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A BIOCHEMICAL MUTANT OF *LYCOPERSICON ESCULENTUM* MILL. ISOLATION AND PROPERTIES OF THE NINHYDRIN-POSITIVE "NORMALIZING FACTOR"

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Abstract—The tomato mutant "chloronerva" is unable to synthesize a ninhydrin-positive substance which is of widespread occurrence among higher green plants. Leaf application of this substance, even in microgram amounts, causes normal growth and development of the otherwise partly chlorophyll-deficient, growth inhibited and flowerless mutant. Isolation of the biologically active substance was accomplished by ion exchange chromatography and gel filtration with Sephadex. The yield from dried alfalfa shoots was about 0·002 per cent. The substance contains C, H, and N and is soluble in water, less so in methanol and ethanol, and insoluble in other organic solvents. Its molecular weight was estimated from gel filtration to be of the order of 350–500. Absorption spectra reveal a weak shoulder at 265 nm and peaks in the i.r., indicating amide linkages. Colour yield after reaction with ninhydrin is remarkably high, the absorption spectrum of the reaction product being similar to that of leucine and triglycine. The "normalizing factor" is resistant to enzymatic degradation, but is readily degraded with 6 N HCl to a number of fragments, which weakly stain with ninhydrin. Fragments have been separated by use of an amino acid analyser but are not identical with any protein amino acids.

INTRODUCTION

THE TOMATO mutant "chloronerva", which spontaneously arose from the variety "Bonner Beste" at the Gatersleben collection, fails to develop normally and is characterized by retarded growth, chlorosis of young leaflets, altered leaf shape and habit as well as lack of generative organs. This phenotype is changed to that of the wild-type both by grafting on "Bonner Beste" rootstocks or by leaf application of water extracts from normal plants.[1,2] The chemical basis of this "phenotypic normalization" was shown to be a water-soluble, heat-stable, ninhydrin-positive substance, which had previously been isolated from tomato and alfalfa.[3] We have now reason to suppose that the "normalizing factor" is widely distributed among higher green plants and represents a general product of plant metabolism. Samples from different sources apparently possess similar biological activity, but this does not inevitably implicate structural identity. In order to obtain better information on the chemical structure it was necessary to isolate ample amounts of the "normalizing factor". The main problem involved careful isolation of the substance existing in minute amounts together with much larger quantities of amino acids exhibiting very similar chemical properties. This paper describes the extraction methods used and the chemical characteristics of the substance isolated.

[1] H. BÖHME and G. SCHOLZ, *Kulturpflanze* **8**, 93 (1960).
[2] G. SCHOLZ and H. BÖHME, *Kulturpflanze* **9**, 181 (1961).
[3] G. SCHOLZ, *Flora (Jena)* **154**, 589 (1964).

RESULTS AND DISCUSSION

The isolation procedure is illustrated in Fig. 1. The extract from powdered oven-dried alfalfa shoots, after decolorization using Wofatit EZ, was desalted with Dowex 50 × 4, H^+. After washing the column with water, elution was effected with 0·3 N pyridine formate buffer, pH 3·5. The "normalizing factor", together with amino acids, is eluted within the first 1·5 l. of ninhydrin-positive fractions. After evaporation, this amino fraction is subjected to gel filtration using Sephadex G-25 fine, with 0·05 N acetic acid as solvent. The "factor" passes down the column in front of other ninhydrin-positive material and so is separated from the main bulk of amino acids. The next step was carried out on Dowex 50 × 2 using pH-gradient technique, which yielded a rather pure sample between pH 3·18 and 3·22. Desalting and further purification was achieved by a second gel filtration step using Sephadex

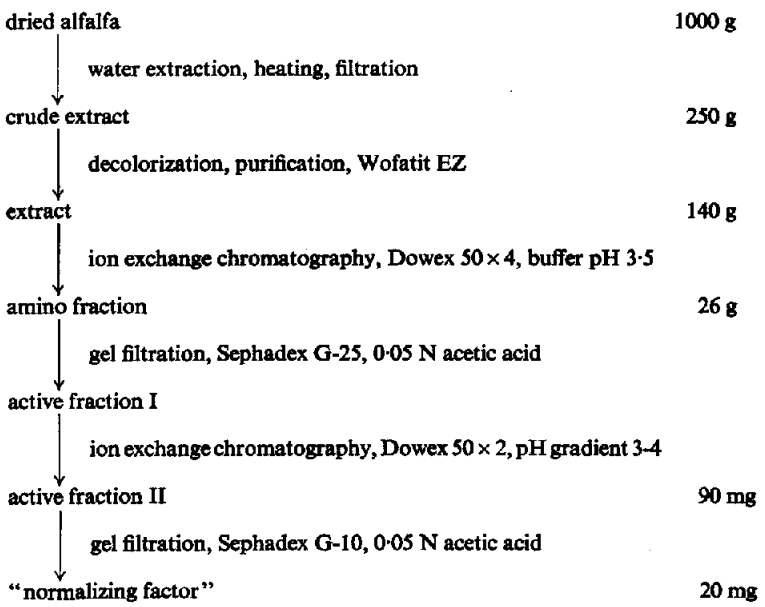

FIG. 1. ISOLATION OF THE "NORMALIZING FACTOR" FROM DRIED ALFALFA SHOOTS

G-10 fine. The substance emerged as a single peak distinctly separated from ninhydrin-positive impurities of smaller molecular size (Fig. 2). Its molecular weight was estimated after calibration of the Sephadex column with glycine, triglycine and pentaglycine to be in the order of 350–500, provided that the shape of the molecule is comparable to that of peptides and that gel filtration is not severely influenced by absorption. The "normalizing factor" thus obtained is a white, amorphous substance, soluble in water, less soluble in methanol and ethanol and insoluble in other organic solvents. Its biological activity is high, 70 μg per plant gave a positive response and alter chlorotic leaflets of mutant seedlings to a normal green pattern. The substance decomposes above 195° and is optically active, $[\alpha]_D^{20} = -46°$ (water, $c = 0{\cdot}28$). The elemental analysis yielded C 44·54, H 6·87 and N 12·77 per cent; S and P were not detected. Its spectra showed a weak shoulder at 265 nm and, among others, peaks in the i.r. regions at 1400 and 1600 cm^{-1}, indicating amide linkages. R_f values on paper chromatograms are below 0·05 in *n*-butanol–acetic acid–water, 4:1:1

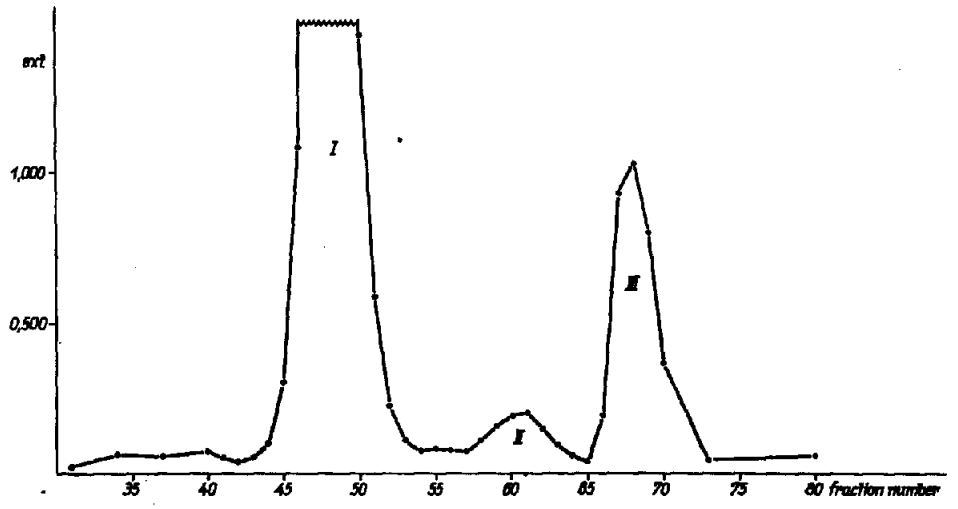

FIG. 2. ELUTION DIAGRAM AFTER GEL FILTRATION WITH SEPHADEX G-10.

Column 1·2 × 180 cm, solvent 0·05 N acetic acid, fraction volume 1·1 ml. Extinction after reaction with ninhydrin-hydrindantin. I = "normalizing factor", II and III = impurities.

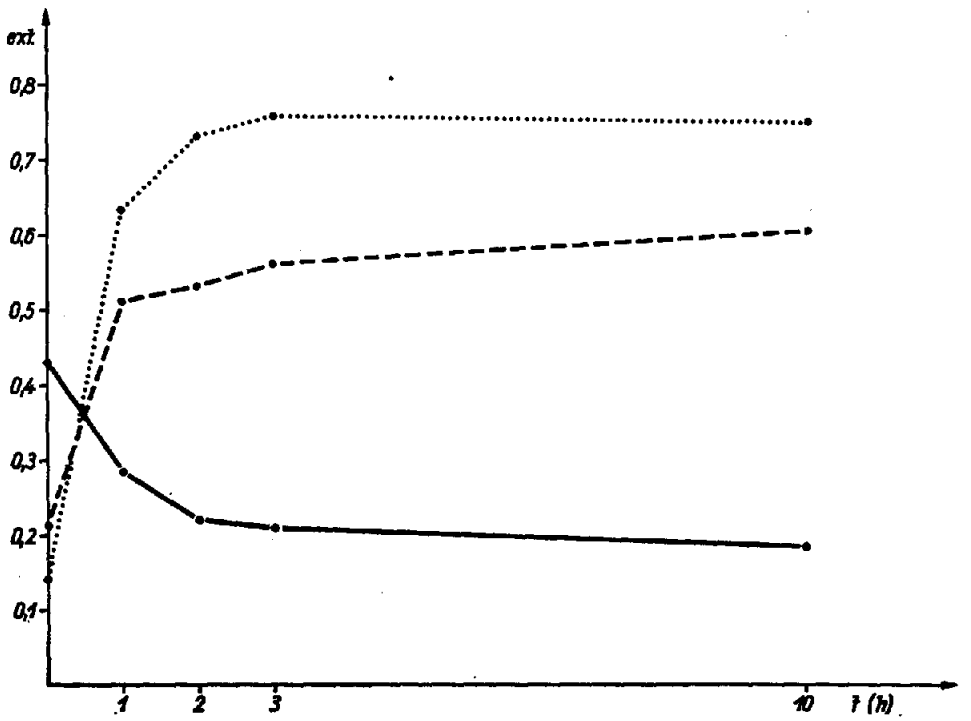

FIG. 3. COLOUR INTENSITY WITH NINHYDRIN-HYDRINDANTIN OF TWO OLIGOPEPTIDES AND THE "NORMALIZING FACTOR" AFTER DIFFERENT TIMES OF HYDROLYSIS.

Hydrolysis with 6 N HCl, 100°. ····· penta-glycine, – – – – leucylglycylglycine, ——— "normalizing factor".

(v/v), and 0·6 in phenol–water, 4:1 (v/v). The "normalizing factor" reacts with ninhydrin, giving a blue product with a remarkably high colour yield, assuming a molecular weight of 440 (Fig. 3). The absorption spectrum of the reaction product with ninhydrin is quite similar to that of leucine and triglycine. After hydrolysis with 6 N HCl, biological activity is lost. Though several ninhydrin-positive substances are produced, colour yield drops sharply with hydrolysis time, in contrast to the behaviour of common peptides (Fig. 3).

TABLE 1. ION EXCHANGE CHROMATOGRAPHIC ANALYSIS OF FRAGMENTS OBTAINED AFTER ACID HYDROLYSIS* OF THE "NORMALIZING FACTOR"

Values from test amino acids for comparison. Numbers represent millilitres of eluant (V_{max}), after which the peak of the respective substance emerges.

Amino acid	V_{max} from test-amino acids	V_{max} of fragments from "normalizing factor"† after hydrolysis
Lys	32·8	19·8
		(27·2)‡
		30·8
		(35·6)
His	39·6	40·6
NH_3	47·6	47·0
		52·6
Arg	68·4	—
$CySO_3H$	26·6	(28·6)
Asp	66·2	(66·0)
Thr	80·8	(80·8)
Ser	85·6	(85·6)
Glu	97·0	(96·8)
Pro	109·6	110·4
Gly	136·8	(137·2)
Ala	146·2	(144·8)
Cys	158·2	—
Val	182·8	185·2
Met	192·8	189·2
Ile	204·8	199·6
Leu	210·8	206·0
		226·0
Tyr	237·6	236·8
Phe	248·8	—
		281·2

* Hydrolysis conditions: 6 N HCl, 100°, 24 hr, sealed tube.
† V_{max} of the unhydrolysed "normalizing factor" = 80·0.
‡ Brackets indicate trace amounts.

Table 1 shows positions of peaks resulting from automatic amino acid analysis subsequent acid hydrolysis. Most of the peaks represent only trace amounts, and none of them proved to be identical with corresponding common amino acids. The substance occupying a peak in the histidine position is Pauly-negative. The peak corresponding to proline differs from it in its absorption spectrum. All other peaks are due to substances which differ from corresponding amino acids with respect to paper chromatography and high voltage electrophoresis.

All characteristics so far examined indicate that most peaks, if not all, do not represent amino acids known from proteins, but represent degradation products of unknown nature.

The "normalizing factor" resists enzymatic hydrolysis by trypsin, chymotrypsin, and subtilisin. Applying the DNP-method and hydrazinolysis for detection of N- and C-terminal amino acids also results in products which, after paper chromatography and high voltage electrophoresis (fingerprint),[4] do not coincide with those from common amino acids. In the light of our present knowledge, our original hypothesis which considers the "normalizing factor" to be a peptide is thus difficult to maintain. Further work is in progress to establish definitely the structure of the "normalizing factor".

Our special interest in the mutant "chloronerva" and the "normalizing factor" derives from the fact that our knowledge of "biochemical mutants" with a well-defined response to chemical substances is slight as far as higher plants are concerned. This is partly a consequence of inherent difficulties in the selection of mutants on a large scale under sterile conditions. Selection methods, well known from microbiological work, are with very few exceptions not applicable to higher plants. Langridge[5,6] and Feenstra,[7] using special devices, gave evidence of thiamine auxotrophic mutants of *Arabidopsis thaliana*. Redei[8] in the same species proved the mutant "immatus" to be cysteine dependent. Eriksson *et al.*[9] and Walles[10] described two chlorotic barley mutants whose normalization is achieved by either a single supply of aspartic acid (albina 7) or a continuous provision of leucine (xantha 23), respectively. Mutants from *Marchantia polymorpha*, deficient in either arginine, methionine, vitamins or growth substances, which have been selected by Miller *et al.*,[11] may also be included into this category. Besides these examples detected by systematic screening, our knowledge is confined to mutants detected more or less by chance. In past years attention has been focused upon mutants of *Arabidopsis* and *Lycopersicon* with metabolic blocks of thiamine biosynthesis. Boynton,[12] in a recent paper giving physiological and genetical details of the three thiamine auxotrophic mutants *te*, *spa*, and *tl*, summarized information concerning thiamine mutants both from micro-organisms and higher plants.

Generally, these mutants are lethal or semi-lethal due to a severe obstruction in chlorophyll synthesis or chlorophyll stability. Since the mutant "chloronerva" is lethal under field conditions and characterized by an abnormal chlorophyll distribution it fits into this pattern.[1] The chemical background of this phenotype remains obscure until the chemical nature of the "normalizing factor" is elucidated, though correlations between phenotype and iron metabolism have been established.[13,14] Extensive investigations performed in order to detect additional mutants lacking the "normalizing factor" among the Gatersleben collection have been without success.[15] They support our observation that the "normalizing factor" is of general occurrence in higher plants.

It should be mentioned for completeness, that another type of biochemical mutants from

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[10] B. Walles, *Hereditas* **50**, 317 (1963).
[11] M. W. Miller, E. D. Garber and P. D. Voth, *Botan. Gaz.* **124**, 94 (1962).
[12] J. E. Boynton, *Heredity* **56**, 238 (1966).
[13] G. Scholz, *Kulturpflanze* **13**, 239 (1965).
[14] G. Scholz, *Kulturpflanze* **15**, 255 (1967).
[15] H. Böhme, unpublished.

higher plants is represented by dwarf mutants lacking growth factors. Phinney[16,17] described several dwarf mutants in *Zea mays* which responded to gibberellic acid with normal growth. The same seems to be true in the case of a mutant of *Solanum tuberosum*.[18] The mutant "Halbzwerg" from *Antirrhinum majus* becomes normal both by grafting upon wildtype rootstock or application of gibberellic acid which is interpreted as counteraction against the influence of an unknown inhibitor produced by the mutant.[19] A gibberellin-induced anther and pollen development in a stamen-less mutant from *Lycopersicon esculentum* was reported by Phatak *et al.*[20]

EXPERIMENTAL

Isolation Procedure

Oven-dried (60°) young shoots from alfalfa (*Medicago sativa* L.) were ground and extracted as already mentioned.[3] For decolorization of the dark-brown crude extract, Wofatit EZ (VEB Farbenfabrik Wolfen) was used. Ion exchange chromatography was performed using Dowex 50 × 4 H^+ and 0·3 N pyridine formate buffer (pH 3·5) as eluant. For pH-gradient technique columns 1·7 × 120 cm were filled with Dowex 50 × 2 and equilibrated with 0·1 N pyridine formate buffer, pH 3·0. The material to be separated was dissolved in the same buffer, adjusted with formic acid to pH 2·85. A linear gradient was arranged between 0·1 N pyridine formate buffer, pH 3·0, and 0·6 N pyridine acetate buffer, pH 4·0. For gel filtration columns 3·4 × 140 cm with Sephadex G-25 "fine" and 1·2 × 180 cm with Sephadex G-10 "fine", respectively, were used with 0·05 N acetic acid as solvent.

Amino Acid Analysis

The substance was heated with 6 N HCl at 100° in sealed tubes for different times. Quantitative determinations were made by means of an automatic amino acid analyser manufactured by the Czechoslovak Academy of Sciences, Prague. Preparative separations were run on a column 0·9 × 20 cm with Amberlite CG-120 for basic and a column 0·9 × 150 cm for neutral and acidic amino acids. Colour yield was determined by heating with ninhydrin-hydrindantin.[21] For two-dimensional paper chromatography Schleicher–Schüll 2043 bMgl paper impregnated with 0·1 per cent Na_2EDTA was used, *n*-butanol–acetic acid–water, 4:1:1 (v/v) in the first, phenol–water, 4:1 (v/v) in the second direction. Fingerprints, end-group determinations and enzymatic hydrolyses have been prepared according to Meloun *et al.*[4]

Spectroscopy in the visible and u.v. regions was performed by the Perkin–Elmer Spectrophotometer 137 u.v. For i.r. spectroscopy the Zeiss-Infrared Spectrophotometer UR 10 was used.

Biological tests were arranged as described earlier.[3] To conserve material, test solutions were applied with a small brush to the youngest leaflets of mutant seedlings.

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[17] B. O. PHINNEY, *Plant Growth Regulation*, p. 489, Ames, Iowa, U.S.A. (1961).
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