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ISOLATION OF GIBBERELLINS A_1, A_3, A_9 AND OF A FOURTH GROWTH SUBSTANCE FROM *ALTHAEA ROSEA* CAV.

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Abstract—From approximately 30 kg of shoot apices from vernalized plants of a biennial strain of *Althaea rosea* four biologically active substances, designated X_1, X_2, Y and Z, were isolated and crystallized. All four stimulate the elongation of *Avena* mesocotyl sections; three of them (X_1, X_2, Y) are active in the dwarf maize (d-3) test. X_1 and X_2 are also active in the lettuce hypocotyl test, and cause the germination of lettuce seeds (var. Grand Rapids) in total darkness, though X_1 is less effective than X_2. The data from physical analyses (mass spectrography, fluorescence, melting point) of these substances, together with the results of the various bioassays, show that the substances X_1, X_2, and Y are identical with gibberellins A_9, A_3 and A_1, respectively. Compound Z has not been identified chemically.

INTRODUCTION

IN RECENT years, a number of growth substances have been isolated from immature seeds or young fruits which are generally rich in these compounds (see review by Nitsch[1]). This has not been the case, however, with growing shoot apices, although they are known to contain a variety of endogenous growth factors. The main difficulty arises from the extremely low concentrations present in these tissues. Nevertheless, in 1959, Sumiki and Kawarada[2] isolated GA_1* from water sprouts of citrus trees, and very recently Tamura *et al.*[3] reported the isolation of a "bamboo gibberellin" from the bleaching water of 44 tons of bamboo shoots.

We have reported earlier (Harada and Nitsch[4]) the presence of several growth substances in the shoot apices of *Althaea rosea*, which undergo quantitative changes during the development of this plant. In particular, there is a marked increase after vernalization in the activity of one factor, called "substance E". Several biological and chemical properties of this substance have been determined but, due to lack of material, its exact chemical nature remained to be defined. The present paper concerns the identification of four of the substances extracted from the apices of *Althaea rosea*.

RESULTS

Four biologically active substances, designated X_1, X_2, Y and Z, were isolated and crystallized from the slightly acid, ethyl-acetate fraction, and three of them were subsequently identified.

* Abbreviation: GA_1 stands for gibberellin A_1, for example.

[1] J. P. NITSCH, *Handbuch der Pflanzenphysiologie* (edited by W. RUHLAND), Vol. 15, p. 1537 (1965).
[2] Y. SUMIKI and A. KAWARADA, *Plant Growth Regulation*, p. 483, Iowa State University Press, Ames, Iowa (1959).
[3] S. TAMURA, N. TAKAHASHI, N. MUROFUSHI, S. IRIUCHIJIMA, J. KATO, Y. WADA, E. WATANABE and T. AOYAMA, *Tetrahedron Letters* **22**, 2465 (1966).
[4] H. HARADA and J. P. NITSCH, *Ann. Physiol. Vég.* **3**, 193 (1961).

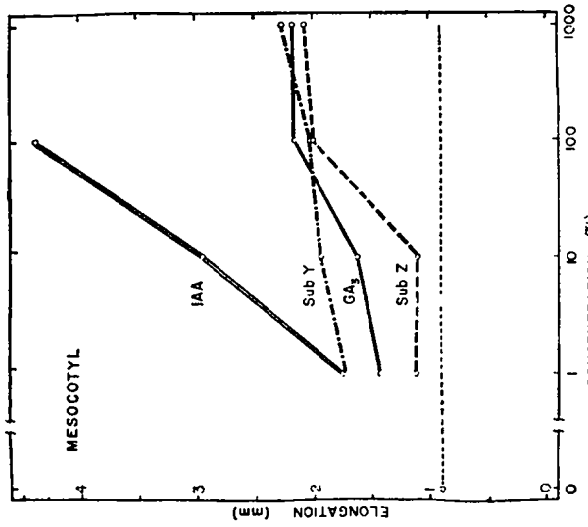

FIG. 2. EFFECT OF SUBSTANCES Y AND Z, AND OF GA_3 AND IAA ON THE ELONGATION OF *Avena* FIRST INTERNODE SECTIONS. EACH POINT REPRESENTS THE AVERAGE OF TEN SECTIONS (INITIAL LENGTH: 4 mm).

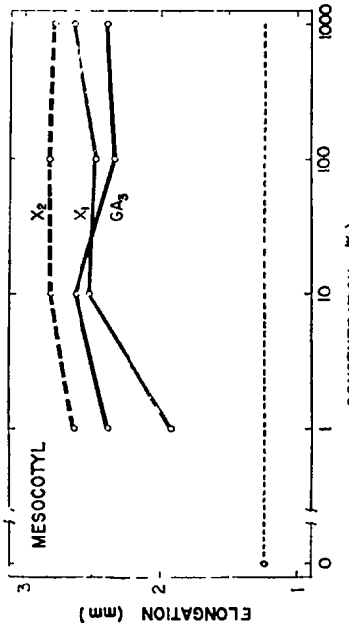

FIG. 1. EFFECTS OF SUBSTANCES X_1 AND X_2 AND OF GA_3 ON THE ELONGATION OF *Avena* FIRST INTERNODE SECTIONS. EACH POINT REPRESENTS THE AVERAGE OF TEN SECTIONS WHICH WERE 4 mm LONG INITIALLY.

Biological Properties of Substances X_1, X_2, Y and Z

The biological properties of the substances X_1, X_2, Y and Z have been determined in four different tests.

(*a*) *Avena mesocotyl test.* As shown in Figs. 1 and 2, substances X_1, X_2 and Y are nearly as active as GA_3 in the *Avena* first-internode test. Substance Z is less active than GA_3 at concentrations below 100 μg/l. The response curves of the four substances are different from that of indoleacetic acid (IAA).

(*b*) *Dwarf maize test.* Mutant "dwarf-3" responded to substances X_1, X_2 and Y, but not to substance Z, within the range of concentrations tested (Figs. 3, 4). The response curve of substance X_2 was nearly parallel to that of GA_3. Substance Z was also inactive on mutant "dwarf-5" (Fig. 5).

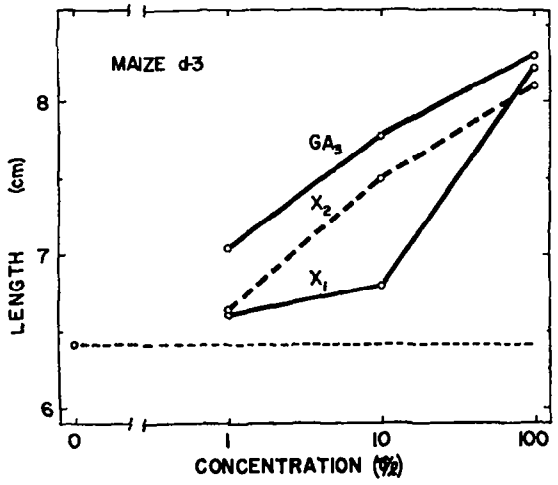

FIG. 3. EFFECTS OF SUBSTANCES X_1 AND X_2, AND OF GA_3 ON THE ELONGATION OF THE FIRST AND SECOND LEAF SHEATHS OF DWARF-3 MAIZE SEEDLINGS. EACH POINT REPRESENTS THE AVERAGE OF TEN PLANTS.

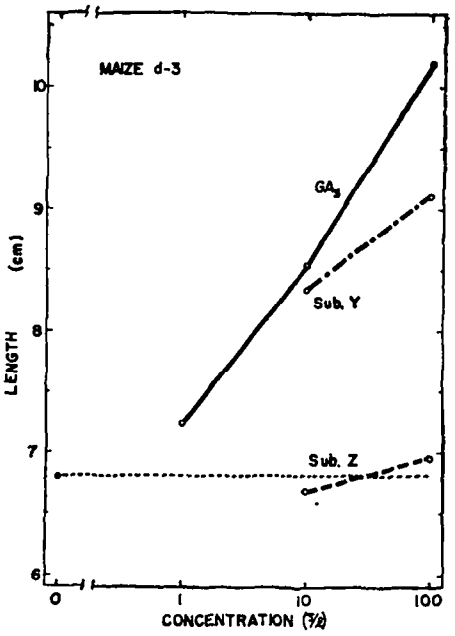

FIG. 4. EFFECTS OF SUBSTANCES Y AND Z, AND OF GA_3 ON THE ELONGATION OF THE FIRST AND SECOND LEAF SHEATHS OF DWARF-3 MAIZE SEEDLINGS.

(*c*) *Lettuce seed germination test.* As seen in Fig. 6, substance X_2 was practically as active as GA_3 in promoting germination in total darkness. The activities of substances Y and X_1 were lower than that of GA_3. Substance Z was inactive, at least at concentrations of 10 mg/l and below. Higher concentrations could not be tried because of the small amounts of substances available.

(*d*) *Lettuce hypocotyl test.* This test was carried out only with substances X_1 and X_2. Both were active, substance X_2 stimulating growth more than X_1 at concentrations of 100 μg/l and above (Fig. 7).

In brief, the results obtained with four biological tests show that substances X_1, X_2 and Y possess gibberellin-like activities, while substance Z does not.

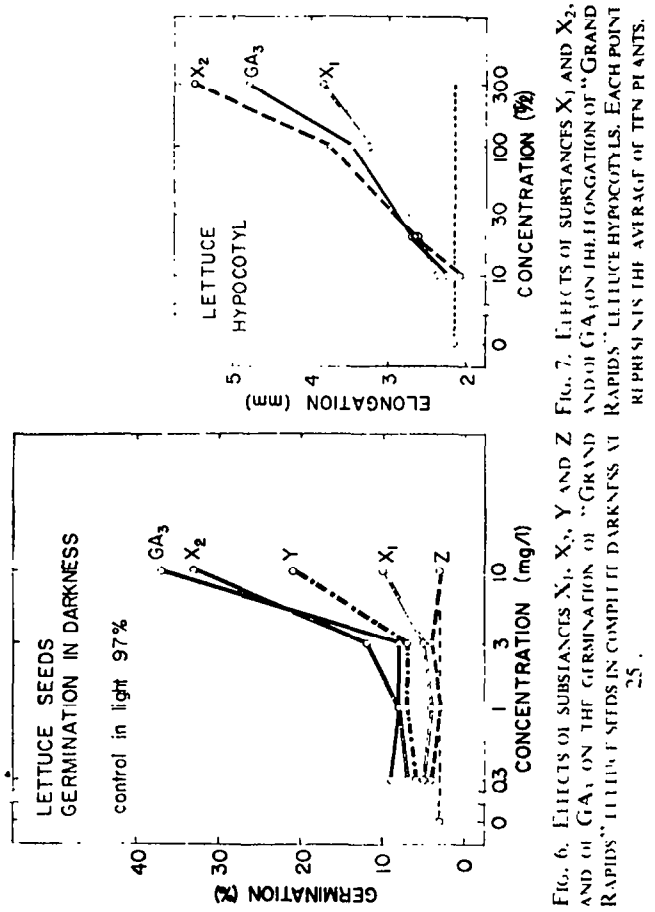

FIG. 5. EFFECTS OF SUBSTANCES Z AND OF GA_3 ON THE ELONGATION OF THE FIRST AND SECOND LEAF SHEATHS OF DWARF-5 MAIZE SEEDLINGS. EACH POINT REPRESENTS THE AVERAGE OF TEN PLANTS.

FIG. 6. EFFECTS OF SUBSTANCES X_1, X_2, Y AND Z AND OF GA_3 ON THE GERMINATION OF "GRAND RAPIDS" LETTUCE SEEDS IN COMPLETE DARKNESS AT 25°.

FIG. 7. EFFECTS OF SUBSTANCES X_1 AND X_2, AND OF GA_3 ON THE ELONGATION OF "GRAND RAPIDS" LETTUCE HYPOCOTYLS. EACH POINT REPRESENTS THE AVERAGE OF TEN PLANTS.

Physical Properties

(*a*) *Aspects of the crystals.* Substance X_1 crystallized in the form of needles, substance X_2 in the form of platelets, substance Y in the form of prisms and substance Z in the form of needles also.

(*b*) R_f *values and fluorescence.* The R_f values of substance Y on silica gel G with the solvent system 1* of MacMillan and Suter[5] and on kieselguhr with their solvent system 3† were 0·05–0·1 and 0·5–0·6, respectively. Both the R_f values and the blue color of the fluorescence which developed after spraying with H_2SO_4 and heating at 110–120° correspond to those of GA_1.

The R_f values of substances X_1 and X_2 chromatographed on silica gel G in 90% isopropanol were 0·55–0·6 and 0·35–0·4, respectively, corresponding to those of GA_9 and GA_3. The R_f values of X_1 and X_2 on silica gel G with solvent system 1 were 0·6–0·7 and 0·15–0·25. These two R_f values are in accordance with those obtained by us with authentic samples of GA_9 and GA_3, respectively, but slightly different from that reported by MacMillan and Suter.[6] With the sulphuric acid reagent, X_1 produced a purple fluorescence (as did GA_9), and X_2 a blue fluorescence (as did GA_3) after heating at 110–120° for 10 min.

(*c*) *Melting points.* The melting points of the four substances are as follows:

Substance	Original fraction*	Quantity obtained (mg)	Melting point
X_1	AE-Ia$_1$	2·5	190–205°
X_2	AE-Ia$_2$	5·0	225–232°
Y	AE-Ib	5·0	234–241°
Z	AE-II	2·5	296–301°
		(decomposes with gas evolution)	

* See Experimental Section.

The melting point of Y is in good accordance with that of GA_1 (235–240°) reported by MacMillan and Suter.[6]

(*d*) *Mass spectrometry and molecular weights.* The mass spectrograph gave 316 as the molecular weight of substance X_1, which is that of GA_9. Both substances produced secondary peaks at 298, 272, 229, 203, etc. Similarly, the molecular weight of substance X_2 was shown to be 346, with other peaks at 328, 300, 284, 136, etc. All these peaks were found with an authentic sample of carefully purified GA_3 (Fig. 8).

In the case of substance Y, the molecular weight was 348, which is the same as that of gibberellin A_1. Other peaks were found at 330, 302, 261, 163, 149, 135–136 and 121 for both substance Y and a sample of GA_1. For substance Z, the mass spectrograph gave a possible molecular weight of 368, although traces of compounds with higher molecular weights were also present; other peaks were located at masses 353, 256, 246–247, 149, etc.

(*e*) *U.V. absorption spectra.* The u.v. absorption curves obtained with substances X_1, X_2 and Y in methanol were practically identical with those of gibberellins A_9, A_3 and A_1, respectively. Substance Z showed practically no absorption in u.v. light.

* Di-isopropyl ether:acetic acid (95:5).

† Benzene:propionic acid:H_2O (8:3:5).

[5] J. MacMillan and P. J. Suter, *Nature* **197**, 790 (1963).

[6] J. MacMillan and P. J. Suter, *Naturwiss.* **45**, 46 (1958).

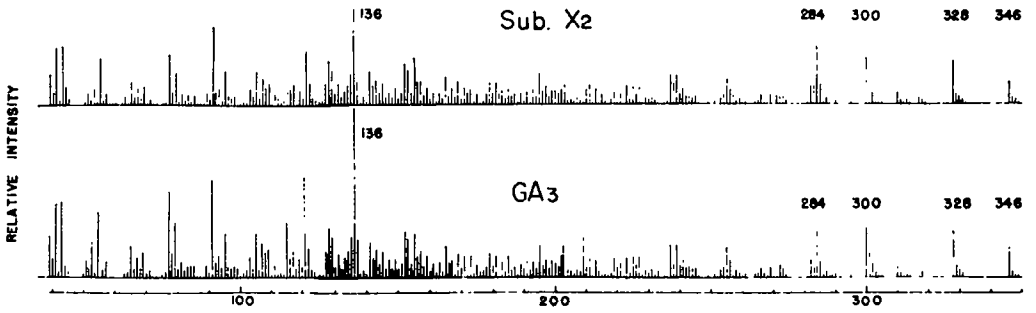

FIG. 8. MASS SPECTRA OF SUBSTANCE X_2 AND GA_3.

CONCLUSIONS

Identity of the isolated substances. Table 1 summarizes the properties, both physico-chemical and biological, of the isolated substances, together with those of known gibberellins. The sum of all these data allows one to conclude that: substance $X_1 = GA_9$; $X_2 = GA_3$; and $Y = GA_1$. The identity of substance Z is still unknown. The biological properties indicate that Z is not a gibberellin.

TABLE 1. SUMMARY OF THE PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF THE COMPOUNDS ISOLATED FROM *Althaea rosea*

	X_1	GA_9	X_2	GA_3	Y	GA_1	Z
Cristalline appearance	Needles		Platelets		Prisms		Needles
Molecular weight: theoretical		316		346		348	
found by mass spectrometry	316	316	346	346	348	348	368
Melting point	190–205°	208–211°	225–232°	233–235°	234–241	235–240	296–301°
R_f in silica gel G							
(1)*	0·6–0·7	0·6–0·7	0·15–0·25	0·15–0·25	0 05–0 15	0 05–0 15	0·0
(2)†	0·55–0·6	0·55–0·6	0·35–0·4	0·35–0·4			
Fluorescence under u.v.‡	Purple	Purple	Blue	Blue	Blue	Blue	0
Biological tests							
Avena mesocotyl	+++	+++§	+++	+++§	+++	++§	+
Dwarf maize d-3	++	++§	+++	+++§	++	++§	0
Dwarf maize d-5		+++§		++§		+++§	0
Lettuce hypocotyl	+	+	++	++		+	
Lettuce germination	+	+	+++	+++	++	++	0

* Solvent: di-isopropyl ether (95), acetic acid (5).
† Solvent: 90% isopropanol.
‡ After spraying with H_2SO_4 and heating at 110–120°.
§ Nitsch and Nitsch.[16]
‖ See P. W. BRIAN, H. G. HEMMING and D. LOWE, *Nature* **193**, 946 (1962).

Other instances of occurrence of GA_1, GA_3 and GA_9 in higher plants. Gibberellin A_1 has been found in immature seeds of *Phaseolus multiflorus* (MacMillan and Suter[6]), in the endosperm of *Echinocystis macrocarpa* (Elson *et al.*[7]) and in shoot tips of *Nicotiana tabacum* (Sembdner and Schreiber[8]). Gibberellin A_3 has been reported in barley (Jones *et al.*[9]), in *Echinocystis* (Elson *et al.*[7]), in *P. multiflorus* (Sembdner *et al.*[10]) and also in shoot tips of tobacco (Sembdner and Schreiber[8]). To our knowledge, in contrast, gibberellin A_9, which has been first extracted from cultures of *Fusarium moniliforme* (Cross *et al.*[11]) has never been isolated from higher plants. Khalifah and co-workers,[14] however, have provided biological and chromatographical evidence for its occurrence in citrus fruits.

Role of the extracted substances in flowering. The presence of three different gibberellins plus substance Z in the extracts of vernalized *Althaea rosea* tips show how complex the spectrum of substances leading to bolting and flowering may be. Up to now we have only tried substance E or GA_3 alone to replace the physiological effect of vernalization: both substances have caused bolting without the formation of flower buds in this species. It is possible that other gibberellins, or a certain combination of gibberellins, may be more stimulatory to flowering. Michniewicz and Lang,[12] for example, have shown that GA_3 was unable to cause flowering in *Myosotis alpestris*, whereas GA_1 and, especially, GA_7 were active in this process.

EXPERIMENTAL

Plant Material

A total of about 30,000 apices of vernalized, biennial hollyhocks (*Althaea rosea* Cav. var. "Fordhook Giant Double Scarlet") were harvested before the plants started to bolt in the spring. The fresh material was immediately frozen in liquid nitrogen and broken into small fragments while in the frozen state. Care was taken that the material remained frozen until it has been thoroughly lyophilized.

Extraction

The method of extraction and separation of growth substances from the plant material is shown in the flow diagram of Fig. 9.

Purification of the Ethyl Acetate Fraction

The purification of "fraction EA" was achieved by means of thin-layer and paper chromatography. The *Avena* mesocotyl test (see Nitsch and Nitsch[13]) was used to detect the biologically-active zones. One mm-thick layers of silica gel G were prepared on glass plates (20 cm × 20 cm); they were streaked with not more than 0·2 ml of fraction EA; the solvent (acetone) was allowed to ascend 15 cm at room temperature (20°–25°). The active zone (R_f 0·0–0·3) of each plate was scraped off and eluted five times with methanol. The combined eluates were concentrated to about 5 ml.

In a second step of purification, this partially purified extract was streaked as 5 mm-wide bands across sheets of Whatman No. 31 paper (extra thick, 46 cm × 57 cm). The solvent (isopropanol:ammonium hydroxide:water, 80:0·05:19, 95, v/v) was allowed to ascend 25 cm at 15°. Two major, active zones, AE-I (R_f 0·5–0·8) and AE-II (R_f 0·3–0·5) were eluted five times with methanol. The eluates were concentrated to about 5 ml each and streaked uniformly as very narrow bands across the length of thin-layer plates (20 cm × 20 cm) coated with silica gel G (1 mm thick). The solvent (di-isopropyl-ether:acetic acid, 95:5, v/v) was run for 15 cm at room temperature. Under these conditions, fraction AE-I was further divided into two major active fractions: AE-Ia (spread between R_f 0·15 and 0·7) and AE-Ib (R_f 0·05–0·15). As was the case for fraction AE-II, the major active substances stayed at the initial spot, thus allowing mobile, inactive substances to be eliminated. The eluate of fraction AE-Ia was further chromatographed on thin-layer plates (silica gel G, 0·5 mm thickness), in isopropanol:water (90:10, v/v). Two active zones separated sharply from one another: EA-Ia_1 (R_f 0·55–0·6) and AE-Ia_2 (R_f 0·35–0·4).

[7] G. W. Elson, D. F. Jones, J. MacMillan and P. J. Suter, *Phytochem.* **3**, 93 (1964).
[8] G. Sembdner and K. Schreiber, *Phytochem.* **4**, 49 (1965).
[9] D. F. Jones, J. MacMillan and M. Radley, *Phytochem.* **2**, 307 (1963).
[10] G. Sembdner, G. Schneider, J. Weiland and K. Schreiber, *Experientia* **20**, 89 (1964).
[11] B. E. Cross, R. H. B. Galt and J. R. Hanson, *Tetrahedron Letters* **23**, 22 (1960).
[12] M. Michniewicz and A. Lang, *Planta* **58**, 549 (1962).
[13] J. P. Nitsch and C. Nitsch, *Plant Physiol.* **31**, 94 (1956).
[14] R. A. Khalifah, L. N. Lewis and C. W. Coggins, Jr., *Plant Physiol.* **40**, 441 (1965).

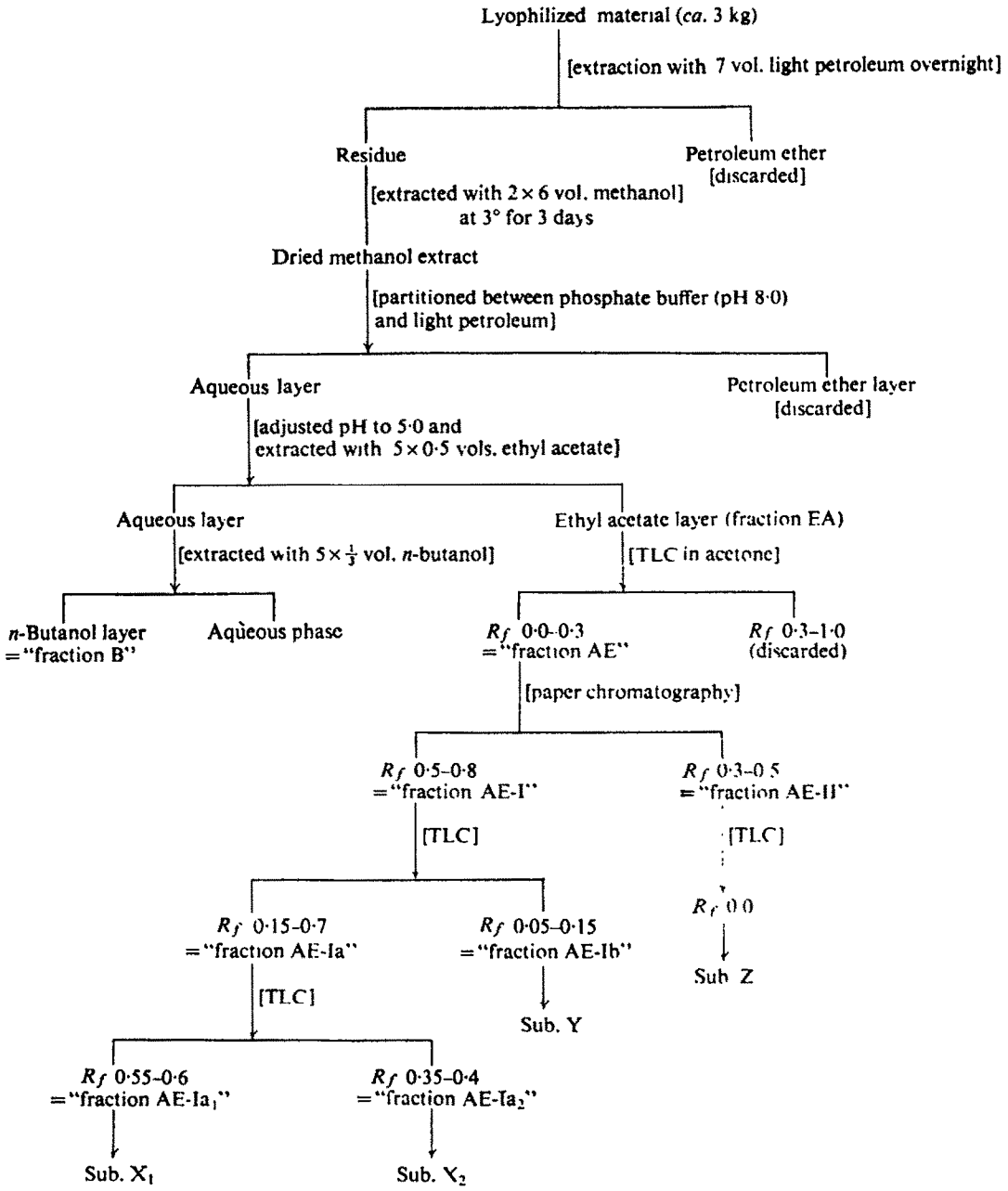

FIG. 9. SUMMARY OF THE EXTRACTION PROCEDURE FOR SUBSTANCES X_1, X_2, Y AND Z FROM *Althaea rosea* SHOOT TIPS.

The four active fractions (AE-Ia$_1$, AE-Ia$_2$, AE-Ib, AE-II) were eluted five times with methanol. After complete evaporation of the methanol, each eluate was dissolved in a small quantity of acetone, and petroleum ether was added until the acetone solution became slightly translucent. Upon cooling crystals of active substances were formed (see text).

Bioassays

Apart from the *Avena* mesocotyl test which was employed to detect active zones on chromatograms, the following biological tests were used to determine some of the biological properties of the isolated substances.

(*a*) *Dwarf maize test.* The procedure used was that of Nitsch and Nitsch,[15] similar to the one described by Phinney and West.[16] The seedlings were treated when the first leaf blade emerged about 2 cm from the coleoptile. Each seedling received 0·1 ml of test solution containing 0·1% of "Tween 80". Seedlings treated with "Tween 80" alone served as controls. The lengths of the first and the second leaf sheaths were measured 7 days after treatment. The sum of these lengths was used as a measure of the response.

(*b*) *Lettuce seed germination test.* One hundred seeds (var. "Grand Rapids") were sown in each Petri dish containing two layers of filter paper imbibed with 5 ml of the solution to be tested. After 4 days at 25° in complete darkness, the percentage of germinated seeds was determined.

(*c*) *Lettuce hypocotyl test.* The seeds (var. "Grand Rapids") were pregerminated at room temperature (20°–25°) for 1 day. Ten seedlings selected for uniformity were placed in small glass vessels (2·5 cm diameter) containing 1 ml of 1% agar and 0·5 ml of test solution. The seedlings were grown under artificial light (*ca.* 7000 lx, 16 hr/day). Four days after the beginning of the treatment, the length of the hypocotyls was measured under a binocular microscope.

Chemical and Physical Analyses

(*a*) *Detection of gibberellins by fluorescence on thin-layer chromatograms.* The thin-layer chromatograms were sprayed with sulphuric acid (5% in ethanol or 30% in water), heated and then observed under the u.v. light (350 mμ).

(*b*) *Melting points.* The melting points were determined with the Kofler apparatus under a microscope.

(*c*) *Mass spectra.* The mass spectra were determined with an MS-9 (A.E.I.) mass spectrometer. The samples were introduced directly. Ionizing voltage: 70 eV. The temperatures used varied from 150° to 250°, according to the sample.

(*d*) *U.v. absorption spectra.* The u.v. spectrum of each substance was determined on methanolic solutions, using a Beckman DK-2 spectrophotometer.

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The oat seeds var. "Brighton" were generously supplied by the Canadian Department of Agriculture, Scott, Saskatchewan, Canada, and the dwarf-maize mutants by Professor B. O. Phinney, Dept. of Botany, University of California, Los Angeles, U.S.A.

[15] J. P. Nitsch and C. Nitsch, *Ann. Physiol. Vég.* **4**, 85 (1962).

[16] B. O. Phinney and C. A. West, *Handbuch der Pflanzenphysiol.* (Edited by W. Ruhland). Vol. 15, p. 1185. (1961).