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ENDOGENOUS GIBBERELLINS AND GIBBERELLIN-LIKE SUBSTANCES IN LONG-DAY AND SHORT-DAY SPECIES OF TOBACCO PLANTS: A POSSIBLE CORRELATION WITH PHOTOPERIODIC RESPONSE

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Abstract—The long-day plant *Nicotiana sylvestris* and short-day plant *Nicotiana tabacum* were grown both under favourable and unfavourable photoperiodic conditions and then extensively analysed for endogenous gibberellins and gibberellin-like substances. GA_3 was found to be the main gibberellin in the leaves of both plants. Its content reached a maximum (12 μg/kg) in *N.sylvestris* grown under favourable long-day conditions. No GA_3 was detected in *N. tabacum* grown under short-day conditions. On the basis of this evidence it is concluded that the biosynthesis of gibberellins is responsible for floral induction only in the case of long-day plants. In the case of short-day plants, this process is not involved in the flowering process. The higher levels of gibberellins observed in plants grown under long-day conditions regardless of their photoperiodic response implies that some steps in the biosynthesis of gibberellins are light-dependent. Four new gibberellin-like substances of unknown nature were found in the ether extract of *N. sylvestris*. Three of them appear to be specific for *N. sylvestris* grown under long-day conditions. They stimulate the flowering of long-day plants under unfavourable photoperiodic regime.

INTRODUCTION

THE ROLE of gibberellins in floral induction has been much discussed in recent years. Soon after 1954 it was found that exogenous gibberellic acid (GA_3) initiates the flowering of long-day plants under unfavourable photoperiodic conditions but does not affect the development of short-day plants.[1-3] The first interpretation of these facts[4,5] stipulated that for the production of the floral stimulus in long-day plants high levels of free gibberellins are required, while short-day plants need only small amounts of gibberellins to start flower formation. Later it was found that the inhibitors of gibberellin biosynthesis, such as CCC and AMO-1618 also suppress the flowering of long-day plants.[6-10] These experimental facts

[1] M. KH. CHAILAKHIAN and V. N. LOZHNIKOVA, *Physiol. Rast.* **7**, 521 (1960).
[2] A. LANG and E. REINHARD, *Advan. Chem.* **28**, 71 (1961).
[3] M. KH. CHAILAKHIAN, T. V. NEKRASOVA, L. P. CHLOPENKOVA and U. N. LOZHNIKOVA, *Physiol. Rast.* **10**, 465 (1963).
[4] P. W. BRIAN, *Nature* **181**, 1122 (1958).
[5] P. W. BRIAN, *Biol. Rev. Can. Phil. Soc.* **34**, 37 (1959).
[6] B. BALDEV and A. LANG, *Am. J. Botany* **52**, 408 (1965).
[7] J. A. D. ZEEVAART, *Planta* **71**, 68 (1966).
[8] J. A. D. ZEEVAART and A. LANG, *Planta* **58**, 531 (1962).
[9] J. A. D. ZEEVAART and A. LANG, *Planta* **59**, 509 (1963).
[10] J. A. D. ZEEVAART, *Plant Physiol.* **39**, 402 (1964).

were summarized in the hypothesis that in the case of long-day plants flower formation is controlled by the biosynthesis of gibberellins.[6,11] However, the validity of the hypothesis depends on whether the mechanisms of action of endogenous and exogenous gibberellins are identical.[6] The first indirect evidence in its favour was obtained from grafting experiments and from studies with crude plant extracts[12,13] but such techniques were unsuitable for elucidation of the role of individual gibberellins in flower induction.

A promising approach to the problem might consist in both qualitative and quantitative determination of endogenous gibberellins produced in the leaves of a pair of plants belonging to the same genus but displaying opposite and well pronounced photoperiodic responses. Such analysis must be carried out not only for plants grown under normal inductive conditions but also for plants grown under unfavourable photoperiods. Following this line, we examined two *Nicotiana* species, the first being the long-day plant *Nicotiana sylvestris* Spegaz and Comes and the other being the short-day plant *Nicotiana tabacum* L. Sp. pl. (Mammoth variety); both species have been previously studied by Chailakhian *et al.*[14]

RESULTS

The analysis of gibberellins in plants is hampered by serious experimental problems. Since the content of gibberellins in the vegetative parts of plants does not exceed $10^{-6}\%$ of their dry weight[15] the isolation of a few milligrams of crystalline individual material amounts to the working up of many tons of vegetative tissue.[16,17] On the other hand, work with crude eluates obtained from chromatography of plant extracts on paper or in thin layers of silica gel is hardly reliable. Such eluates, if obtained from the extracts of leaves, contain large amounts of polyphenols. The latter often have the same R_fs as gibberellins, which prevents the chromatographic identification of gibberellins and may seriously distort the results of bioassays. A compromise was found by using an amount of material which would allow the identification of gibberellins as chromatographically individual spots.[18]

Extraction of each of the four variants (*N. sylvestris*-LD, *N. tabacum*-SD, *N. sylvestris*-SD and *N. tabacum* LD) was carried out as shown in Chart 1. Some 4–5 kg of fresh leaves from the 4-month old plants were lyophilized with liquid nitrogen, ground and extracted with 95 per cent ethanol. The bulk of chlorophyll and lipids was precipitated upon concentration of the ethanolic extract. Bioassays on the dwarf pea (Pioneer variety) in all four cases showed no gibberellin-like activity in the chlorophyll–lipid precipitate. The aqueous concentrate was extracted with ether, than acidified and extracted with ethyl acetate. All physiological activity, as determined by bioassays on the Pioneer dwarf pea, was associated with acidic fractions of both extracts.

1. *Physiologically Active Substances Extracted with Ethyl Acetate*

Acidic substances extracted with ethyl acetate were chromatographed on a column with silica gel impregnated with phosphate buffer (pH 6·2). As was shown earlier,[19] these con-

[11] M. KH. CHAILAKHIAN, *The factors of generative development* Nauka, Moscow (1964)
[12] M. KH. CHAILAKHIAN, *Usp. Sovr. Biol.* **63**, 202 (1967)
[13] M. KH. CHAILAKHIAN, *Ann. Rev. Plant Physiol.* **19**, 1 (1962).
[14] M. KH. CHAILAKHIAN and A. N. BOJARKIN, *Dokl. Acad. Nauk. SSSR* **105**, 592 (1955).
[15] D F JONES, J. MACMILLAN and M. RADLEY, *Phytochem.* **2**, 307 (1963).
[16] S. TAMURA, N. TAKAHASHI, N. MUROFUSHI and S. IRIUCHIJIMA, *Tetrahedron Letters*, 2465 (1966).
[17] F. HAYASHI and L. RAPPORT, *Plant Physiol* **41**, 53 (1966).
[18] N. J. GRIGORIEVA, V. F. KUTCHEROV, U N. LOZHNIKOVA and M. KH CHAILAKHIAN, *Khim. Prirodn. Soedin.* **4**, 296 (1969).
[19] V. F. KUTCHEROV, I. A. GURVICH, A. V. SIMOLIN and I M. MILSTEIN, *Dokl Acad. Nauk. SSSR* **163**, 765 (1965).

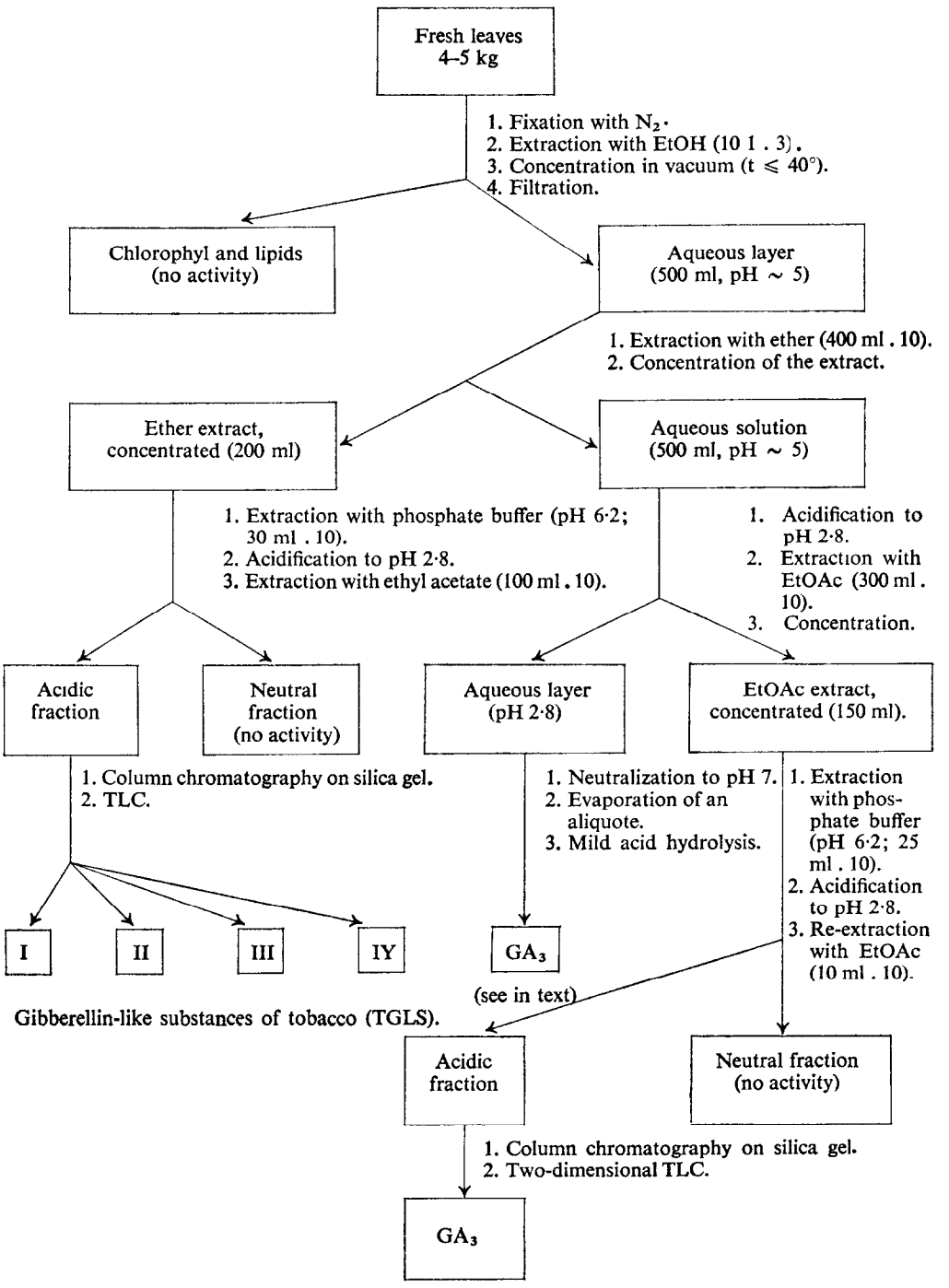
Fresh leaves
4–5 kg
1. Fixation with N_2.
2. Extraction with EtOH (10 l . 3).
3. Concentration in vacuum (t ⩽ 40°).
4. Filtration.
Chlorophyl and lipids
(no activity)
Aqueous layer
(500 ml, pH ~ 5)
1. Extraction with ether (400 ml . 10).
2. Concentration of the extract.
Ether extract,
concentrated (200 ml)
Aqueous solution
(500 ml, pH ~ 5)
1. Extraction with phosphate buffer (pH 6·2; 30 ml . 10).
2. Acidification to pH 2·8.
3. Extraction with ethyl acetate (100 ml . 10).
1. Acidification to pH 2·8.
2. Extraction with EtOAc (300 ml . 10).
3. Concentration.
Acidic
fraction
Neutral
fraction
(no activity)
Aqueous layer
(pH 2·8)
EtOAc extract,
concentrated (150 ml).
1. Column chromatography on silica gel.
2. TLC.
1. Neutralization to pH 7.
2. Evaporation of an aliquote.
3. Mild acid hydrolysis.
1. Extraction with phosphate buffer (pH 6·2; 25 ml . 10).
2. Acidification to pH 2·8.
3. Re-extraction with EtOAc (10 ml . 10).
I
II
III
IY
GA_3
(see in text)
Gibberellin-like substances of tobacco (TGLS).
Acidic
fraction
Neutral fraction
(no activity)
1. Column chromatography on silica gel.
2. Two-dimensional TLC.
GA_3

CHART 1.

ditions allow a neat separation of GA_1 and GA_3 and the bulk of physiologically active substances was associated with exactly those fractions which usually contained GA_3. When the active fractions were analysed by TLC using the specially prepared highly disperse KSK silica gel[18] their active principle was identified as GA_3. Thus, the active fraction from *N. sylvestris* grown under long-day conditions displays the spot of this gibberellin with typical green fluorescence after spraying the plate with H_2SO_4 and heating at 100° for 1 min (see Fig. 1). This spot has exactly the same R_fs in alkaline, neutral and acidic solvent systems

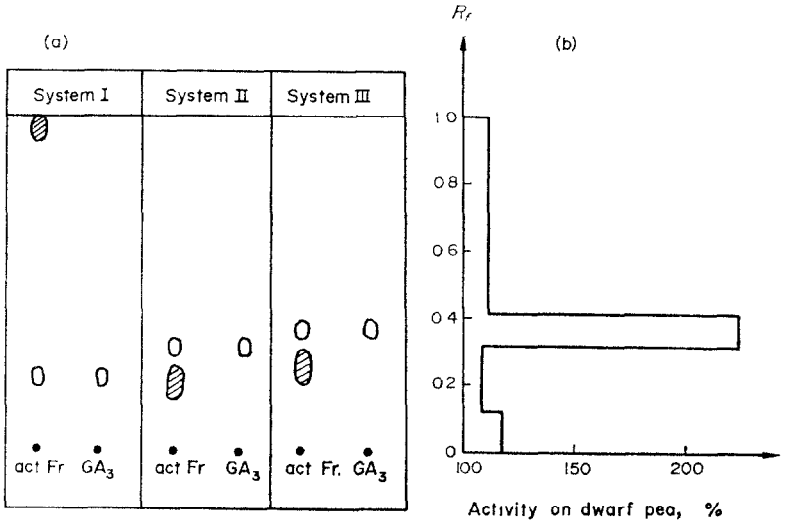

FIG. 1. (a) THE IDENTIFICATION OF GA_3 IN THE PHYSIOLOGICALLY ACTIVE FRACTION (COLUMN CHROMATOGRAPHY OF THE ETHYL ACETATE EXTRACT) OBTAINED FROM THE LEAVES OF *N. sylvestris* GROWN UNDER LONG-DAY CONDITIONS. SOLVENT SYSTEMS: 1. *n*-C_4H_9OH–3N NH_4OH (5:1); 2. $CHCl_3$–CH_3OH (95:5). 3. EtOAc–$CHCl_3$–HOAc (40:60 1). (b) HISTOGRAM OF THIS FRACTION IN SYSTEM 3.

as pure GA_3. Ethanolic eluate from this spot contains practically all gibberellin-like activity of this fraction. When treated with H_2SO_4 according to the known procedure[15,18] this eluate displays a fluorescence spectrum identical with that of pure GA_3 (see Fig. 2). The treatment of the eluate with diazomethane in ether followed by TLC-analysis gave a spot corresponding to the methyl ester of GA_3. All this evidence confirms the presence of GA_3 in the leaves of *N. sylvestris* grown under long-day conditions. The presence of GA_3 in the leaves of *N. sylvestris* grown under short-day conditions and in the leaves of *N. tabacum* grown under long-day conditions was demonstrated in the same manner.

Chromatographically pure GA_3 was obtained by means of two-dimensional chromatography in the thin layer of highly disperse KSK silica gel[18] after successive developments with ethyl acetate–chloroform–acetic acid (40.60:1) and *n*-butanol–3N ammonia (5:1). The amount of GA_3 eluted from the corresponding spots was estimated by (1) by comparing the yield of fluorescence in the spectrum of the eluate (after treatment with H_2SO_4 and heating) with that observed for a given amount of authentic GA_3[18,20] and (2) by comparing the physiological activity of the eluate with that observed for a given amount of authentic GA_3 in bioassays on the Pioneer dwarf pea in the range of concentrations 10^{-4}–10^{-8} g/l.

[20] D. F. JONES, *Nature* **202**, 1309 (1964).

Both methods gave similar results. The highest content of GA_3 in leaves (12 μg/kg of fresh leaves) was observed in the case of the long-day plant, *N. sylvestris*, grown under long-day conditions.* When the same plant was grown under non-inductive, short-day conditions, the content of GA_3 fell to 4 μg/kg.

A different situation was observed in the case of the short-day plant. *N. tabacum*. The content of GA_3 of this plant grown under non-inductive long-day conditions was only

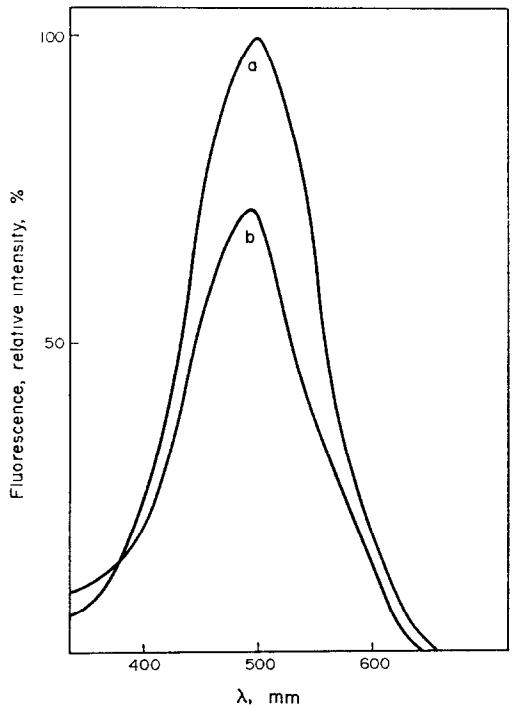

FIG. 2. THE FLUORESCENCE SPECTRA OF AUTHENTIC GA_3 (A) AND OF THE SPECIMEN ISOLATED FROM *N. sylvestris*, GROWN UNDER LONG-DAY CONDITIONS (B).

1·4 μg/kg of fresh leaves, i.e. it was nearly three times lower than in the case of *N. sylvestris* grown under unfavourable conditions. When *N. tabacum* was grown under short-day conditions, no GA_3 at all could be detected. Thus, whereas the bioassays on dwarf pea in the case of *N. sylvestris* revealed the presence of GA_3 in the aliquots corresponding to 10 g of fresh leaves, in the case of *N. tabacum* grown under short-day conditions no gibberellin-like activity was found even in the aliquots corresponding to 1 kg of fresh leaves (aliquots were taken from the fractions eluted from the column with chloroform–ethyl acetate 4:1 mixture). At first glance, this finding seemed to be best explained by the assumption that GA_3 exists in the leaves of *N. tabacum* grown under short-day conditions in its 'bound' form.

* The error in the estimation of GA_3 content in all cases is a sum of two errors of different origin. The first one is inherent in the method of analysis (about 5 per cent for fluorescent spectroscopy), the second is due to the losses inevitable in the course of isolation. However, since all operations were carried out under identical conditions the relative error must be nearly permanent and hence the relative content of GA_3 in leaves can be reliably estimated.

Since gibberellins are known to form glycosides[21–24] or peptide complexes[25,26] it was supposed that such highly polar substances could not be extracted with ethyl acetate and therefore must remain in the aqueous layer (see Chart 1). Consequently, the dry residues obtained from the aqueous layers were subjected to enzymic hydrolysis with emulsin (β-glucosidase) in acetate buffer at pH 5·4 (37°, 44 hr). However, this hydrolysis afforded GA_3 only in the case of *N. sylvestris* grown under long-day conditions. Since the blank experiment under the same conditions also gave GA_3, it seems likely that the latter is released not by enzymic but rather by mild acidic hydrolysis; therefore, in our case the bound form of GA_3 is not a β-glucoside. The same results were observed when the dry residues were hydrolysed with 10 per cent AcOH in acetone or *n*-butanol (25°, 48 hr); here also GA_3 was found only in the hydrolysates obtained from *N. sylvestris* grown under long-day conditions. Hence the absence of GA_3 in the leaves of *N. tabacum* grown under favourable, short-day conditions cannot be explained by the existence of a bound form of this gibberellin.

2. *Physiologically Active Substances Extracted with Ether*

When the ether extract of *N. sylvestris* grown under long-day conditions was chromatographed on KSK silica gel four fractions exhibiting high activity in bioassays on dwarf pea were obtained (see Chart 1); the active principles of these fractions will subsequently be referred to as TGLS-I, TGLS-II, TGLS-III and TGLS-IV, respectively (TGLS=gibberellin-like substance of tobacco). Certain characteristics of the active fractions are given in Table 1.

TABLE 1. ETHER-SOLUBLE PHYSIOLOGICALLY ACTIVE PRINCIPLES OF *Nicotiana sylvestris* GROWN UNDER LONG-DAY CONDITIONS

	Isolation (by column chromatography)		Bioassays (water = 100%)	
	Fractions, NN	Eluent (% of ethyl acetate in benzene)	Pioneer dwarf pea (activity corresponds to 500 g of fresh leaves)	d − 1 dwarf maize (in brackets—the amount of leaves corresponding to the assayed aliquote)
TGLS—I	14, 15	20	175	—
TGLS—II	23–25	60	210	150(15 g)
TGLS—III	27, 28	70	210	inactive
TGLS—IV	37, 38	100	240	260(7 g)

Well pronounced activity in the bioassays on dwarf maize was found in the cases of TGLS-II and TGLS-IV, the responses of d-1, d-3 and d-5 mutants being of the same order.

The most interesting property, common to TGLS-II, TGLS-III and TGLS-IV, is their ability to induce the same photoperiodic response of higher plants as that caused by GA_3.

[21] G. SEMBDNER, J. WEILAND, O. AURICH and K. SCHREIBER, in *Plant Growth Regulators*, p. 70, London, (1968).
[22] Y. MURAKAMI, *Botan. Mag. Tokyo* **74**, 424 (1961).
[23] E. REINHARD and R. SACHER, *Experientia* **23**, 415 (1967).
[24] S. TAMURA, N. TAKAHASHI, T. YOKOTA, N. MUROFUSHI and Y. OGAWA, *Planta* **78**, 208 (1968).
[25] G. B. MAXIMOV, V. V. POLEVOI, G. K. PADKEVICH and L. P. LOGVENKOVA, in *Plant Growth Regulators*, p. 53, Nauka, Moscow, 1964.
[26] G. SEMBDNER, G. SCHNEIDER, J. WEILAND and K. SCHREIBER, *Experientia* **20**, 89 (1964).

When a long-day plant, *Rudbeckia bicolor* L., was exposed to short-day conditions and then treated with corresponding active fractions, normal reproductive development was re-established.

Ether extracts from *N. sylvestris* grown under short-day conditions as well as those from *N. tabacum* grown both under inductive and non-inductive conditions contained only the photoperiodically inactive TGLS-I.

DISCUSSION

The analysis of endogenous gibberellins in leaves from the long-day and short-day species of tobacco grown under inductive and non-inductive photoperiods reveals remarkable differences both in the nature and content of these phytohormones.

As can be seen from Table 2, the level of endogeneous GA_3 in leaves of the long-day

TABLE 2. ENDOGENOUS GA_3 AND GIBBERELLIN-LIKE SUBSTANCES IN LEAVES OF TWO SPECIES OF TOBACCO GROWN UNDER DIFFERENT PHOTOPERIODS

Species	Photoperiod	Free GA_3 (μg/kg fr. wt.)*	Gibberellin-like substances of tobacco
N. sylvestris (LD—plant)	Long day	12	TGLS—I, II, III, IV
	Short-day	4	TGLS—I
N. tabacum Mammoth variety (SD—plant)	Long day	1·4	—
	Short-day	0	TGLS—I

* See footnote, p. 513.

plant, *N. sylvestris*, considerably increases when the plant is transferred to the inductive long-day conditions. On the other hand, it is well known that exogenous GA_3 induces the flowering of long-day plants grown under unfavourable photoperiods,[1,2] while the inhibitors of biosynthesis of gibberellins suppress the flower formation of these plants even under flower-inducing long-day conditions.[6–10] A comparison of these facts leads to the conclusion that the mode of action of exogenous and endogenous GA_3 are fairly similar. Our results agree with the hypothesis that the flowering of long-day plants is controlled by the biosynthesis of gibberellins.

A different situation is observed in the case of the short-day plants. As shown in Table 2, leaves of *N. tabacum* (Mammoth variety) do not contain GA_3—at least, in amounts detectable by bioassays (see p. 513). This can be explained by two alternative assumptions. Either the biosynthesis of GA_3 is blocked in this short-day plant, or the rate of metabolization and/or translocation of GA_3 is greater than the rate of its biosynthesis. The latter alternative seems less probably since exogenous GA_3 does not initiate flowering of *N. tabacum*. In our opinion, the absence of GA_3 in leaves of *N. tabacum* grown under favourable short-day conditions is more likely to be caused by the low level of its biosynthesis.* This means that the biosynthesis of GA_3 in leaves of the short-day plants is not related to flowering.

* The verification of this hypothesis requires special tracer experiments with radioactive GA_3.

The next point deserving attention is the marked increase of GA_3 content in plants grown under long-day conditions regardless of their photoperiodic response. This implies that the biosynthesis of gibberellins includes certain light-dependent stages. The same conclusion was arrived at by Stoddart and Lang in their studies of gibberellin-like substances of red clover.[27] The importance of light is further evidenced by experiment with *Brassica oleracea*[28] where about 16% of gibberellin-like activity was associated with chloroplasts. The latter are able to transform (—)-kaur-16-en-19-oic acid into gibberellins.[29] Moreover, the biosynthesis of gibberellins in the culture of *Gibberella fujikuroi* Saw. is also likely to contain some light-dependent stages for it is considerably accelerated by light.[30] The exact nature of such light-dependent steps cannot be elucidated without special tracer experiments. Perhaps, one of the oxidative stages leading from (—)-kaurene to (—)-kaur-16-en-19-oic acid is light-dependent since West and co-workers demonstrated that the inhibition of these stages induced by carbon monoxide can be reversed by illumination.[31]

The gibberellin-like substances of tobacco extracted with ether at pH 5 are also of interest, especially the photoperiodically active TGLS-II, TGLS-III and TGLS-IV which are specific for the long-day plant, *N. sylvestris*, grown under favourable conditions. The occurrence of such substances in tobacco is reminiscent of the results of Stoddart[32] who detected two gibberellin-like substances in *Trifolium pratense* L. of which one was observed only in flowering plants.

EXPERIMENTAL

All tobacco plants were grown in the open air (Moscow, summer 1968) receiving 16 hr of daylight under long-day conditions or 8 hr of daylight under short-day conditions. Bioassays on the dwarf pea (Pioneer variety) and *Rudbeckia bicolor* were carried out as described in our earlier publications.[33,34]

Silica gel KSK used in this work was freed from the traces of iron and treated according to the previously described procedure.[18] Column chromatography of the acidic part of the EtOAc extracts was carried out using silica gel KSK as described earlier.[18] In all four variants the ratio between the adsorbed material and the adsorbent was 1:35 (w/w) and the height of the column was about 12·5 times as large as its diameter.

Fluorescence spectra were recorded on a Jobin and Ivon spectrofluorometer (France), the excitation wavelength being at 380 nm. The samples were prepared according to Jones, MacMillan and Radley.[15]

Column chromatography of the acidic part of the ether extracts was carried out as described below for a typical case (*N. sylvestris*, long-day conditions). The dry residue obtained after evaporation of the acidic ether extract (2 51 g) was chromatographed on a column packed with 251 ml of silica gel KSK (40 × 3 cm). The column was successively eluted with pure petroleum, petroleum–benzene (4:1, 1:1 and 1:4 mixtures) and pure benzene, the volume of fractions being 2 l each. Then elution was continued with benzene containing successively 10, 15, 20, 30, 40, 60, 70, 80 and 90% EtOAc; finally the column was washed with pure EtOAc For each solvent type, three fractions were collected (3 × 250 ml) Physiologically active fractions were disclosed by bioassays on dwarf pea.

The hydrolysis of water-soluble 'bound gibberellins' was carried out as described below for the case of *N. sylvestris* grown under long-day conditions: the aqueous layer remaining after the extraction with EtOAc (see Chart 1) was immediately neutralized to pH 7 with $NaHCO_3$. A part of this solution, corresponding to 300 g of fresh leaves, was evaporated to dryness to give a residue (12·3 g) which was dissolved in 240 ml of 0·05 N NaOAc buffer pH 5·4 A solution of emulsin (595·5 mg) in the same buffer (125 ml) was then

[27] J. L. Stoddart and A. Lang, *Biochemistry and Physiology of Plant Growth Substances* (edited by F. Wightman and G. Satterfield), p. 1371, Runge Press, Ottawa (1968)
[28] J. L. Stoddart, *Planta* **81,** 106 (1968).
[29] J. L. Stoddart, *Phytochem.* **8,** 831 (1969).
[30] D. Mertz and W. Henson, *Physiol. Plantarum* **20,** 187 (1967).
[31] C. A. West, M. O. Osler, D. Robinson, F. Lew and P. J. Murphy, *Biochemistry and Physiology of Plant Growth Substances* (edited by F. Wightman and G. Satterfield), p. 313, Runge Press, Ottawa (1969).
[32] J L. Stoddart, *J. Exptl. Botany* **17,** 96 (1966)
[33] V. N. Lozhnikova, L. P. Chlopenkova and M. Kh. Chailakhian, *Agrochimija* 132 (1967)
[34] M. Kh. Chailakhian, L. P. Chlopenkova and V. N. Lozhnikova, *Dokl. Acad. Nauk Arm. SSR* **38,** 45 (1964).

added to the above solution and the resulting mixture was kept at 38° for 48 hr (with stirring, in two portions). The hydrolysate was extracted with ether (250 ml × 5), acidified to pH 2·8 and extracted with EtOAc (200 ml × 5). Both ether and EtOAc extracts were active in the bioassays on dwarf pea. TLC analysis of the EtOAc extract revealed the presence of GA_3.

In the case of *N. sylvestris* grown under short-day conditions and in the case of *N. tabacum* grown under both short-day and long-day conditions neither ether nor EtOAc extracts displayed any gibberellin-like activity.

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