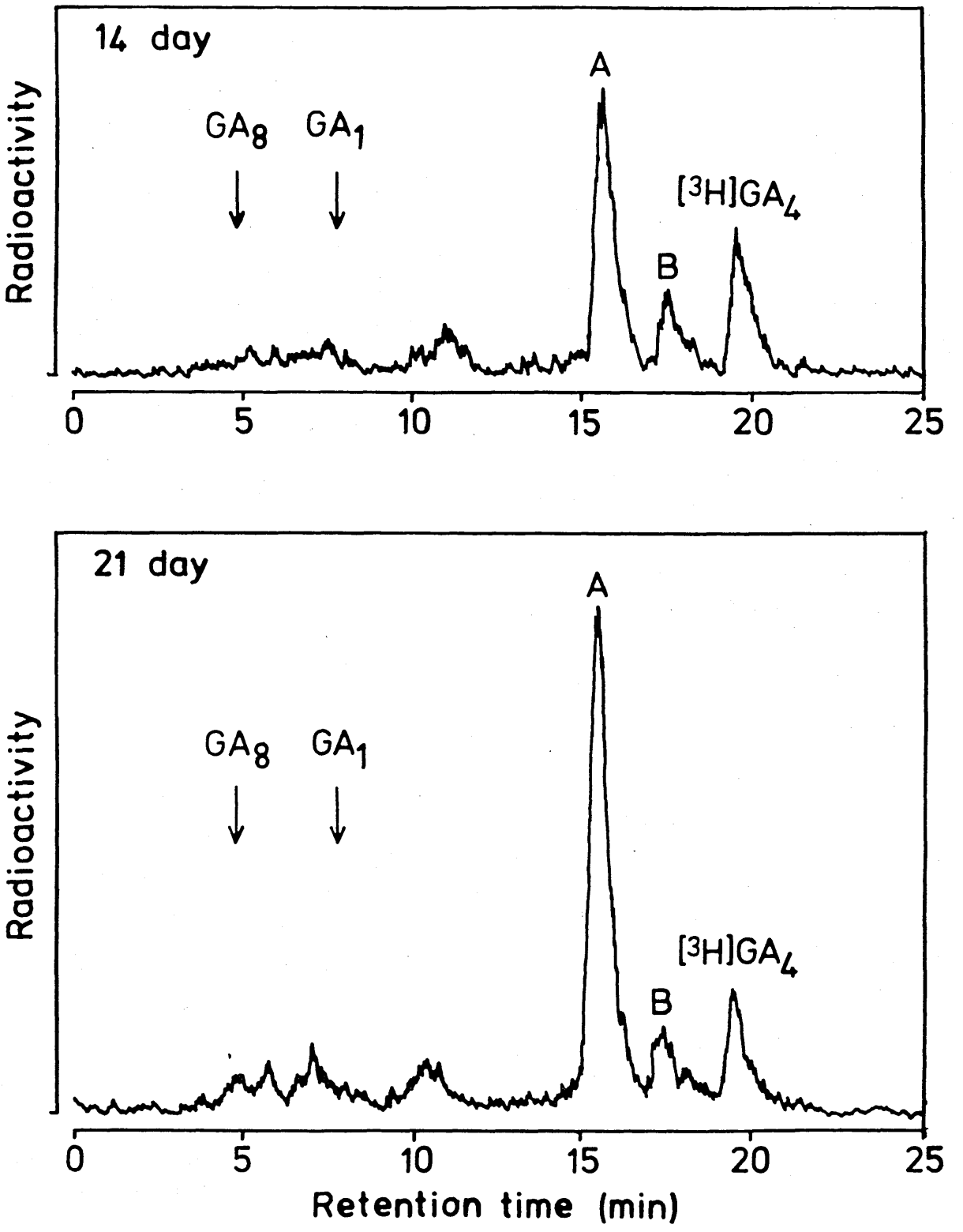

4 day
GA8
GA1
A
B
[3H]GA4
Radioactivity
0
5
10
15
20
25
7 day
GA8
GA1
[3H]GA4
A
B
Radioactivity
0
5
10
15
20
25
Retention time (min)

FIGURE 5

HPLC profiles of the 14 and 21 day EtOAc extracts from the 6 weeks 5°C [^{3}H]GA_4 feeds.


14 day
A
GA8
GA1
[3H]GA4
B
Radioactivity
0
5
10
15
20
25
21 day
A
GA8
GA1
[3H]GA4
B
Radioactivity
0
5
10
15
20
25
Retention time (min)

12 week 5°C Samples

The control sample was analysed using a gradient of 60-100% MeOH over 15 min and so the retention times were different from those in the other samples. As can be seen in Figure 6 the background was raised early on in the trace indicating the presence of other substances, but the majority of the [^{3}H]GA_4 was left unmetabolised (Table 7).

Four days incubation resulted in both peaks A and B being produced but B was the major metabolite (Table 7, Figure 7). The 7 day trace would have been more useful if a larger amount of sample had been available but [^{3}H]GA_4 and peak A were evident along with other more polar metabolites (Table 7, Figure 7). A peak at R_t 4.8 min had the same retention time as [^{3}H]GA_8 and an aliquot was run using a 20 min, 20-100% MeOH gradient to give a longer retention time. On this gradient [^{3}H]GA_8 had a retention time of 10.6 min but the nearest peak in the sample eluted about 2 min later than this.

In the 14 day sample [^{3}H]GA_4 and peak A were the major constituents with early eluting polar metabolites again being present (Table 7, Figure 8). Peak A was also the major metabolite in the 33 day sample, being larger than the [^{3}H]GA_4 peak (Table 7, Figure 8). To try and achieve better resolution of the more polar metabolites in the 33 day sample an aliquot was run on a 30 min gradient of 20-100% MeOH (Figure 9). Peak A and [^{3}H]GA_4 are labelled, along with the retention times of [^{3}H]GA_8 and [^{3}H]GA_1 which were 11.0 and 17.3 min, respectively. There was

improved resolution of the earlier peaks one of which had a retention time of 17.2 min, comparable to the retention time of 17.3 min for [^{3}H]GA_1.

Initially therefore, peak B appeared to be the major metabolite but after longer incubation times it decreased and peak A accumulated to a much greater extent. However, even after 33 days a substantial amount of the [^{3}H]GA_4 substrate remained.

TABLE 7 [^{3}H]GA_4 Metabolism - Percentage distribution of radioactivity between peaks A and B and [^{3}H]GA_4 in the 12 week 5°C samples. N.D. = Not detected.

Incubation time	% radioactivity		
	Peak A	Peak B	[^{3}H]GA_4
0 day	N.D.	N.D.	100.0
4 day	15.0	43.0	42.0
7 day	49.0	N.D.	51.0
14 day	49.0	N.D.	51.0
33 day	69.0	N.D.	31.0

FIGURE 6

HPLC profile of the control (0 day) EtOAc extract from the 12 weeks 5°C [^{3}H]GA_4 feed.

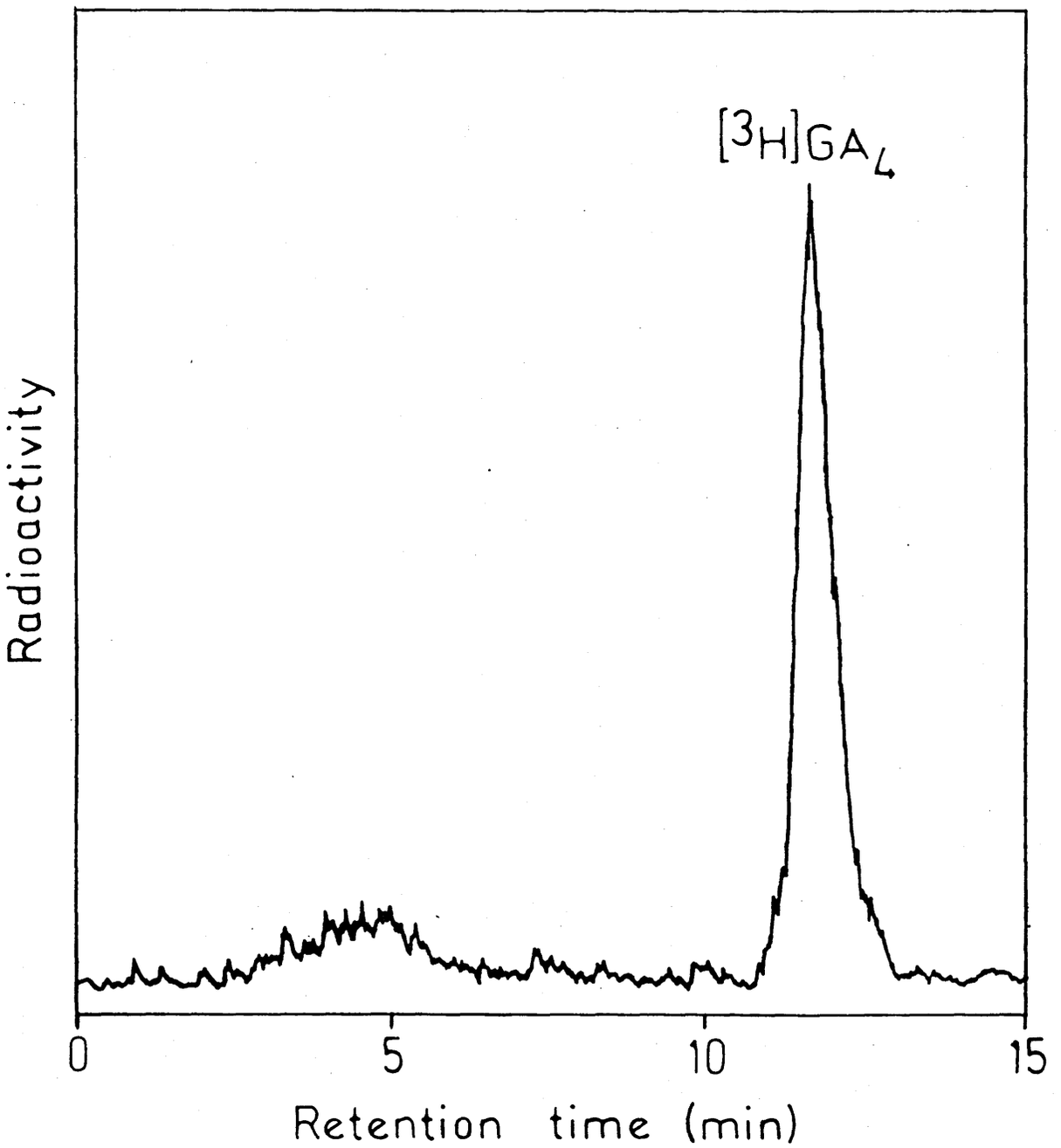

[3H]GA4
Radioactivity
0
5
10
15
Retention time (min)

FIGURE 7

HPLC profiles of the 4 and 7 day EtOAc extracts from the 12 weeks 5°C [^{3}H]GA_4 feeds.

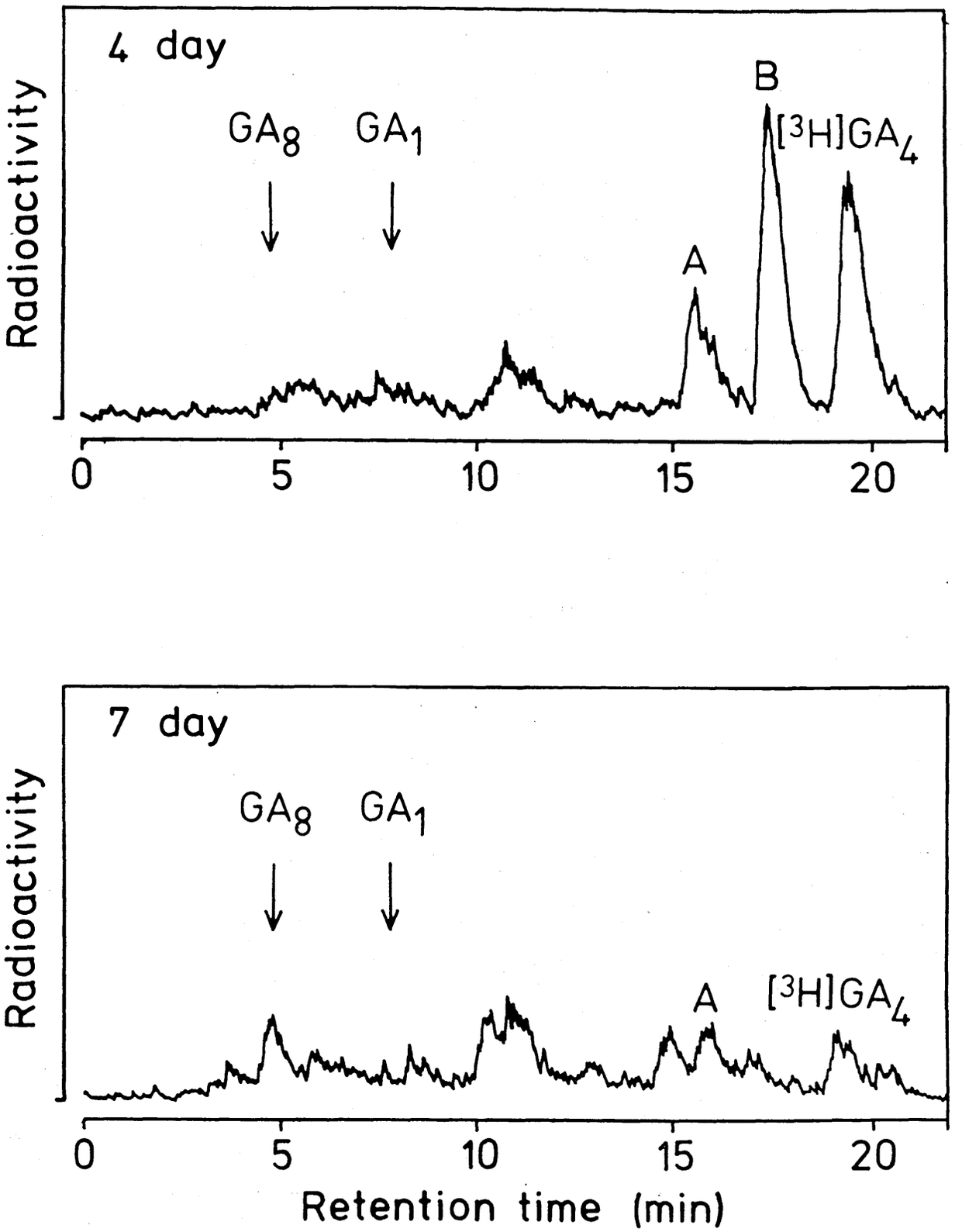

4 day
GA8
GA1
A
B
[3H]GA4
Radioactivity
0
5
10
15
20
7 day
GA8
GA1
A
[3H]GA4
Radioactivity
0
5
10
15
20
Retention time (min)

FIGURE 8

HPLC profiles of the 14 and 33 day EtOAc extracts from the 12 weeks 5°C [^{3}H]GA_4 feeds.

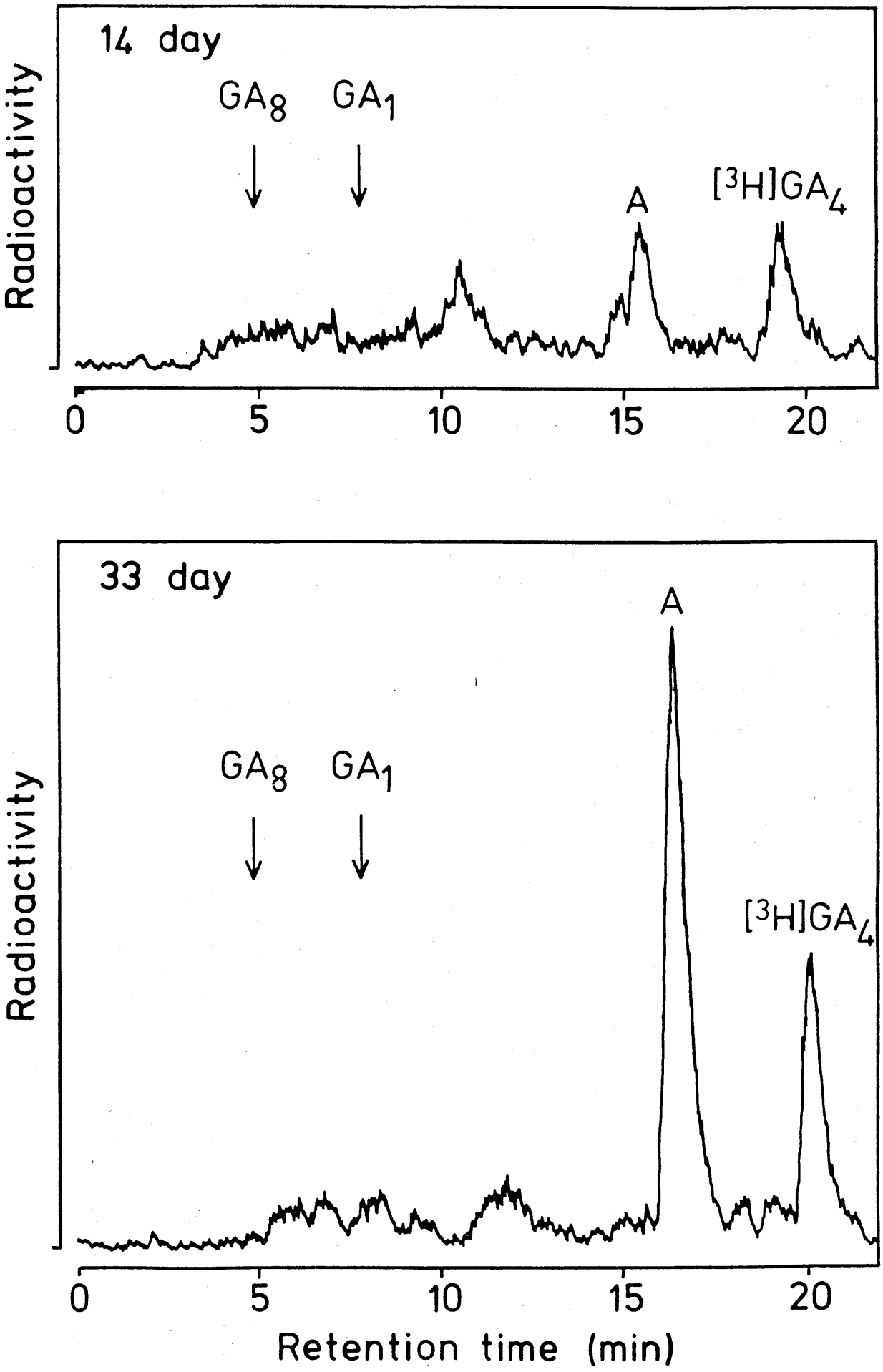
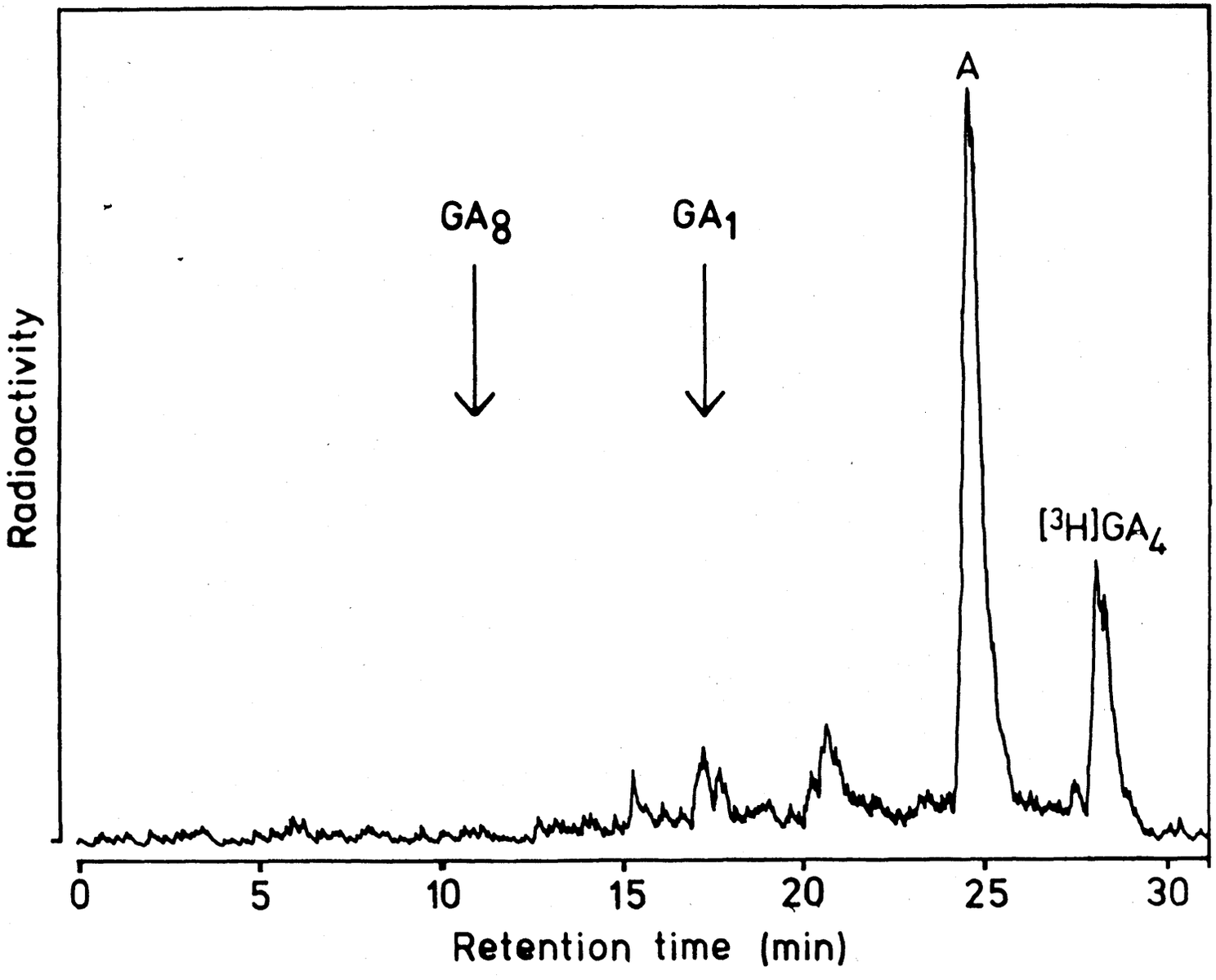

14 day
GA8
GA1
A
[3H]GA4
Radioactivity
0
5
10
15
20
33 day
A
GA8
GA1
[3H]GA4
Radioactivity
0
5
10
15
20
Retention time (min)

FIGURE 9

HPLC profile of the 33 day EtOAc extract from the 12 weeks 5°C [^{3}H]GA_4 feeds.

Gradient : 20-100% MeOH, 0-30 min.


A
GA8
GA1
[3H]GA4
Radioactivity
0
5
10
15
20
25
30
Retention time (min)

Bulk Extracts

In addition to the individual samples extracted and analysed as previously described, other samples were combined and extracted together to give three separate bulk extracts of the 0, 6 and 12 week samples. After HPLC analysis they were combined with the individual extracts from the appropriate storage period, prior to gel permeation chromatography (GPC).

The recovery and distribution of radioactivity in the aqueous and EtOAc fractions of the bulk extracts was relatively similar in all three samples, and no significant differences were evident (Table 8).

TABLE 8 [^{3}H]GA_4 Metabolism - Recovery and distribution of radioactivity in the aqueous and EtOAc fractions of the bulk extracts. Data are expressed as percentages of the applied and recovered radioactivity respectively.

Sample		Aqueous	EtOAc	Total Recovery
0W5°C	Recovery	15.6	44.9	60.5
	Distribution	25.8	74.2	
6W5°C	Recovery	22.1	46.3	68.4
	Distribution	32.3	67.7	
12W5°C	Recovery	17.5	42.1	59.6
	Distribution	29.4	70.6	

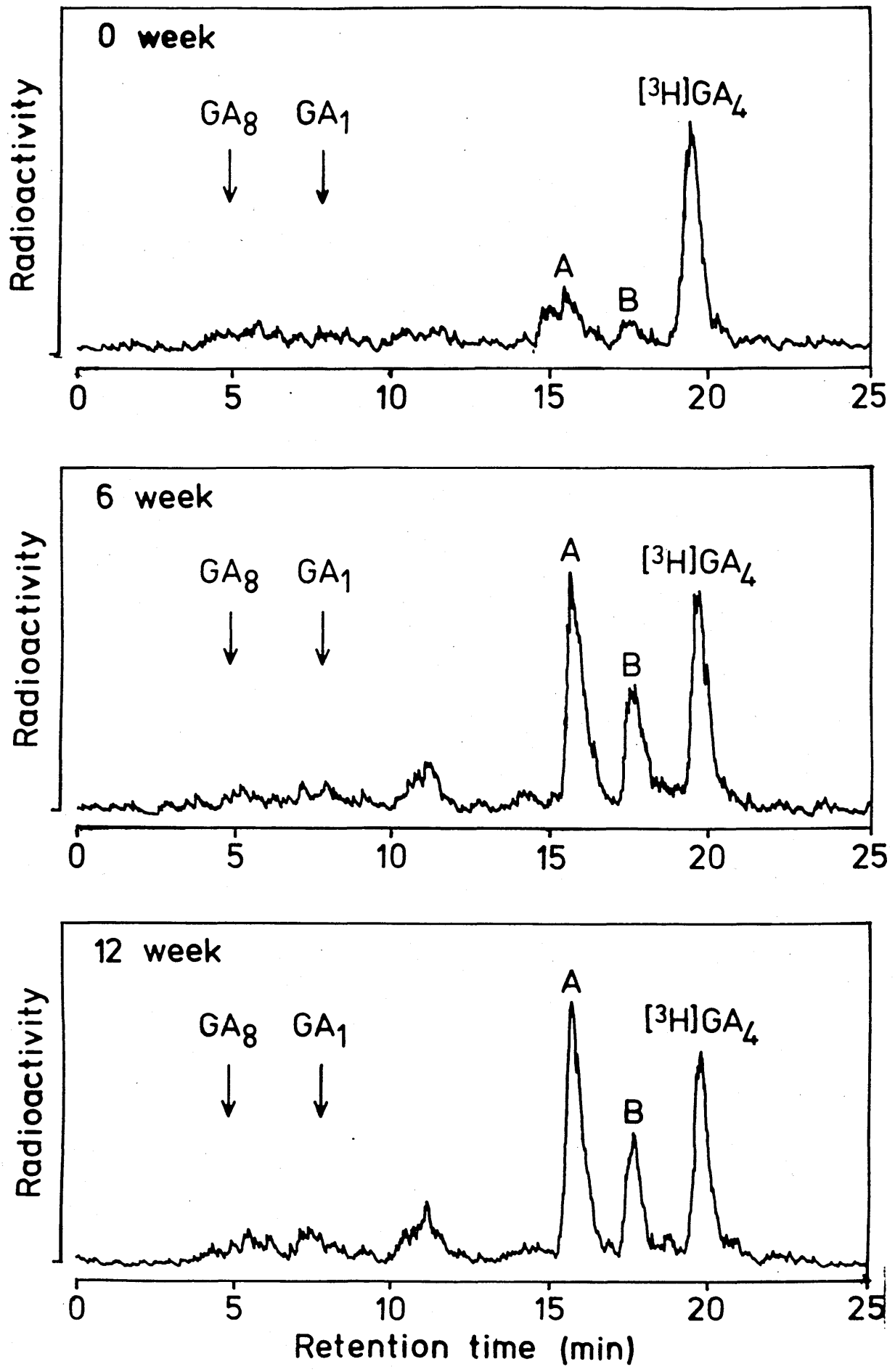
The bulk extracts were analysed by reversed-phase gradient HPLC as before, using the 40-100% MeOH gradient over 20 min. The percentage of [^{3}H]GA_4 decreased as the percentage of peak A increased, with increasing length of prior 5°C storage (Table 9, Figure 10). In the 6 week sample [^{3}H]GA_4 and peak A were of equivalent size and peak B, which had been very small in the 0 week sample, was also a major metabolite. Peak A had increased relative to [^{3}H]GA_4 in the 12 week sample and peak B, although slightly smaller, was still a major component.

TABLE 9 [^{3}H]GA_4 Metabolism - Percentage distribution of radioactivity between peaks A and B and [^{3}H]GA_4 in the 0, 6 and 12 week 5°C bulk extracts.

Sample	% radioactivity		
	Peak A	Peak B	[^{3}H]GA_4
0W5°C	14.0	6.0	80.0
6W5°C	37.0	26.0	37.0
12W5°C	48.0	21.0	31.0

FIGURE 10

HPLC profiles of the 0, 6 and 12 week bulk EtOAc extracts from the [^{3}H]GA_4 feeds.


0 week
Radioactivity
GA8
GA1
[3H]GA4
A
B
0
5
10
15
20
25
6 week
Radioactivity
GA8
GA1
A
[3H]GA4
B
0
5
10
15
20
25
12 week
Radioactivity
GA8
GA1
A
[3H]GA4
B
0
5
10
15
20
25
Retention time (min)

GEL PERMEATION CHROMATOGRAPHY (GPC)

After gradient HPLC analysis the 0, 6 and 12 week bulk EtOAc extracts were combined with the appropriate individual EtOAc extracts. The resultant 0 week 5°C sample was subjected to GPC first, separately, but because the main metabolites appeared to be the same the 6 and 12 week 5°C samples were combined and subjected to GPC together. Three areas of radioactivity were found in the GPC fractions and the fractions in each area were combined to give Fractions 1, 2 and 3 (Table 10). From the 0 week 5°C sample fraction 1 accounted for the highest percentage

TABLE 10 The elution volume and percentage of recovered radioactivity of the GPC fractions. The figures in brackets refer to the individual GPC fractions which were combined to give one bulk fraction.

Sample	Fraction	GPC elution volume (ml)	% of recovered radioactivity
0W5°C	1 (18-22)	349-378	37.0
	2 (26-31)	395-430	29.6
	3 (32-36)	430-459	33.4
6W5°C plus 12W5°C	1 (17-23)	343-383	33.4
	2 (25-33)	389-441	19.6
	3 (34-37)	441-465	47.0

of the recovered radioactivity followed by fractions 3 and 2. Fraction 3 had the highest percentage in the 6 and 12 week 5°C sample followed by fractions 1 and 2 (Table 10). As can be seen in Table 10 the areas of radioactivity occurred in similar fractions in both GPC runs and so fractions 1, 2 and 3 from the 0 week 5°C sample were combined with the corresponding fraction from the 6 plus 12 week 5°C sample.

The elution volume of fraction 1 was within the high molecular weight range where GA conjugates elute but fraction 2 seemed to elute slightly late for this. Fraction 3 eluted in the low molecular weight range where free GAs are found. It seemed probable that fraction 3 was [^{3}H]GA_4 whilst fraction 2 contained more polar metabolites and fraction 1 was possibly a GA_4 conjugate.

Isocratic HPLC

Each fraction was analysed by isocratic HPLC and the peaks were compared with unlabelled standards of GA conjugates plus [^{3}H]GA_4, but only small amounts of sample were available precluding extensive analysis (Table 11, Figure 11). The retention times varied as can be seen from the differing retention times of the [^{3}H]GA_4 standard and fraction 3 which was the sample [^{3}H]GA_4. This variation made comparison of standard and sample peaks difficult but fraction 1 contained one component comparable to peak A. It eluted at 9.9 min and as such was separate from GA_4 glucosyl ester which had a

retention time of 10.4 min. Fraction 2 contained several peaks including peaks which eluted early and were not properly separated and probably consisted of the more polar metabolites seen in some of the samples during gradient analysis. The last peak of fraction 2 eluted at 13.9 min and probably corresponded with peak B. It had a retention time similar, but not identical, to that of GA_4-3-O-glucoside which eluted at 14.7 min.

TABLE 11 Isocratic HPLC of [^{3}H]GA_4 metabolites - retention times of GPC fractions and free GA and GA conjugate standards.

Fraction	R_t (min)	Substance	R_t (min)
1 (Peak A)	9.9*[1]	GA_4-GE	10.4[1]
2	5.0[1]		
	6.7[1]		
	12.7[1]		
(Peak B)	13.9[1]	GA_4-3-O-Gluc	14.7[1]
3 ([^{3}H]GA_4)	10.6[2]	[^{3}H]GA_4	11.1[2]

* - Isocratic HPLC conditions. 1 = 55% MeOH

2 = 60% MeOH

GA_4-GE = GA_4-glucosyl ester

GA_4-3-O-Gluc = GA_4-3-O-glucoside

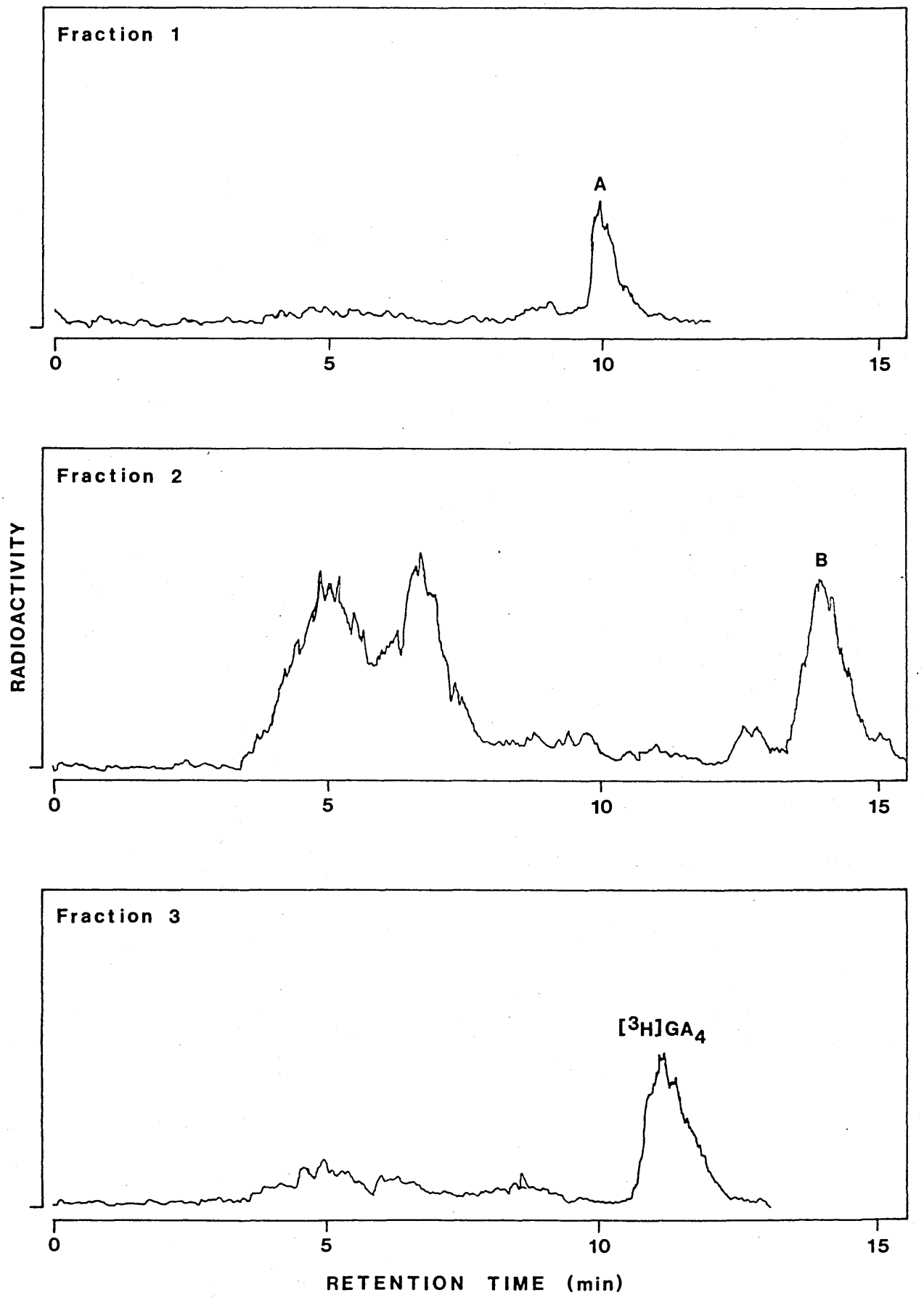
FIGURE 11

HPLC profiles of the GPC fractions 1-3 from the [^{3}H]GA_4 feeds.

Isocratic HPLC conditions :

Fractions 1 and 2 - 55% MeOH

Fraction 3 - 60% MeOH


Fraction 1
A
0
5
10
15
Fraction 2
B
RADIOACTIVITY
0
5
10
15
Fraction 3
[3H]GA4
0
5
10
15
RETENTION TIME (min)

Discussion

After 14 days incubation the [^{3}H]GA_4 substrate was present in reduced amounts, whether prior storage had been for 0, 6 or 12 weeks, but the extent of the reduction was less with longer periods of 5°C storage. Peak B was generally the largest initial metabolite but in later samples peak A was the major metabolite. Comparing the 4 day samples, after 0 weeks prior 5°C storage peak A was the largest product, but after 6 or 12 weeks 5°C storage peak B was the major metabolite and peak A only increased after longer incubation times. The extent of metabolism varied and in some samples the [^{3}H]GA_4 peak appeared to increase and decrease, probably due to sample variation rather than any real change.

Due to their proximity to [^{3}H]GA_4 it was thought possible that peaks A and B could be GA_4 conjugates. One peak was found in the high molecular weight fraction after GPC but did not correspond exactly to any of the GA_4 conjugates available. Another peak eluted later in the low molecular weight area.

The conversion of [^{3}H]GA_4 to [^{3}H]GA_{34} by 2β hydroxylation has been found in germinating pollen grains of *Pinus attenuata* (Kamienska *et al.*, 1976) and shoots of *Pseudotsuga menziesii* seedlings (Wample *et al.*, 1975). The conjugate GA_{34}-O-glucoside has also been found to be a major metabolite of [^{3}H]GA_4 (Turnbull *et al.*, 1986). However, considering the HPLC retention times of these substances (Turnbull *et al.*, 1986), the retention times of

the GPC peaks seem too late, in relation to the two GA_4-conjugates, to be GA_{34} and GA_{34}-O-glucoside.

[^{3}H]GA_4 has been found to be metabolised to GA_1, GA_8 and GA_1-glucoside and GA_8-glucoside in immature and mature seeds of *Phaseolus vulgaris* (Yamane *et al.*, 1975, 1977) and to GA_1, GA_8 and GA_8-2-O-glucoside in *P. coccineus* (Turnbull *et al.*, 1986). These did not seem to be metabolic products of [^{3}H]GA_4 in tulip bulbs, the only evidence of GA_1 being a minor peak with a similar retention time to GA_1 in the 12 week 33 day sample.

Within the separate storage times the percentage radioactivity remaining in the aqueous phase after partitioning with EtOAc at pH 2.5 increased with time. Considering the different storage times, there was little difference between them after the longer incubation times but after 0 and 4 days the 12 week samples had lower percentages in the aqueous phase than the other samples. The increase of radioactivity remaining in the aqueous phase could be an indication that the [^{3}H]GA_4 was progressively conjugated. During partitioning with EtOAc at pH 2.5 most free GAs will migrate into the EtOAc whilst GA conjugates will tend to stay in the aqueous phase (Schneider, 1983), although Turnbull *et al* (1986) found [^{3}H]GA_4 metabolites of *P. coccineus* seedlings, including free GAs and conjugates, in both the EtOAc fraction and the butanol fraction (from partitioning of the aqueous phase against butanol). In this case only the EtOAc fractions were analysed and any future studies should also

analyse the aqueous fractions. The aqueous phase would probably contain the peaks detected in the EtOAc fraction giving greater quantities of sample for analysis, plus other additional metabolites. This would allow further analysis of the individual peaks, possibly using the methods developed by Turnbull *et al* (1986), leading to eventual identification. These methods were for the identification of radiolabelled free and conjugated GAs using reversed-phase HPLC, GPC, enzymatic hydrolysis and GA derivatisation.

DISCUSSION

DISCUSSION

The growth analysis confirmed that tulips are not dormant during storage, with growth of the shoot, daughter bulbs and basal plate continuing at 5°C and 17°C at the expense of the scales. The overall RGR of each component was slightly higher at 17°C than 5°C and it was higher for the shoot than the other components at both temperatures. During 17°C storage the shoot RGR decreased, and storage at 5°C resulted in a marked decrease between 4 and 6 weeks, but the RGR then increased until it reached a level similar to that at 17°C between 8 and 12 weeks. Gilford and Rees (1973) stored bulbs at 4°C for 6 weeks followed by planting and storing at 9°C until forcing. They found that the 4°C treatment slowed down the RGR whilst at 9°C the RGR increased 2 weeks prior to transfer to the glasshouse. Bulbs stored at 9°C for the whole of the cold treatment, with and without a prior 35°C treatment, did not display this final increase in RGR until the bulbs were growing at 18°C in the glasshouse (Gilford and Rees, 1973). Therefore the bulbs stored dry at 5°C underwent a similar increase during storage although it began earlier and was evident during the last 6 weeks of storage.

Initial assays of the 0, 6 and 12 week whole bulb samples indicated the presence of three main peaks in all the samples, one of which was possibly GA_1 and another either GA_5 or GA_{20}. The possible identity of the other peak was unknown. GA_1 and GA_5 have been tentatively

identified in tulips (Aung *et al.*, 1971b; De Hertogh *et al.*, 1971).

The results suggested that the overall level of these GAs in the whole bulbs fell during cold storage. This contrasts with previous findings which suggest that GAs increase during cold storage (Aung and De Hertogh, 1967, 1968) or that levels do not change (Van Bragt, 1971). Unfortunately later assays of the whole bulb samples gave very different results and it appeared that the endogenous GAs in the bulb tissue had deteriorated during storage. The experiments were carried out at the Glasshouse Crops Institute in Littlehampton but analysis could only be carried out in Glasgow necessitating storage of the tissue. Additional delays were caused by shortage of antiserum and difficulties in raising further stocks. In future it would be best to at least extract tissue as soon as possible.

GA_1-like and GA_5/GA_{20}-like peaks were detected in the original 12 week 5°C scale and basal plate extracts and peaks were detected in these areas plus a GA_4-like peak in some of the later extracts of the individual bulb components. GA_1 and GA_5 have previously been tentatively identified in extracts from shoots (Aung *et al.*, 1971b; De Hertogh *et al.*, 1971) and scales (Aung *et al.*, 1971b).

The GA_1 HPLC zone was assayed in all the samples and a GA_1-like peak was generally found in the basal plate and daughter bulb samples whilst in many of the scale and shoot samples no such peak was detected. This contrasts

with earlier findings that shoots have comparatively high activity, but those findings consider the total activity detected by bioassay, whereas this is the result for only one peak. The GA-like activity has been found to be highest in the shoot and daughter bulbs (De Hertogh *et al.*, 1971; Einert *et al.*, 1972) and lowest in the scales and roots (De Hertogh *et al.*, 1971).

The total GA level detected in the whole bulb samples appeared to decrease during storage. However, in the basal plate the level of the GA_1-like peak decreased between 0 and 8 weeks, but then increased after 12 weeks at both temperatures, although to a greater extent at 17°C. The basal plate is the part of the bulb which the roots develop in and emerge from when it is planted. Correspondent with the apparent increase in the GA_1-like peak at 17°C the RGR of the basal plate was higher during the 8-12 week period than during the 4-6 and 6-8 week periods. However, at 5°C the RGR was at its lowest at this point and thus the GA rise did not correlate with increased growth at 5°C. The daughter bulbs also exhibited a 12 weeks increase in the GA_1-like peak following a decrease, but only at 17°C. The RGR of the daughter bulbs was higher and less variable from 8-12 weeks than in the preceding 2 week period at 17°C but at 5°C where no GA increase was found there was no RGR increase although it was again less variable than before. Einert *et al* (1972) found an increase in GA activity in the daughter bulbs after a decrease in the combined scales

and daughter bulbs during cooling, but the increase did not occur until the greenhouse phase of forcing at 18°C. These bulbs were rooted and cooled rather than dry cooled and so the results are not directly comparable in any case.

Metabolism of [^{3}H]GA_4 appeared to occur to a greater extent than that of [^{14}C]GA_3. In the 14 day samples, after 0, 6 and 12 weeks storage, the GA_3 peak accounted for 45.0%, 66.0% and 73.0% of the total area respectively, whilst the GA_4 peak accounted for only 8.0%, 29.0% and 51.0% respectively. The main [^{14}C]GA_3 metabolite appeared to be a free GA rather than a conjugate. One of the [^{3}]GA_4 metabolites eluted in the high molecular weight range after GPC but the other eluted later, indicating that it was a free GA.

Radiolabelled GA_4 is commonly metabolised to GA_6 via GA_1 and to their respective conjugates (Schneider, 1983; Sponsel, 1983) but this did not seem to occur in tulip bulbs. The only evidence of GA_1 was a minor peak with a similar retention time to GA_1 in the 12 week 33 day sample. It is not known whether GA_1, GA_4 and GA_6 are native to tulips and thus whether this would be a normal metabolic pathway. GA_1-like peaks were detected in this study and tentatively identified by other researchers (Aung *et al.*, 1971b; De Hertogh *et al.*, 1971) and a GA_4-like peak was present in a 0 week whole bulb sample.

REFERENCES

REFERENCES

A.D.A.S. (AGRICULTURAL DEVELOPMENT AND ADVISORY SERVICE) (1981). Tulip forcing. Booklet 2300. Ministry of Agriculture, Fisheries and Food (Publications), Alnwick, NE66 2PF, U.K.

ALGERA, L. (1936). Concerning the influence of temperature treatment on the carbohydrate metabolism, the respiration and the morphological development of the tulip I-III. Proc. Kon. Ned. Akad. Wet. (Amst.). 39: 846-855, 971-981, 1106-1114.

ALGERA, L. (1947). Over den invloed van de temperatuur op de koolhydraatstofwisseling en ademhaling bij de tulp en de hyacinth en de beteekenis daarvan voor de ontwikkeling der plant. Meded. LandbHoogesch. Wageningen. 48: 87-183.

ALPI, A., CECCARELLI, N., TOGNONI, F. and GREGORINI, G. (1976). Gibberellin and inhibitor content during *Iris* bulb development. Physiol. Plant. 36: 362-367.

ALPI, A. and DE HERTOGH, A.A. (1975). Extractability of gibberellin-like substances from tulips grown at two temperatures in either the light or dark. Acta Hort. 47: 297-305.

ATZORN, R. AND WEILER, E.W. (1983). The immunoassay of gibberellins. I. Radioimmunoassays for the gibberellins A_1, A_3, A_4, A_7, A_9 and A_{20}. Planta 159: 1-6.

AUNG, L.H. AND DE HERTOGH, A.A. (1967). The occurrence of

gibberellin-like substances in tulip bulbs (*Tulipa* sp.). Plant and Cell Physiol. 8: 201-205.

AUNG, L.H. AND DE HERTOGH, A.A. (1968). Gibberellin-like substances in non-cold and cold treated tulip bulbs (*Tulipa* sp.). In: Biochemistry and Physiology of Plant Growth Substances, pp. 943-956, Wightman, F. and Setterfield, G., eds., Runge Press, Ottowa.

AUNG, L.H. and DE HERTOGH, A.A. (1979). Temperature regulation of growth and endogenous abscisic acid-like content of *Tulipa gesneriana* L. Plant Physiol. 63: 1111-1116.

AUNG, L.H., DE HERTOGH, A.A. and STABY, G.L. (1969a). Gibberellin-like substances in bulb species. Can. J. Bot. 47: 1817-1819.

AUNG, L.H., DE HERTOGH, A.A. and STABY, G.L. (1969b). Temperature regulation of endogenous gibberellin activity and development of *Tulipa gesneriana* L. Plant Physiol. 44: 403-406.

AUNG, L.H., DE HERTOGH, A.A. and STABY, G.L. (1971a). The alteration of bulb hormones content by environmental stimuli. Acta Hort. 23: 156-161.

AUNG, L.H., DE HERTOGH, A.A. and STABY, G.L. (1971b). Possible identification of gibberellins in *Tulipa gesneriana* by gas-liquid chromatography. Phytochem. 10: 215-217.

AUNG, L.H. and REES, A.R. (1974). Changes in endogenous gibberellin levels in *Tulipa* bulblets during ontogeny. J. Exp. Bot. 25: 745-751.

BALDEV, B. and LANG, A. (1965). Control of flower formation by growth retardants and gibberellin in *Samolus parviflorus*, a long-day plant. Amer. J. Bot. 52: 408-417.

BRIGGS, J.B. (1975). The effects on growth and flowering of the chemical growth regulators ethephon on narcissus and ancymidol on tulip. Acta Hort. 47: 287-296.

BYLOV, W.N. and SMIRNOVA, Z.I. (1979). Possibilities of gibberellin application for early forcing *Tulipa gesneriana* cv. 'London'. Acta Hort. 91: 173-177.

CHARLES-EDWARDS, D.A. and REES, A.R. (1975). An analysis of the growth of forced tulips. 2. Effects of low-temperature treatments during development on plant structure at anthesis. Scientia Hort. 3: 373-381.

CLELAND, C.F. and ZEEVART, J.A.D. (1970). Gibberellins in relation to flowering and stem elongation in the long-day plant *Silene armeria*. Plant Physiol. 46: 392-400.

COCOZZA TALIA, M. and CAPUTO, V. (1980). A research on the possibility of using gibberellin for tulip forcing. Acta Hort. 109: 163-168.

COCOZZA TALIA, M. and STELLACCI, P. (1977). A research on early tulip flowering induced by applying plant growth regulators. Acta Hort. 68: 133-143.

COCOZZA TALIA, M. and STELLACCI, P. (1979). A research of the effect of gibberellin upon tulip flowering. Acta Hort. 91: 167-172.

COOLBAUGH, R.C. and HAMILTON, R. (1976). Inhibition of *ent*-kaurene oxidation and growth by α-cyclopropyl-α-(p-methoxyphenyl)-5-pyrimidine methyl alcohol. Plant Physiol. 57: 245-248.

COOLBAUGH, R.C., HIRANO, S.S. and WEST, C.A. (1978). Studies on the specificity and site of action of α-cyclopropyl-α-[p-methoxypropyl]-5-pyrimidine methyl alcohol (ancymidol), a plant growth regulator. Plant Physiol. 62: 571-576.

CROZIER, A., SANDBERG, G., MONTEIRO, A.M. and SUNDBERG, B. (1986). The use of immunological techniques in plant hormone analysis. In: Plant Growth Substances 1985, pp. 13-21, Bopp, M., ed., Springer-Verlag, Berlin, Heidelberg.

DE HERTOGH, A.A., AUNG, L.H. and BENSCHOP, M. (1983). The Tulip: Botany, Usage, Growth and Development. Horticultural Reviews 5: 45-125.

DE HERTOGH, A.A., AUNG, L.H., EINERT, A.E. and STABY, G.L. (1971). Aspects of extractable gibberellin-like substances from *Tulipa gesneriana* L. Acta Hort. 23: 90-95.

DE HERTOGH, A.A. and BREG, T. (1980). Internodal growth of the tulip during the greenhouse phase of standard forcing. Acta Hort. 109: 157-162.

DE MUNK, W.J. (1973). Flower-bud blasting in tulips caused by ethylene. Neth. J. Plant Pathol. 79: 41-53.

DE MUNK, W.J. (1979). Influence of plant growth regulators on the development of bulbous plants with

special reference to organ relationships. Acta Hort. 91: 207-219.

DE MUNK, W.J. and GIJZENBERG, J. (1977). Flower-bud blasting in tulip plants mediated by the hormonal status of the plant. Scientia Hort. 7: 255-268.

DE MUNK, W.J. and HOOGETERP, P. (1975). Flower-bud blasting in tulips. Acta Hort. 47: 149-155.

EINERT, A.E., STABY, G.L. and DE HERTOGH, A.A. (1972). Gibberellin-like activity from organs of *Tulipa gesneriana* L. Can. J. Bot. 50: 909-914.

GIANFAGNA, T., ZEEVART, J.A.D., and LUSK, W.J. (1983). Effect of photoperiod on the metabolism of deuterium-labelled gibberellin A_{53} in spinach. Plant Physiol. 72: 86-89.

GILFORD, J.MCD. AND REES, A.R. (1973). Growth of the tulip shoot. Scientia Hort. 1: 143-156.

GRAEBE, J.E. and ROPERS, H.J. (1978). Gibberellins. In: Phytohormones and Related Compounds: A Comprehensive Treatise, Vol. 1, pp. 107-204, Letham, D.S., Goodwin, P.B. and Higgins, T.J.V., eds., Elsevier/North-Holland Biomedical Press, Amsterdam.

HANKS, G.R. (1979). Potential uses of plant growth regulators in bulbous ornamentals. News Bulletin, British Plant Growth Regulator Group 3(1): 5-16.

HANKS, G.R. (1982a). Can GA replace cold? Grower 97(16): 44, 46-48.

HANKS, G.R. (1982b). The response of tulips to gibberellins following different durations of cold

storage. J. Hort. Sci. 57: 109-119.

HANKS, G.R. (1984). Factors affecting the response of tulips to gibberellin. Scientia Hort. 23: 379-390.

HANKS, G.R. and MENHENETT, R. (1979). Response of potted tulips to new and established growth-retarding chemicals. Scientia Hort. 10: 237-254.

HANKS, G.R. and REES, A.R. (1977). Stem elongation in tulip and narcissus: the influence of floral organs and growth regulators. New Phytol. 78: 579-591.

HANKS, G.R. and REES, A.R. (1980a). Growth substances of tulip: The activity of gibberellin-like substances in field-grown tulips from planting until flowering. Z. Pflanzenphysiol. 98: 213-224.

HANKS, G.R. and REES, A.R. (1980b). Partly cooled tulips: response to GA_3 and other gibberellins. Acta Hort. 109: 169-176.

HANKS, G.R. and REES, A.R. (1983). The response of partly cooled tulips to vacuum infiltration with gibberellins. Scientia Hort. 21: 159-172.

HARTSEMA, A.M. (1961). Influence of temperatures on flower formation and flowering of bulbous and tuberous plants. In: Encyclopaedia of Plant Physiology, Vol. 16, pp. 123-167, Ruhland, W., ed., Springer-Verlag, Berlin.

JONES, M.G. and ZEEVART, J.A.D. (1980a). Gibberellins and the photoperiodic control of stem elongation in the long-day plant *Agrostemma githago* L. Planta 149: 269-273.

JONES, M.G. and ZEEVART, J.A.D. (1980b). The effect of photoperiod on the levels of seven endogenous gibberellins in the long-day plant *Agrostemma githago* L. Planta 149: 274-279.

JONES, M.G. and ZEEVART, J.A.D. (1982). Effect of photoperiod on metabolism of [^{3}H]gibberellins A_1, 3-*epi*-A_1, and A_{20} in *Agrostemma githago* L. Plant Physiol. 69: 660-662.

JONES, S.K. and HANKS, G.R. (1984). Treatment of tulips with gibberellic acid by vacuum infiltration. J. Hort. Sci. 59: 241-252.

KAMERBEEK, G.A., BEIJERSBERGEN, J.C.M. and SCHENK, P.K. (1970). Dormancy in bulbs and corms. Proc. 18th Int. Hort. Congr. 5: 233-239.

KAMIENSKA, A., DURLEY, R.C. and PHARIS, R.P. (1976). Endogenous gibberellins of pine pollen. III. Conversion of 1,2-[^{3}H]GA_4 to gibberellins A_1 and A_{34} in germinating pollen of *Pinus attenuata* Lemm. Plant Physiol. 58: 68-70.

KNOX, J.P., BEALE, M.H., BUTCHER, G.W. and MACMILLAN, J. (1987). Preparation and characterisation of monoclonal antibodies which recognise different gibberellin epitopes. Planta 170: 86-91.

METZGER, J.D. and ZEEVART, J.A.D. (1980a). Identification of six endogenous gibberellins in spinach shoots. Plant Physiol. 65: 623-626.

METZGER, J.D. and ZEEVART, J.A.D. (1980b). Effect of photoperiod on the levels of endogenous gibberellins

in spinach as measured by combined gas chromatography-selected ion current monitoring. Plant Physiol. 66: 844-846.

METZGER, J.D. and ZEEVART, J.A.D. (1982). Photoperiodic control of gibberellin metabolism in spinach. Plant Physiol. 69: 287-291.

MOE, R. AND WICKSTRØM, A. (1973). The effect of storage temperature on shoot growth, flowering and carbohydrate metabolism in tulip bulbs. Physiol. Plant. 28: 81-87.

MOE, R. AND WICKSTRØM, A. (1979). Effect of precooling at 5 or -1°C on shoot growth, flowering and carbohydrate metabolism in tulip bulbs. Scientia Hort. 10: 187-201.

OHKAWA, K. (1976). The role of gibberellins in shoot emergence and elongation, flower differentiation and development of *Lilium speciosum rubrum*. J. Japan. Soc. Hort. Sci. 45: 289-299.

OKUBO, H. and UEMOTO, S. (1984a). Effects of darkness on stem elongation in tulip. Scientia Hort. 23: 391-398.

OKUBO, H. and UEMOTO, S. (1984b). The application of dark treatment to cut tulip production. Scientia Hort. 24: 75-82.

OKUBO, H. and UEMOTO, S. (1985). Changes in endogenous gibberellin and auxin activities during first internode elongation in the tulip flower stalk. Plant Cell Physiol. 26: 709-719.

OP DEN KELDER, P., BENSCHOP, M. and DE HERTOGH, A.A. (1971). Factors affecting floral stalk elongation of flowering tulips. J. Amer. Soc. Hort. Sci. 96: 603-605.

PENGELLY, W.L. (1986). Validation of immunoassays. In: Plant Growth Substances, Bopp, M., ed., Springer-Verlag, Berlin, Heidelberg.

PHARIS, R.P. and KING, R.W. (1985). Gibberellins and reproductive development in seed plants. Ann. Rev. Plant Physiol. 36: 517-568.

RAKHIMBAEV, I.R. and SOLOMINA, V.F. (1975). Natural cytokinins and dormancy of tulip bulbs. Translated from Fiziol. Rast. 22: 615-618.

RAKHIMBAEV, I.R., SYRTANOVA, G.A. and SOLOMINA, V.F. (1978). Effect of cold treatment on the level of biological activity of endogenous growth regulators in tulip bulbs. Translated from Fiziol. Rast. 25: 249-253.

REES, A.R. (1969). Effects of duration of cold treatment on the subsequent flowering of tulips. J. Hort. Sci. 44: 27-36.

REES, A.R. (1972). The Growth of Bulbs. Academic Press, London.

REES, A.R. (1973). Effects on tulip bulbs of warm storage following low-temperature treatment. J. Hort. Sci. 48: 149-154.

REES, A.R. (1984). Dormancy, flowering and periodicity in ornamental bulbs. The Plantsman 6: 33-41.

REES, A.R. AND BRIGGS, J.B. (1975). Cold requirements of tulips for earliest flowering. Acta Hort. 47: 209-214.

REES, A.R. and HANKS, G.R. (1979). Potential uses of plant growth regulators in bulb growing and forcing. Acta Hort. 91: 153-159.

REEVE, D.R. and CROZIER, A. (1975). Gibberellin bio-assays. In: Gibberellins and Plant Growth, pp. 35-64, Krishnamoorthy, H.N., ed., Wiley Eastern, New Delhi.

REEVE, D.R. and CROZIER, A. (1976). Purification of plant hormone extracts by gel permeation chromatography. Phytochem. 15: 791-793.

REEVE, D.R. and CROZIER, A. (1980). Quantitative analysis of plant hormones. In: Hormonal Regulation of Development 1. Molecular Aspects of Plant Hormones. Encyclopedia of Plant Physiology New Series, Vol. 9, pp. 203-280, MacMillan, J., ed., Springer-Verlag, Heidelberg, New York.

RODBARD, D. (1974). Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clin. Chem. 20: 1255-1270.

RODRIGUES PEREIRA, A.S. (1964). Endogenous growth factors and flower formation in Wedgwood Iris bulbs. Acta Bot. Neerl. 13: 302-321.

RUDNICKI, R.M., NOWAK, J. and SANIEWSKI, M. (1976). The effect of gibberellic acid on sprouting and flowering of some tulip cultivars. Scientia Hort. 4: 387-397.

SANIEWSKI, M. and DE MUNK, W.J. (1981). Hormonal control of shoot elongation in tulips. Scientia Hort. 15: 363-372.

SANO, Y. (1975). Effect of cooling, forcing temperature and gibberellin treatment on the growth and flowering in Iris 'Wedgwood'. J. Japan. Soc. Hort. Sci. 44: 59-65.

SCHNEIDER, G. (1983). Gibberellin Conjugates. In: The Biochemistry and Physiology of Gibberellins, Vol. 1, pp. 389-456, Crozier, A., ed., Praeger, New York.

SHOUB, J. and DE HERTOGH, A.A. (1974). Effects of ancymidol and gibberellins A_3 and A_{4+7} on *Tulipa gesneriana* L. cv. 'Paul Richter' during development in the greenhouse. Scientia Hort. 2: 55-67.

SHOUB, J. and DE HERTOGH, A.A. (1975). Growth and development of the shoot, roots and central bulblet of *Tulipa gesneriana* L. cv. 'Paul Richter' during standard forcing. J. Amer. Soc. Hort. Sci. 100: 32-37.

SOLOMINA, V.F., RAKHIMBAEV, I.R., KEFELI, V.I. and KOMIZERKO, E.I. (1976). Isolation and identification of a natural cytokinin - zeatin ribonucleoside - from growing tulip bulbs. Translated from Doklady Akad. Nauk SSSR. 229: 1274-1276.

SPONSEL, V.M. (1983). *In vivo* Gibberellin Metabolism in Higher Plants. In: The Biochemistry and Physiology of Gibberellin, Vol. 1, pp. 151-250, Crozier, A., ed., Praeger, New York.

SUGE, H. (1970). Changes of endogenous gibberellins in vernalised radish plants. Plant Cell Physiol. 11: 729-735.

SUGE, H. and RAPPAPORT, L. (1968). Role of gibberellins in stem elongation and flowering in radish. Plant Physiol. 43: 1208-1214.

SUH, J.K., KIM, K.S., BYUN, J.K. and ROH, S.M. (1983). The influence of temperature treatments on the level of endogenous growth substances in tulip (*Tulipa gesneriana* L. 'Purrissima') bulb. J. Kor. Soc. Hort. Sci. 24: 214-220.

SYRTANOVA, G. and RAKHIMBAEV, I. (1973). Endogenous gibberellins during dormancy and growth of tulip bulbs. Translated from Fiziol. Rast. 20: 721-724.

SYRTANOVA, G.A., TURETSKAYA, R.Kh. and RAKHIMBAEV, I. (1973). Natural auxins and inhibitors in dormant and growing tulip bulbs. Translated from Fiziol. Rast. 20: 1133-1136.

TERRY, P.H., AUNG, L.H. and DE HERTOGH, A.A. (1982). Identification of abscisic acid in *Tulipa gesneriana* L. by gas-liquid chromatography with electron capture and combined gas-liquid chromatography and mass spectrometry. Plant Physiol. 70: 1574-1576.

TURNBULL, C.G.N., CROZIER, A. and SCHNEIDER, G. (1986). HPLC-based methods for the identification of gibberellin conjugates: metabolism of [^{3}H]Gibberellin A_4 in seedlings of *Phaseolus coccineus*. Phytochem. 25: 1823-1828.

TYMOSZUK, J., SANIEWSKI, M. and RUDNICKI, R.M. (1979). A possible use of the infiltration method for application of growth regulators into bulbs. Acta Hort. 91: 179-183.

VAN BRAGT, J. (1971). Endogenous gibberellin levels and floral stalk elongation in tulip cv. 'Apeldoorn'. Mededelingen van de Rijksfaculteit Landbouwwetenschappen te Gent 36: 1301-1307.

VAN BRAGT, J. and VAN AST, K.J. (1976). Substitution of the cold requirement of tulip cv. 'Apeldoorn' by GA_3. Scientia Hort. 4: 117-122.

VAN BRAGT, J. and ZIJLSTRA, F.A. (1971). Effects of gibberellins on flowering of tulip cv. 'Apeldoorn'. Z. Pflanzenphysiol. 64: 139-144.

VAN VUNAKIS, H. (1980). Radioimmunoassays: an overview. Methods Enzymol. 70: 201-209.

WAMPLE, R.L., DURLEY, R.C. and PHARIS, R.P. (1975). Metabolism of gibberellin A_4 by vegetative shoots of Douglas fir at three stages of ontogeny. Physiol. Plant. 35: 273-278.

WEILER, E.W. (1982). Plant hormone immunoassay. Physiol Plant. 54: 230-234.

WEILER, E.W. (1984). Immunoassay of plant growth regulators. Ann. Rev. Plant Physiol. 35: 85-95.

YAMANE, H., MUROFUSHI, N., OSADA, H. and TAKAHASHI, N. (1977). Metabolism of gibberellins in early immature bean seeds. Phytochem. 16: 831-835.

YAMANE, H., MUROFUSHI, N. and TAKAHASHI, N. (1975). Metabolism of gibberellins in maturing and germinating bean seeds. Phytochem. 14: 1195-1200.

YOKOTA, T., REEVE, D.R. and CROZIER, A. (1976). The synthesis of [^{3}H]-gibberellin A_9 with high specific activity. Agr. Biol. Chem. 40: 2091-2094.

ZEEVART, J.A.D. (1968). Vernalisation and gibberellin in *Lunaria annua* L. In: Biochemistry and Physiology of Plant Growth Substances, pp. 1357-1370, Wightman, F. and Setterfield, P., eds., Runge Press, Ottawa.

ZEEVART, J.A.D. (1971). Effects of photoperiod on growth rate and endogenous gibberellins in the long-day rosette plant spinach. Plant Physiol. 47: 821-827.

ZEEVART, J.A.D. (1983). Gibberellins and Flowering. In: The Biochemistry and Physiology of Gibberellins, Vol. 2, pp. 333-374, Crozier, A., ed., Praeger, New York.

ZEEVART, J.A.D. (1985). Inhibition of stem growth and gibberellin production in *Agrostemma githago* L. by the growth retardant tetcyclacis. Planta 166: 276-279.