Phytochemistry, 1968, Vol. 7, pp. 1923 to 1931. Pergamon Press. Printed in England

BIOLOGICAL AND CHROMATOGRAPHIC PROPERTIES OF TWO GIBBERELLIN-LIKE COMPOUNDS FROM ETIOLATED *PHASEOLUS MULTIFLORUS* SEEDLINGS

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(*Received* 11 *May* 1968)

Abstract—Two gibberellin-like compounds, Phaseolus I and Phaseolus II, were detected in extracts of etiolated *Phaseolus multiflorus* seedlings. Phaseolus I shows activity in the barley endosperm and lettuce bioassays. Phaseolus II also is active in the barley endosperm assay but induces only a marginal response in the lettuce bioassay. Both Phaseolus I and II have thin-layer chromatographic properties similar to gibberellins A_1 and A_3 from which they can be separated by column partition chromatography. Although it is impossible at this time to identify either Phaseolus I or Phaseolus II, it appears that they are different from at least nineteen of the twenty-three gibberellins at present characterized.

INTRODUCTION

THE IMMATURE seed of *Phaseolus multiflorus* is one of the most productive sources of gibberellins. MacMillan *et al.*[1,2] identified gibberellins A_1, A_5, A_6 and A_8. Cavell *et al.*,[3] also detected gibberellins A_1, A_5, A_6 and A_8, in addition to the possible presence of gibberellins A_4 and A_{13}. Later they used combined gas chromatography–mass spectrometry to confirm the presence of gibberellins A_1, A_4, A_5, A_6 and A_8 and to show that what had previously been thought to be gibberellin A_{13} was in fact an isomer of A_{13}.[4] The isomer was subsequently named gibberellin A_{17}.[5] More recently, gibberellin A_{19} (bamboo gibberellin) was identified in extracts of immature *P. multiflorus* seed,[6] and what was originally thought to be A_4 was shown to be gibberellin A_{20} (Pharbitis gibberellin).[7]

Sembdner and co-workers[8] found gibberellins A_1, A_5, A_6 and A_8 in both pod and seed extracts, and gibberellin A_3 in pod extracts. They also detected five gibberellin-like substances. "Phaseolus α to Phaseolus ϵ", all more polar than gibberellin A_3. "Phaseolus ϵ" has recently been identified as gibberellin A_8-glucoside.[9] Jones[10] found gibberellins A_1, A_3, A_5, A_6 and A_8, plus two unknown gibberellin compounds. Although present throughout the seed, 80 per cent of the total gibberellin was concentrated in the seed coat. Jones made a further comparison between gibberellin content of seeds of different sizes and hence at different stages of maturity and found that there was an apparent sequential production of gibberellins as the seed matured.

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1 J. MACMILLAN, J. C. SEATON and P. J. SUTER, *Tetrahedron* **11**, 60 (1960).
2 J. MACMILLAN, J. C. SEATON and P. J. SUTER, *Tetrahedron* **18**, 349 (1962).
3 B. D. CAVELL, J. MACMILLAN, R. J. PRYCE and A. C. SHEPPARD, *Phytochem.* **6**, 867 (1967).
4 J. MACMILLAN, R. J. PRYCE, G. EGLINTON and A. MCCORMICK, *Tetrahedron Letters* 2241 (1967).
5 R. J. PRYCE and J. MACMILLAN, *Tetrahedron Letters* 4173 (1967).
6 R. J. PRYCE and J. MACMILLAN, *Tetrahedron Letters* 5009 (1967).
7 J. MACMILLAN and R. J. PRYCE, *Tetrahedron Letters* 1537 (1968).
8 G. SEMBDNER, G. SCHNEIDER, J. WEILAND and K. SCHREIBER, *Experientia* **15**, 83 (1964).
9 K. SCHRIEBER, J. WEILAND and G. SEMBDNER, *Tetrahedron Letters* 4285 (1967).
10 D. F. JONES, *Nature* **202**, 1309 (1964).

In the light of this work on the seed it was decided to examine the native gibberellins of the *P. multiflorus* seedling. The present paper reports the biological and chromatographic properties of two gibberellin-like compounds extracted from etiolated seedlings.

RESULTS

Partition Column Chromatography

The acid ethyl acetate-soluble fraction, extracted from 1000 *Phaseolus multiflorus* seedlings, was chromatographed on a phosphate buffered celite column. Barley endosperm bioassays

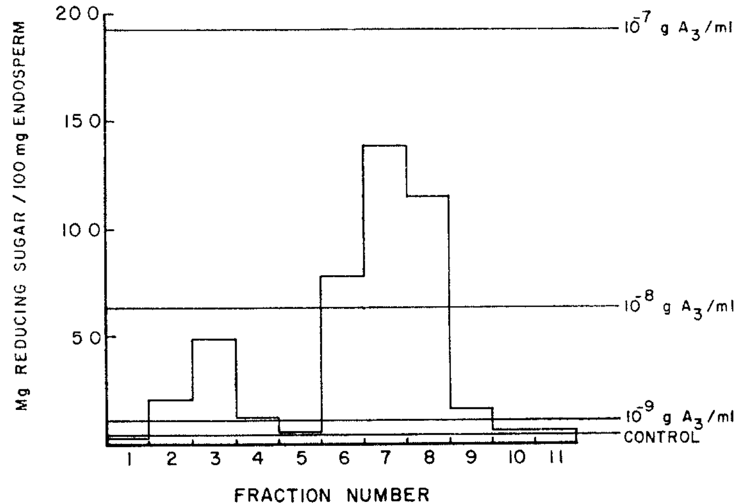

FIG. 1. BARLEY ENDOSPERM BIOASSAY OF A PARTITION COLUMN CHROMATOGRAM OF THE ACID FRACTION EXTRACTED FROM 1000 ETIOLATED *Phaseolus multiflorus* SEEDLINGS.

Fractions diluted 37·5 fold prior to bioassay. Elution pattern of standard gibberellins: fraction 1, A_4, A_7 and A_9; fractions 2–3, A_5; fraction 9, A_1; and fraction 11, A_3 and A_8.

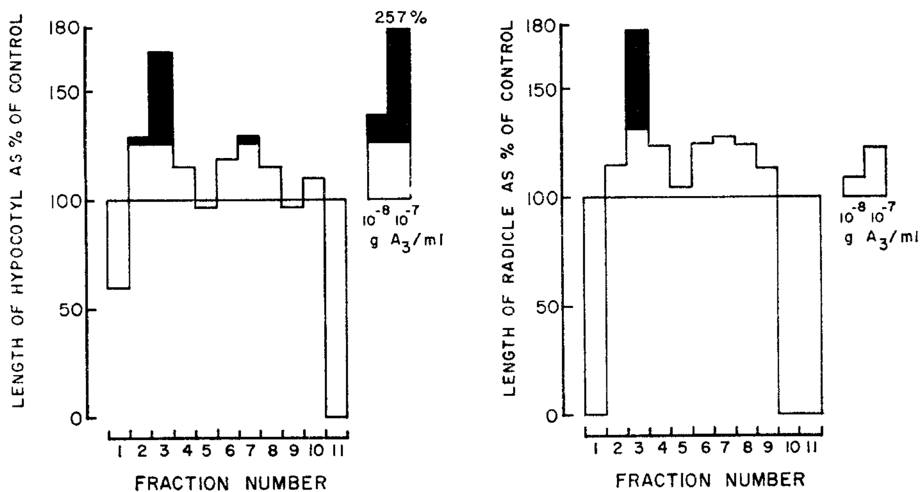

FIG. 2. LETTUCE BIOASSAY OF A PARTITION COLUMN CHROMATOGRAM OF THE ACID FRACTION EXTRACTED FROM 1000 ETIOLATED *Phaseolus multiflorus* SEEDLINGS.

Fractions diluted 15 fold prior to bioassay. Shaded portions of the histograms indicate stimulation. above controls that is significant at the 5 per cent level

of the successive eluates from the column, revealed two distinct areas of gibberellin-like activity (Fig. 1). The peak centered over fraction 3 has been designated "Phaseolus I" and the peak over fraction 7, "Phaseolus II". The elution pattern of available standard gibberellins from the celite column is given under Fig. 1. When the eluates were further tested with the lettuce bioassay the same peaks of activity were apparent (Fig. 2). However, whereas Phaseolus I strongly promoted both hypocotyl and radicle growth, Phaseolus II induced no more than a slightly significant response in either system. Estimates on the quantities of the two gibberellin-like substances present in the extract are presented in Table 1.

TABLE 1. LEVELS OF ENDOGENOUS GIBBERELLIN-LIKE SUBSTANCES EXTRACTED FROM ETIOLATED *Phaseolus multiflorus* SEEDLINGS*

	μg A_3 equivalents/1000 plants*	
Bioassay system	Phaseolus I	Phaseolus II
Barley endosperm	0·27	3·6
Lettuce hypocotyl	0·25	—

* Estimates are based on barley endosperm and lettuce hypocotyl bioassays of extracts eluted from a phosphate buffered partition column chromatogram.

Thin-Layer Chromatography

Following their separation on a celite column, Phaseolus I and II were rechromatographed using five different thin-layer chromatogram solvent systems (see Table 2). In all five, both Phaseolus I and Phaseolus II had mobilities that were either equivalent to or only slightly more polar than the mobilities of gibberellins A_1 and A_3 (Fig. 3A and B, Table 2).

TABLE 2. THIN-LAYER CHROMATOGRAM R_f VALUES OF PHASEOLUS I AND PHASEOLUS II

	R_f value in solvent system*				
Compound	1	2	3	4	5
Phaseolus I	0·0–0·1	0·0–0·1	0·3–0·4	0·5–0·6	0·4–0·5
Gibberellin A_1	0·07–0·12	0·0–0·02	0·34–0·42	0·57–0·64	0·42–0·56
Gibberellin A_3	0·06–0·10	0·0–0·02	0·34–0·40	0·56–0·61	0·30–0·36
Phaseolus II	0·0–0·1	0·0–0·1	0·3–0·4	0·5–0·6	0·4–0·5
Gibberellin A_1	0·08–0·11	0·0–0·02	0·32–0·39	0·61–0·72	0·47–0·57
Gibberellin A_3	0·06–0·09	0·0–0·02	0·32–0·37	0·62–0·69	0·36–0·44

* Solvent: 1, Di-isopropyl ether/acetic acid (95:5)[a]; 2, carbon tetrachloride/acetic acid/water (8:3:5), equilibriate in upper phase for 36 hrs, develop in lower phase[b]; 3, benzene/*n*-butanol/acetic acid (80:15:5)[b]; 4, benzene/*n*-butanol/acetic acid (75:20:5)[b]; and 5, carbon tetrachloride/acetic acid/water (8:3:5), equilibriate in upper phase for 36 hr, develop in lower phase plus 20% ethyl acetate[b]. Solvents 1 to 4 were run on silica gel G, solvent 5 on kieselguhr G.

[a] J. MACMILLAN and P. J. SUTER, *Nature* **197**, 790 (1963).

[b] T. KAGAWA, T. FUKINBARA and Y. SUMIKI, *Agr. Biol. Chem. (Tokyo)* **27**, 598 (1963).

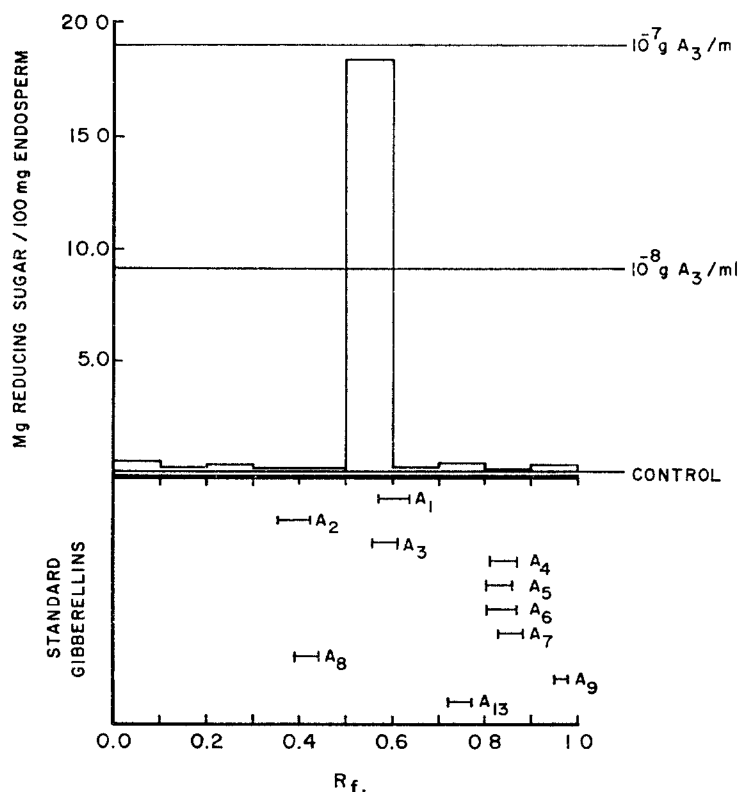

FIG. 3. (A) BARLEY ENDOSPERM BIOASSAY OF "PHASEOLUS I" FOLLOWING CHROMATOGRAPHY ON A THIN LAYER OF SILICA GEL G WITH SOLVENT SYSTEM 4.

Unsuccessful attempts were made to detect Phaseolus I and II on thin-layer chromatograms as fluorescent spots after heating with ethanol/sulphuric acid, using the methods of MacMillan and Suter.[11] As fluorescence, particularly on chromatograms developed in solvent system 5, was usually limited to the point of origin, possible explanations for this failure are that the extracts did not contain sufficiently large quantities of Phaseolus I or II for visual detection and/or that Phaseolus I and II give only weakly fluorescent reactions.

DISCUSSION

Although one can eliminate many of the characterized gibberellins, it is not possible at this stage to identify either Phaseolus I or Phaseolus II. Comparisons of mobilities on partition columns and thin-layer chromatograms reveal that neither Phaseolus I or II is identical with gibberellins A_1, A_2, A_3, A_4, A_5, A_6, A_7, A_8, A_9 or A_{13}. Although the *Phaseolus* seedling gibberellins have thin-layer chromatographic properties very similar to those of gibberellins A_1 and A_3, they behave differently on a phosphate buffered celite column. Phaseolus I appears in fractions 3, Phaseolus II in fraction 7 whereas gibberellins A_1 and A_3 appear in fractions 9 and 11 respectively (Fig. 1).

[11] J. MACMILLAN and P. J. SUTER, *Nature* **197**, 790 (1963).

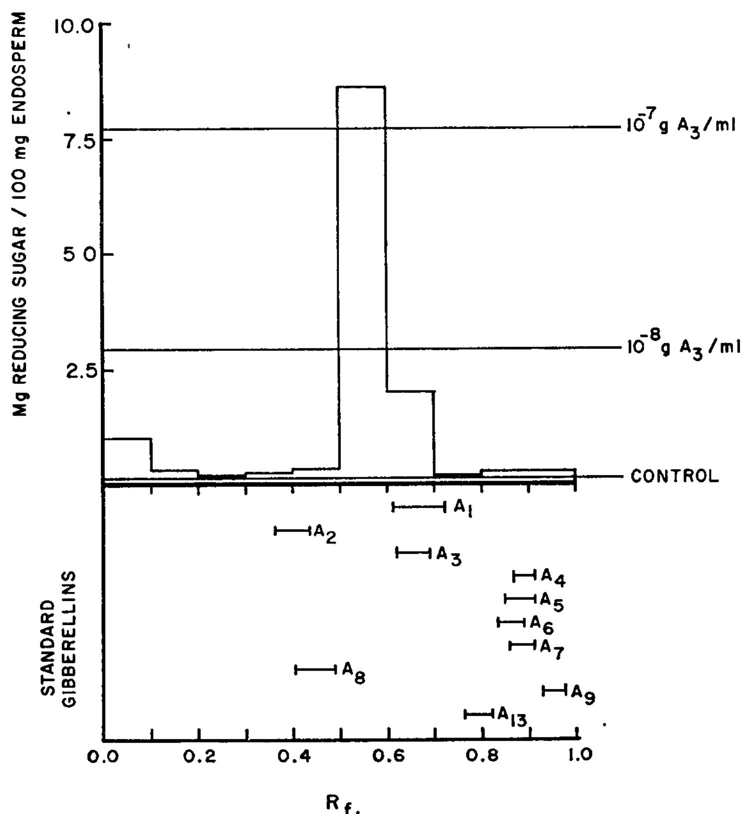

FIG. 3. (B) BARLEY ENDOSPERM BIOASSAY OF "PHASEOLUS II" FOLLOWING CHROMATOGRAPHY ON A THIN LAYER OF SILICA GEL G WITH SOLVENT SYSTEM 4.

Attempts to make a comparison between the properties of Phaseolus I and II and those of other characterized gibberellins were hampered because the latter were not available for use as standards. However, one can go some way towards it by drawing on published data.

Thus an examination of Table 3 reveals that gibberellins A_{10}, A_{11}, A_{12}, A_{14}, A_{15}, A_{18}, A_{20} and A_{23} have thin-layer chromatographic properties distinctly different from those of Phaseolus I and II. Tamura *et al.*[12] quote gibberellins A_{21} and A_{22} as having R_f's of 0·2 and 0·3 in solvent system 4 (Table 2). Unfortunately those values were not compared with those of gibberellin A_3. Both gibberellins of the *Phaseolus* seedling move to a position similar to that of A_3 in this solvent system, and the R_f cited for A_3 is 0·54.[13] This value will no doubt vary from chromatogram to chromatogram, but it is likely that A_{21} is much too polar a molecule to be either Phaseolus I or II. The properties of gibberellins A_{21} and A_{22} on a celite column and in the barley endosperm and lettuce assay systems are unknown. Comparisons of Phaseolus I and II with gibberellin A_{16}, which has been isolated only in the form of a methyl ester from *Gibberella fujikuroi*,[14] and gibberellin A_{17}, which was detected in immature *P. multiflorus* seed extracts and characterized by gas chromatography–mass spectrometry,[5] are impossible because of a complete lack of suitable data. Gibberellin A_{19}

[12] S. TAMURA, N. TAKAHASHI, N. MUROFUSHI, T. YOKOTA, J. KATO and Y. SHIOTANI, *Planta* **75**, 279 (1967).
[13] T. KAGAWA, T. FUKINBARA and Y. SUMIKI, *Agr. Biol. Chem.* (*Tokyo*) **27**, 598 (1963).
[14] R. H. B. GALT, *Tetrahedron* **24**, 1337 (1968).

behaves in a manner very similar to that of Phaseolus I and II in solvent systems 1, 2 and 4. Unfortunately the similarity stops here, as there is no published information on the mobility of gibberellin A_{19} on a celite column or of its activity in the barley endosperm and lettuce bioassays.

TABLE 3. THIN-LAYER CHROMATOGRAM MOBILITIES OF PHASEOLUS I, PHASEOLUS II AND CHARACTERIZED GIBBERELLINS

Compound	Mobility in solvent system* 1 (R_f)	2† (R_f)	3 (R_f or RA_3)
Gibberellin			
A_3	0·06[a]	0·00[a]	R_f 0 54[b]
A_{10}	0·06[a]	0·36[a]	—
A_{11}	0·49[a]	1·00[a]	—
A_{12}	0·67[a]	1·00[a]	—
A_{14}	0·26[a]	0·75[a]	—
A_{15}	0·44[a]	1·00[a]	—
A_{16}	—	—	—
A_{17}	—	—	—
A_{18}	0·04[a]	0 02[a]	RA_3 0·68[cd]
A_{19}	0·08[a]	0·00[a]	RA_3 0·98[e]
A_{20}	—	—	RA_3 1 60[f]
A_{21}	—	—	R_f 0·2[g]
A_{22}	—	—	R_f 0·3[g]
A_{23}	—	—	RA_3 0 68[d]
Phaseolus I	0·0–0·1	0·0–0·1	0 5–0·6
Phaseolus II	0·0–0·1	0·0–0 1	0·5–0·6
A_3	0·09	0·00	0 62

* See Table 2.

† Characterized gibberellins run in benzene/acetic acid/water (8:3:5), Phaseolus I and II run in carbon tetrachloride/acetic acid/water (8:3:5).

[a] B. D. CAVELL, J. MACMILLAN, R. J. PRYCE and A. C. SHEPPARD, *Phytochem.* **6**, 867 (1967).

[b] T. KAGAWA, T. FUKINBARA and Y SUMIKI, *Agr. Biol. Chem.* (*Tokyo*) **27**, 598 (1963).

[c] Y. OGAWA, *Bot. Mag.* (*Tokyo*) **79**, 197 (1966).

[d] K. KOSHIMUZU, M. FUKUI, M. INUI, Y. OGAWA and T. MITSUI, *Tetrahedron Letters* 1143 (1968).

[e] N. MUROFUSHI, S. IRIUCHIJAMA, N. TAKAHASHI, S. TAMURA, J. KATO, Y. WADA, E. WATANABE and T. AOYAMA, *Agr. Biol. Chem.* (*Tokyo*) **30**, 917 (1966).

[f] N. TAKAHASHI, N. MUROFUSHI, T. YOKOTA and S. TAMURA, *Tetrahedron Letters* 1065 (1967).

[g] S. TAMURA, N. TAKAHASHI, N. MUROFUSHI, T. YOKOTA, J. KATO and Y. SHIOTANI, *Planta* **75**, 279 (1967).

Thus comparisons of the biological and chromatographic properties reveal that neither Phaseolus I nor Phaseolus II is identical with nineteen of the twenty-three gibberellins at present characterized. Lack of published information prevents similar comparisons with the remaining four.

EXPERIMENTAL

Plant Material

Seeds of *Phaseolus multiflorus* var. Prizewinner* were soaked in aerated running tap water at approximately 25° for 24 hr. The turgid seeds were then placed between sheets of dry filter paper and stored in darkness at 25°. 3 days later, seeds with adequate radicle growth were transferred to aerated water culture in total darkness. After 8 days, by which time the second internode had started to elongate, the seedlings were frozen with liquid N_2 and then lyophilized.

Extraction, Purification and Chromatography

The extraction and purification of gibberellin-like compounds from the plant material is outlined in the flow diagram of Fig. 4. The ethanolic extract was initially purified by running it through a column of cation exchange resin and then through a column of anion exchange resin.[10] Elution from the latter with formic acid gave acidic material which was further purified by the addition of a slurry of basic lead acetate.[15] The solution was filtered, the aqueous supernatant adjusted to pH 2·8 and then extracted with ethyl acetate. The acid ethyl acetate-soluble components were then taken to dryness and subjected to partition chromatography on a phosphate-buffered celite column.[16]

Eluates from the column were tested for gibberellin-like activity with the barley endosperm and lettuce bioassays. Fractions containing gibberellin-like activity were then rechromatographed on 0·25 mm layers of silica gel G and Kieselguhr G using the solvent systems listed in Table 2. Developed chromatograms were air-dried and the silica gel or Kieselguhr scraped off the plates in zones corresponding to 0·1 R_f units. Each zone was then eluted twice with 5 ml of wet ethyl acetate and once with 5 ml of methanol. The combined eluates were taken to dryness and dissolved in distilled water prior to being tested for gibberellin-like activity with the barley endosperm assay. For reference purposes standard gibberellins were run alongside extracts on the same chromatogram. After scraping off the chromatographed extracts the plates were sprayed with ethanol/H_2SO_4 (95:5), heated at 120°, the standard gibberellins thus appearing at the appropriate R_f, as fluorescent spots, visible under u.v. light (350 nm).[11]

Bioassays

Lettuce bioassay. Conditions were similar to those employed by Brian *et al.*[17] Lettuce seed cv. Arctic King† was placed on damp filter paper in 3-in. glass petri dishes and left in darkness at 25° for 36 hr. After this time, groups of ten seedlings at a uniform stage of germination, were placed in similar petri dishes containing filter paper dampened with 3 ml of test solution plus 0·1 ml of conc. nutrient solution.[18] After incubation at 25° for 3 days in a 16 hr photoperiod, length of hypocotyls and radicles[19] were measured, using a photographic enlarger. With each bioassay several control dishes were included, along with dishes containing known concentrations of gibberellic acid. The mean values per dish were calculated in mm and then expressed as a percentage of growth of the distilled water controls. Mean hypocotyl and radicle growth above the 0·05 probability level in "Students" *t* tests were regarded as significant stimulation.

Barley endosperm bioassay. The techniques used were basically those of Nicholls and Paleg,[20] and the barley used was the husked variety Cambrinus.‡ After dehusking with H_2SO_4 the seeds were washed and cut to remove the embryo. The endosperm pieces were weighed in groups of eight and placed in 2-in. glass petri dishes containing 3 ml of test solution plus 500 μg streptomycin sulphate. Incubation was for 30–34 hr at 30°. Following incubation the ambient solutions were assayed for reducing sugar content using the method of Somogyi.[21] Extracts were also tested for reducing sugar content prior to incubation, the results were invariably negative. Extracts were bioassayed in triplicate, and included with each test were at least ten distilled water controls and a range of standard concentration of gibberellin A_3. Reducing sugar values are expressed in mg produced per 100 mg of endosperm. With some bioassays the ambient solutions were tested for protein nitrogen[19] or α-amylase.[22] The results were qualitatively identical to those obtained from reducing sugar estimates.

* Purchased from Messrs. Carters Tested Seeds Ltd., Raynes Park, London, S.W.20.

† Purchased from Dickson, Brown and Tait Ltd., Timperley, Altrincham, Cheshire, U.K.

‡ Purchased from B. Finney and Sons, 94 Grainger Street, Newcastle upon Tyne 1, U.K.

[15] M. Radley, *Ann. Botany* **27**, 373 (1963).

[16] H. Kende and A. Lang, *Plant Physiol.* **39**, 435 (1964).

[17] P. W. Brian, H. G. Hemming and D. Lowe, *Ann. Botany* **28**, 369 (1964).

[18] N. A. Clark, *Plant Physiol.* **1**, 273 (1926).

[19] L. Paleg, D. Aspinall, B. Coombe and P. Nicholls, *Plant Physiol.* **39**, 286 (1964).

[20] P. B. Nicholls and L. G. Paleg, *Nature* **199**, 823 (1963).

[21] M. Somogyi, *J. Biol. Chem.* **195**, 19 (1952).

[22] R. L. Jones and J. E. Varner, *Planta* **72**, 155 (1967).

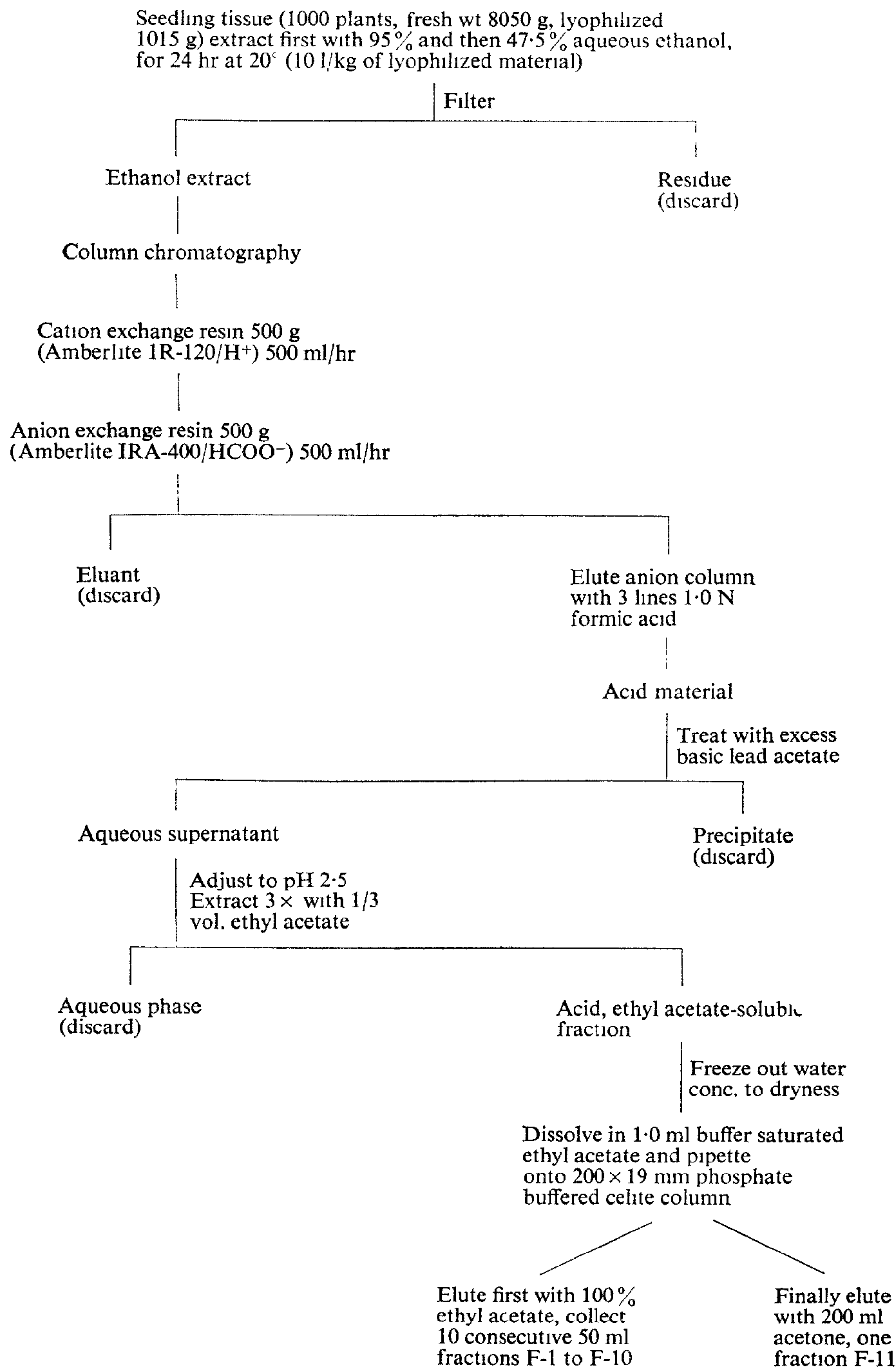

FIG. 4. FLOW DIAGRAM SHOWING THE PROCEDURE FOR EXTRACTION, PURIFICATION AND SEPARATION OF GIBBERELLINS FROM *Phaseolus multiflorus* SEEDLINGS.

Acknowledgements.—The majority of this data was accumulated while one of us (A. C.) was the holder of an S.R.C. studentship, however a portion of the work was supported by N.R.C. (Canada) Grant Number A-2585 to Dr. R. P. Pharis. We would like to thank Dr. D. Broadbent, I.C.I. Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, for the gift of samples of gibberellins A_1, A_4, A_5, A_7, A_8 and A_9; and Dr. R. P. Pharis, Biology Department, University of Calgary, for the use of his facilities and samples of gibberellins A_2, A_6 and A_{13}.