The Preparation of ^{14}C -Labelled Gibberellic Acid

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SUMMARY

Gibberella fujikuroi was allowed to grow on unlabelled medium until gibberellic acid (GA) was being produced, and samples of mycelium were then transferred to a substrate containing ^{14}C -acetate. Pure ^{14}C -GA was isolated from the medium. The specific activity of the ^{14}C -GA was increased by transferring mycelium to a second labelled substrate 6 hr after the first transfer. The specific activity was not increased when unlabelled glucose was present in the transfer medium, or when anaerobic conditions were maintained after transfer. Most of the ^{14}C -GA was produced during the 48 hr following transfer. A large proportion of the ^{14}C was lost as $^{14}\text{CO}_2$, and most of this was produced during the 40 hr after transfer. After this period little GA was produced, but the evolution of unlabelled CO_2 continued.

INTRODUCTION

The work described below was done in order to devise a satisfactory technique for preparing gibberellic acid (GA) labelled with ^{14}C , for use in physiological experiments with higher plants. Several publications describe the isolation of ^{14}C -GA from cultures of *Gibberella fujikuroi* containing labelled substances (Watanabe, 1957; Watanabe & Scully, 1957*a*; Zweig, DeVay & Cosens, 1958; Zweig & DeVay, 1959; Zweig, Yamaguchi & Mason, 1961; Birch, Richards & Smith, 1958; Birch *et al.* 1959; Redemann & Meuli, 1959). Much of this published work was concerned with possible paths of synthesis of GA within the fungus, but Watanabe & Scully (1957*b, c*) and Zweig *et al.* (1961) used some of the ^{14}C -GA produced by the fungus in experiments with higher plants. In these cases the fungus was grown from the time of inoculation on a medium containing labelled substrate, a method which is without doubt the most satisfactory when labelled GA of very high specific activity is required. However, much of the labelled carbon is inevitably lost with such a technique, since the amount of GA produced is high only after the fungus has been growing for a considerable time (Borrow *et al.* 1955). Further, the concentration of carbon should be high initially, as the best conditions for GA production are those in which nitrogen and not carbon becomes the limiting factor for growth (Brian, Radley, Curtis & Elson, 1955). A more practicable means of producing ^{14}C -GA is to allow the fungus to grow on an unlabelled substrate until GA is being produced, and then to transfer mycelium to a labelled substrate. A transfer technique of this kind was apparently first tried by Zweig *et al.* (1958), and a similar technique was devised for the following work.

^{14}C -acetate was chosen as a carbon source. Zweig & DeVay (1959) used several

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carbon sources, and found that acetate was relatively efficient for ^{14}C -GA production. Birch *et al.* (1958) found that alternate carbon atoms of the gibbane ring system of GA (Brian, Grove & MacMillan, 1960) became labelled when ^{14}C -1-acetate was used as a precursor.

METHODS

Organism. *Gibberella fujikuroi* (Saw.) Wr.; conidial state *Fusarium moniliforme* (Sheld.) emend. Snyder & Hansen was used. A culture of the organism, Strain 917, was kindly supplied by Professor P. W. Brian.

Chemicals. Sodium ^{14}C -1-acetate, specific activity 7.23 mc./mm. (88.1 $\mu\text{c.}/\text{mg.}$), was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Unlabelled GA was obtained from L. Light & Co. Ltd. (Slough).

Medium and growth of Gibberella fujikuroi

Medium. The following medium was used for the growth of the fungus before transfer of mycelium to samples of labelled substrate (% w/v): glucose 10; KH_2PO_4 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2; NH_4NO_3 0.24. The substances were dissolved in distilled water, and to every litre of culture solution 2 ml. of the following minor element concentrate were added (g.): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.59; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.075; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.059; $(\text{NH}_4)_2\text{MoO}_4$ 0.079, dissolved in 500 ml. distilled water, with a few drops of HCl added to clear the solution. (The medium is essentially the same as one recommended by Professor P. W. Brian.) A standard volume (100 ml.) of culture solution was used for the preparation of samples of mycelium. Except where otherwise stated, the labelled medium was identical in composition with that described above, except that acetate replaced glucose as the only carbon source. Medium and apparatus were autoclaved at 120° for 5 min.

Culture apparatus. The cultures were aerated. The air was first passed through concentrated H_2SO_4 , and then through sterile water to remove acid fumes and to humidify the air stream. In experiments where CO_2 was collected, the air was passed through a soda-lime tower before entering the H_2SO_4 , and after escaping from the culture vessel was allowed to bubble through 0.2N-NaOH held in Pettenkofer tubes of 20 ml. capacity. Different types of culture vessels have been used, but the most satisfactory was a round-base tube, 22 cm. long and 3 cm. diameter, held vertically, and provided with a ground joint and glass cap. The air passed through a narrow tube sealed into the cap, and bubbled into the culture from a simple orifice near the base of the vessel. Another tube sealed into the cap provided the outlet for the air stream. In a preliminary experiment air was passed into the culture from a sintered-glass bubbler, but the fungus grew on the sinter and eventually cut off the air supply. The culture vessels and the flask of sterile water were kept in a water bath at 25° .

Inoculation and growth. Subcultures were maintained on nutrient agar slopes. At the time of inoculation of the main culture about 1 ml. sterile water was added to one of the subcultures, and hyphae dislodged from the surface into the water with a sterile loop. The suspension of hyphae was poured into the main culture vessel. In one experiment samples (1 or 2 ml.) of culture liquor were withdrawn from time to time after inoculation, and any GA present was extracted and assayed as described below. The fungus was found to be producing GA at a rate of about 8 mg./l.

culture/day, from the 9th to the 14th days (Fig. 1). On subsequent occasions cultivation was discontinued some 14 days from the time of inoculation, so that samples of mycelium, known to be producing GA actively, could be prepared for transfer to samples of labelled medium. In each case a sample of the culture was withdrawn and the presence of GA confirmed before the transfer was made.

Preparation of samples of mycelium for transfer to labelled media. The unlabelled culture was filtered on a Büchner funnel through a 7 cm. diam. Whatman No. 50 filter paper which had previously been subdivided with pencil lines into a convenient number of segments. After filtration the paper and mycelial mat were turned over and cut along the pencil lines with sterile scissors. Each sample of mycelium obtained in this way was used to inoculate a quantity of labelled medium comparable in volume to that from which it was derived. For example, half of the mat was used to inoculate 50 ml. fresh medium, a quarter, 25 ml., and so on. Segments not used immediately were stored at 6° in sterile tubes for use later. A segment stored in this way for 29 weeks liberated about $140\text{ }\mu\text{g}$. GA in 72 hr after being transferred to 10 ml. of medium, but in the experiments reported here much shorter times of storage were used. These operations were carried out in a sterile room.

Measurement of respiration and estimation of specific activity of $^{14}\text{CO}_2$

A study was made of the rate of liberation of $^{14}\text{CO}_2$ from cultures to obtain some measure of the rate of utilization of the labelled substrate. CO_2 trapped in the NaOH was precipitated as BaCO_3 by shaking with excess 0.05 M- BaCl_2 solution. The precipitate was removed by centrifugation, shaken with distilled water, and again centrifuged. The mass of BaCO_3 precipitate (and from this the amount of CO_2 collected) was determined by titrating the excess alkali contained in measured samples of the bulked supernatant liquid against HCl of known strength (about 0.01 N), with phenolphthalein as indicator. A sample of the BaCO_3 precipitate was suspended in water and transferred to a 20 mm. diameter disc of Whatman No. 42 filter paper by using the apparatus described by Calvin *et al.* (1949). Ethanol and then chloroform were passed through the sample and the dry disc of BaCO_3 , supported by filter paper, was stored in a CO_2 free atmosphere ready for counting with a thin-window Geiger-Müller tube; counts were taken at infinite thickness. The product of the mass of BaCO_3 in the whole precipitate and the counts/min. of the samples was used to obtain an estimate of the radioactivity of the whole precipitate, and hence of the evolved CO_2 . This product is referred to as the 'relative counts'. It is an important property of this estimate that it is independent of any contamination of the air stream by extraneous unlabelled CO_2 .

The extraction and assay of gibberellic acid

The methods used for extracting GA were largely based upon those described by Curtis & Cross (1954), Borrow *et al.* (1955) and Brian *et al.* (1955). Small volumes of culture (less than 10 ml.) were filtered, adjusted to pH 2.8–3.0 with dilute HCl, and extracted three times by shaking with equal volumes of ethyl acetate. The lower phase was discarded, and the bulked upper ethyl acetate-rich phase evaporated *in vacuo* at not more than 30° to leave a solid residue. The crude extract was then either taken up in 30% (v/v) ethanol in water and assayed for GA in the manner described below, or transferred in ethyl acetate or acetone to chromatography

paper. Larger volumes of culture (10 to 100 ml.) were filtered, adjusted to pH 2.8–3.0 with dilute HCl, and shaken with activated carbon in the ratio of 2 g. carbon to 100 ml. culture. The carbon was removed on a Büchner funnel and shaken with acetone. The acetone + carbon mixture was filtered, and the dry carbon shaken with a small volume of distilled water and again shaken with acetone; this was repeated a third time. The bulked extract was evaporated *in vacuo* to an aqueous residue, which was adjusted to pH 2.8–3.0 and extracted three times with an equal volume of ethyl acetate. The whole extraction procedure was repeated three times, and the bulked ethyl acetate extract was evaporated to a small volume *in vacuo* and transferred to chromatography paper.

Chromatography. This was done by the descending technique, the solvent being the upper phase of a mixture of 3 vol. *n*-butanol + 1 vol. aqueous 1.5 N-NH₄OH, unless stated otherwise. Spots of GA were detected either by their blue-green fluorescence under ultraviolet (u.v.) radiation after treatment with a mixture of ethanol + sulphuric acid (Curtis & Cross, 1954; Radley, 1956), or, along with other organic substances, by dipping in 0.4 % KMnO₄ and washing in tap water (Bird & Pugh, 1958). On other occasions spots or bands of GA were eluted in 'elution tubes' (Canny, 1960). In earlier work acetone was used as a solvent for elution, but it was later found that a mixture of acetone and water was more effective. The acetone was evaporated away, and the aqueous residue extracted with ethyl acetate. Radioactive spots were detected by standard autoradiographic technique, or by scanning with an automatic scanning device.

Assay by the use of dwarf pea seedlings. This test was used as a routine bioassay for gibberellin. The method was essentially that described by McComb & Carr (1958) except that seedlings of the dwarf pea variety 'Meteor' were raised in a greenhouse from seed sown in John Innes' No. 2 compost (without fertilizer) instead of perlite.

Assay using Callitriche rosettes. Callitriche rosettes elongate strongly after treatment with GA (McComb, 1959), and unknown amounts of GA in solution may be assayed through the response of this plant. Rosettes of Callitriche were cut from their parent shoots and placed with their upper surfaces downwards into 0.2 ml. of aqueous solution to be assayed, ten replicates/treatment. After at least 12 hr the rosettes were transferred to tap water and allowed to grow for 3 days under continuous illumination. The shoots were measured, and an estimate of the amount of GA present in the experimental solution made by reference to the response of shoots treated with known amounts of GA. The test does have some advantage over the pea assay in that no initial measurement need be taken, and the time of assay is relatively short. However, the response of the rosettes is quite variable, the test is not useful over a wide range of concentrations, and plant material can be difficult to maintain. For these reasons the pea assay is to be preferred as a routine method.

RESULTS

The effects of glucose and anaerobic conditions on the production of labelled gibberellic acid

The possibility was investigated that the amount of ¹⁴C incorporated into GA would be increased if unlabelled glucose were provided at the same time as labelled acetate (so as to act as an alternative carbon source), or if the fungus were grown

under anaerobic conditions from the time of transfer to labelled medium. Three test tubes, A, B, and C, were used as culture vessels. A 10 ml. sample of medium containing 10 μC . labelled acetate/ml. was added to each tube. Glucose (0.1 g.) was added to tube B. Each tube was inoculated with a similar sample of mycelium taken from a culture of the fungus known to be actively producing GA. Tubes A and B were aerated, but pure nitrogen instead of air was passed into tube C. After 43 hr a 1 ml. sample was withdrawn from each vessel; the experiment was terminated at 88 hr after inoculation, when further 1 ml. samples were withdrawn. All the samples were extracted and chromatographed on Whatman No. 1 paper. Autoradiographs of the chromatograms revealed apparently identical results for the two sets of samples withdrawn at 43 and 88 hr. In all cases spots could be seen at the same R_F as known GA, but it was clear that the samples from tube A had much more labelled carbon at this R_F than those from the other tubes (Pl. 1, fig. 1). The chromatograms were then dipped in ethanolic sulphuric acid and examined under u.v. radiation, when the greenish fluorescence characteristic of GA could be seen in all cases, at the same R_F as known GA.

The GA present in tube A was extracted with activated carbon and purified by chromatography. Assay with Callitriche revealed that the culture contained about 30 μg . of GA. Samples of the ^{14}C -GA were chromatographed in six different solvent systems, including that used above, selected from the work of Radley (1956), Takahashi *et al.* (1956), Van Overbeek, Racusen, Togami & Hughes (1957) and Phinney, West, Ritzel & Neely (1957). In each case only one spot of labelled compound was detected by autoradiography, coincident in R_F with a spot of known GA.

The GA in tubes B and C was extracted with ethyl acetate, and assayed by using dwarf pea seedlings. B was estimated to contain 34 and C 26 μg of GA. Clearly the most satisfactory method of the three for producing ^{14}C -GA was that in which labelled acetate was provided as the only carbon source in aerated culture. CO_2 was collected during the experiment, and it was found that most of the $^{14}\text{CO}_2$ produced by the fungus was evolved during the first 24 hr of cultivation. It seemed possible that most of the ^{14}C -GA might also be produced during that time. It was important that the ^{14}C -GA obtained should have a specific activity high enough to be of use in physiological experiments. A simple test was made to see whether ^{14}C could be detected in Callitriche plants after they had been treated with the ^{14}C -GA isolated from the culture of tube A. A number of rosettes were each treated with about 2 μg . labelled GA in the same way as in the assay test, and were then washed and allowed to grow in tap water. The plants were dissected, pressed as herbarium specimens at different times after treatment and autoradiographs taken of the dried specimens. Radioactivity was detected (Pl. 1, fig. 2) and it was concluded that ^{14}C -GA of this specific activity could be used in physiological work.

The rate of formation of ^{14}C -gibberellic acid

An experiment was made to determine the rate of production of ^{14}C -GA, and to see whether this was related to the production of $^{14}\text{CO}_2$. One sample of 10 ml. of culture was used, containing ^{14}C -acetate, 5 μC ./ml. and this was inoculated as described. The apparent respiration rate remained fairly constant, some variation being introduced because the culture vessel was opened from time to time to with-

draw samples (Table 1). On the other hand, the rate of evolution of $^{14}\text{CO}_2$ was far from constant (Fig. 2).

Samples of medium were withdrawn from time to time, at first of 1 ml. but later 0.5 ml. These were extracted with ethyl acetate and chromatographed. Autoradiographs revealed spots at the same R_F as known GA, and these were counted with a Geiger tube. Counts were adjusted to the same sample size and plotted against the time of sampling (Fig. 3a). When the experimental data were plotted against the

Fig. 1

Fig. 2

Fig. 1. Estimates of the amount of gibberellic acid present in a culture at different times after inoculation.

Fig. 2. The evolution of $^{14}\text{CO}_2$ from a culture of *Gibberella fujikuroi* containing ^{14}C -acetate. Samples were collected during the times indicated by the horizontal lines. The relative count of each sample was divided by the sample time $\times 10^4$, to give an estimate of the relative rate of $^{14}\text{CO}_2$ production.

Table 1. Estimates of the rate of production of CO_2 at different times, following the transfer of mycelium from a culture of *Gibberella fujikuroi* containing glucose to one containing acetate as the only carbon source

Time from inoculation (hr.)	mg. CO_2 evolved/hr./ml. culture	Time from inoculation (hr)	mg. CO_2 evolved/hr./ml. culture
0-6	0.12	34-43	0.16
6-12	0.10	43-70	0.08
12-21	0.10	70-94	0.11
21-28	0.13	94-118	0.08
28-34	0.12	118-138	0.14

logarithm of the time of sampling, they were found to approximate to a straight line with a correlation coefficient of 0.97. The curve shown in Fig. 3a was calculated from the regression line, as was a curve showing the rate of labelled GA formation (Fig. 3b). It can be seen that most of the GA was produced during the first 48 hr, and the amount produced after about 90 hr was negligible. The spots of labelled GA were eluted, and the eluates assayed with dwarf-pea seedlings. From the assay data and the counts/min. recorded from the chromatograms, estimates were made of the relative specific activities of the various samples (Table 2). The results,

though variable, suggest that an increase in the specific activity of the GA took place with time. It seemed possible that higher specific activity might be achieved if the fungus were placed in a labelled medium for some hours, and then transferred to a second batch of labelled medium.

The effect of transferring mycelium to a second batch of labelled substrate

Mycelium derived from 50 ml. of unlabelled culture was used to inoculate 50 ml. of culture containing 0.5 mc. labelled acetate (medium A). Six hr later the mycelium was transferred to another 50 ml. of culture containing 0.5 mc. labelled acetate

Fig. 3(a). Relative amounts of ^{14}C -gibberellic acid present in a culture of *Gibberella fujikuroi* containing ^{14}C -acetate. The estimates are based on counts/min. recorded from chromatograms at the R_F of known gibberellic acid.

Fig. 3(b). An estimate of the rate of production of ^{14}C -gibberellic acid, based on the data shown in Fig. 3(a). The rate has been calculated as the increase in number of counts/min. with each hour of incubation.

Table 2. Estimates of the amount and relative specific activity ^{14}C -gibberellic acid present in a culture of *Gibberella fujikuroi* at different times after inoculation

Time after inoculation (hr)	Amount of gibberellic acid ($\mu\text{g./ml. culture}$)	Relative specific activity*
6	62	0.3
12	39	1.0
21	49	1.0
28	50	1.3
34	108	0.6
43.2	60	1.5
69.7	70	1.3
138	25	4.3

* $\frac{\text{Counts/min. at } R_F \text{ of gibberellic acid}}{\text{Mass of gibberellic acid on chromatogram}}$

(medium B). A 2 ml. sample was withdrawn from medium A, which was then inoculated with a further sample of hyphae, derived from 50 ml. of culture. The relative counts of $^{14}\text{CO}_2$ evolved from the three cultures are shown in Fig. 4. As comparatively little was evolved from medium A after the second inoculation, it is assumed that most of the ^{14}C -acetate was taken up by the fungus during the first inoculation of that medium.

The incubation was discontinued 120 hr after the first inoculation of medium A, and 2 ml. samples were withdrawn from the culture media. Thus, three samples were available for study: A, withdrawn from medium A when mycelium was transferred to medium B; B, withdrawn from medium B at the end of the experiment; and A¹, withdrawn from medium A at the end of the experiment. Ethyl acetate

Fig. 4. The evolution of ¹⁴CO₂ from cultures of *Gibberella fujikuroi* containing ¹⁴C-acetate. Samples were collected during the times indicated by the horizontal lines. The relative counts of each sample was divided by the sample time $\times 10^7$. At the time indicated by the broken line, the mycelium in one medium, (A), was transferred to a second medium, (B). Medium A was then inoculated with a further sample of mycelium, and the ¹⁴CO₂ evolved from the culture is represented by the line, (A¹), which begins at the time of this inoculation.

extracts of the samples were chromatographed, and the chromatograms scanned. Radioactivity was estimated by measuring the areas under the traces at the R_F of known GA. The GA spots were eluted and assayed in the same batch of assay plants; the results are given in Table 3. It is clear that sample B contained the most GA, and also GA of the highest specific activity. The lower specific activity of the GA

in sample A_1 is attributed to the introduction of unlabelled GA (or GA precursors) at the time of the first inoculation. A further decrease in specific activity accompanied the introduction of more mycelium into medium A (sample A_2). In addition, there was less radioactivity in A_2 than in A_1 , suggesting that some of the ^{14}C -GA present in A had disappeared. It is possible that during the period without an external carbon source (e.g. glucose, acetate) some of the GA present in the medium was broken down by the fungus. Samples of the assayed sample B were counted on 5 mm. squares of filter paper, and the count rate compared with that of a known amount of ^{14}C -acetate under the same counting conditions. The specific activity of the labelled GA was determined as about $4 \mu\text{c./mg.}$ The ^{14}C -GA present in medium B was extracted with activated carbon, and has been used in experiments with higher plants.

Table 3. *Estimates of the amount and relative specific activity of ^{14}C -GA present in chromatograms of extracts from cultures of *Gibberella fujikuroi**

Sample (2 ml.)	Mass gibberellic acid in sample ($\mu\text{g.}$)	Radioactivity.	Relative specific activity. (Area divided by mass)
		Area under trace drawn by scanning machine (mm. ²)	
A_1	4.9	328	67
B	14.1	1149	82
A_2	7.3	192	26

DISCUSSION

The ^{14}C -gibberellic acid obtained by the method described has a specific activity high enough to allow studies to be made on the distribution and breakdown of GA in higher plants, but further increase in specific activity would be advantageous. This might be achieved by simply supplying more labelled acetate, and also by supplying acetate of higher specific activity, labelled in both the methyl and carboxyl groups. The rates of production of $^{14}\text{CO}_2$ and ^{14}C -GA had fallen to a relatively low value within 40 hr after the transfer of mycelium to the labelled substrate; this can be attributed to exhaustion of the supply of labelled carbon in the medium. After this time respiration continued, the unlabelled CO_2 being derived from carbon reserves which had accumulated in the mycelium before transfer (Borrow *et al.* 1961). Since the specific activity of the GA did not fall with time (Table 2), it is assumed that GA was not produced from these unlabelled reserves. This is important as far as the production of ^{14}C -GA is concerned, for any further diminution in specific activity would be undesirable. It seems that carbon derived from reserves within the mycelium passed into the pathways of 'endogenous respiration' (Cochrane, 1958) rather than into those of GA synthesis.

I wish to record my debt to Dr M. J. Canny for advice and encouragement during the course of these experiments. Thanks are also due to Professor P. W. Brian, F.R.S. for the gift of a culture of *Gibberella fujikuroi*, and for information about the growth of the organism. This work was carried out during the tenure of an Overseas Scholarship awarded by the Royal Commission for the Exhibition of 1851, and forms part of the requirements for the Degree of Doctor of Philosophy at the University of Cambridge.

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Fig. 1

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Fig. 2

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EXPLANATION OF PLATE

Fig. 1. Autoradiographs from chromatograms of extracts taken from cultures of *Gibberella fujikuroi* containing ^{14}C -acetate 48 hr after inoculation. A: Aerated culture containing ^{14}C -acetate as sole carbon source. B: Aerated culture containing unlabelled glucose and ^{14}C -acetate. C: Culture containing ^{14}C -acetate as sole carbon source. Nitrogen instead of air was bubbled through the medium. SL: Start line. GA: R_f of known gibberellic acid. SF: Solvent front. Exposure time 7 days.

Fig. 2. Autoradiographs from *Callitriche* plants treated with ^{14}C -gibberellic acid. Rosettes were each treated for 12 hr with about 2 $\mu\text{g.}$ of ^{14}C -gibberellic acid, and transferred to tap water. The autoradiographs shown above are from plants harvested (A) 24 hr, (B) 72 hr after transfer. Radioactivity at the leaf tips is in the region of the hydathodes. Exposure time 35 days.