Chemical Characterization of a Hormone That Promotes Cell Arrest in G_2 in Complex Tissues

Abstract. *A G_2 factor in the cotyledons of* Pisum sativum, *which arrests the growth of cells in both roots and shoots in the G_2 stage of the cell cycle, has been isolated and identified as trigonelline (*N*-methylnicotinic acid). To our knowledge, trigonelline is the first hormone that effects cell arrest in complex tissues of plants and animals to be chemically identified.*

The search for natural substances that regulate the cell proliferative cycle in complex tissues began with the investigations of Bullough and co-workers in the early 1960's (*1, 2*). Since these initial investigations several studies of these substances have focused on the chalones, which regulate cell proliferation (*3*). Understanding the chemical structures and physiological activities of these substances is important in cancer research.

Recently, a factor present in the cotyledons of peas (*Pisum sativum*) has been shown to arrest the growth of cells in both roots and shoots in the G_2 stage of the cell cycle (*4, 5*). Many of the physiological roles of the G_2 factor resemble those of chalones. We have now isolated this G_2 factor and have chemically characterized it as *N*-methylnicotinic acid, or trigonelline.

Trigonelline was first isolated from *Trigonella foenum-graecum* and was subsequently isolated from other biological sources (*6*). Except for its role as a partial substitute for the vitamin nicotinic acid, no independent function has been assigned to it.

Aqueous extracts of cotyledons from 3-day-old pea seedlings were prepared and evaporated under reduced pressure. The extracts were chromatographed with Dowex 50W-X4 and then with Sephadex LH-20 (*7*). The biologically active fraction was then passed through a column of Sephadex G-10, subjected to preparative thin-layer chromatography (TLC) in a mixture of $CHCl_3$, methanol, and water (65:65:10 by volume), and then purified by cellulose TLC in a mixture of acetonitrile, isopropanol, and water (4:3:3 by volume). An extract of 3200 cotyledons yielded 2 mg of material. Further purification was achieved by passing it through a column of Sephadex G-15, which yielded 50 μg of pure G_2 factor.

The general methods used in the bioassay have been described (*5*). Seeds of *Pisum sativum* were surface-sterilized and germinated in sterile vermiculite. Under sterile conditions, excised root tips were placed in medium with sucrose. After 3 days, root meristems were transferred to medium without carbohydrate for 5 days, so that cells were present only in the G_1 and G_2 stages of the cell cycle. Arrest of the cells under these conditions is a nonrandom process (*8*) that reflects the proportions of cells in G_1 and in G_2 during natural cell maturation (*9*). Extracts from cotyledons of peas were tested for their ability to promote cell arrest in G_2. Extracts were added during the culture period in which roots were exposed to sucrose before carbohydrate deprivation. An extract was judged to be effective if the percentage of cells that was arrested in G_2 was at least 40 percent of the entire cell population.

Roots were fixed in a mixture of ethanol and acetic acid (3:1 by volume). Relative amounts of DNA per nucleus were determined on Feulgen-stained nuclei from 0- to 2-mm terminal meristems by microfluorimetry (*10*). Measurements of nuclei were normalized with readings of one-half telophase and prophase figures taken to be 2C (G_1) and 4C (G_2) values, respectively (*11*). The biological activities of various fractions are shown in Table 1.

Chemical characterization of the purified G_2 factor was completed (*12*). A 50-μg batch of G_2 factor was submitted to electron impact-mass spectrometry at 190°C, 70 eV, which gave peaks at mass to charge ratios of 138 (M^+, $C_7H_8NO_2$), 123 ($M - CH_3$), 94 ($M - CO_2$), and 79 (C_5H_5N). The ultraviolet spectrum showed a maximum wavelength at 265 nm with a shoulder at 271 nm. Fourier-transformed infrared analysis suggested the presence of some "hydroxylic" function (bands around 3400 cm^{-1}), a heteroaromatic nucleus, and three adjacent aromatic hydrogens (780 cm^{-1}). Fourier-transformed proton magnetic resonance showed proton resonance signals at 4.35 parts per million (3H, singlet), 8.20 (1H, doublet of doublets, 6.50, and 7.50 Hz), 8.75 (1H, doublet, 7.50 Hz), 8.87 (1H, doublet, 6.50 Hz), and 9.20 (1H, singlet). Several lots of the G_2 factor were combined (250 μg) for a measurement of the carbon magnetic resonance spectrum (repetition rate 3.0 seconds, pulse angle 26°, 239,400 transients, 9 days) which provided evidence for five aromatic carbons, a carboxylate carbon, and an $N^+ - CH_3$ carbon. Taken together, these data establish the structure of the G_2 factor to be *N*-methylnicotinic acid (trigonelline), which was confirmed by comparison with authentic material (*12*). To our knowledge, trigonelline is the first natural hormone effecting cellular arrest in either G_1 or G_2 in complex tissues to be isolated and characterized.

Table 1. Promotion of cell arrest in G_2 by extracts of cotyledons of *Pisum* in a standard bioassay. The bioassay is used to test the effectiveness of various eluates of gel filteration. In experiment 1, the eluates were taken from the Sephadex LH-20 column. The volumes for eluates 1 through 4 were dried and dissolved in 10.0 ml of White's medium (*13*) with sucrose. One milliliter of this medium was used for each 50 ml of medium. In experiment 2, the fraction obtained from the Sephadex G-15 column usually yielded 50 μg of pure G_2 factor. This was derived from about 1600 cotyledons. One-half of this yield was dried and dissolved in 150 ml of White's medium with sucrose.

Treatment	Percentage of cells arrested*	
	G_1	G_2
Experiment 1. Eluates from Sephadex LH-20		
No eluate added	80.1 ± 3.2	19.9 ± 3.2
Eluate No. 1 added	78.0 ± 4.3	22.0 ± 4.3
Eluate No. 2 added	60.3 ± 2.1	39.7 ± 2.1
Eluate No. 3 added	78.1 ± 3.7	21.9 ± 3.7
Eluate No. 4 added	75.3 ± 4.8	24.7 ± 4.8
Experiment 2. Eluate from Sephadex G-15		
Without eluate added	80.0 ± 5.1	20.0 ± 5.1
With eluate added	54.7 ± 6.9	45.3 ± 6.9

*Mean and standard error of three slides per treatment. Fifty cells per slide were examined.

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Multiple Sclerosis: Presence of Lymphatic Capillaries and Lymphoid Tissue in the Brain and Spinal Cord

Abstract. *Thin-walled channels resembling lymphatic capillaries and containing lymphocytes were observed in perivascular spaces throughout the central nervous system of patients with various neurological disorders. This suggests that immunological surveillance in the central nervous system may normally involve lymphocyte circulation through the perivascular compartment. In some old multiple sclerosis plaques, perivascular lymphoid tissue was found which was organized in a manner similar to the antibody-producing medullary region of lymph nodes. This may indicate continuous processing of the putative multiple sclerosis antigen in such lesions.*

Perivascular infiltrates of lymphocytes and plasma cells are a prominent histological feature of the discrete lesions (plaques) that develop periodically in different parts of the brain and spinal cord in typical cases of multiple sclerosis (MS) (*1*, *2*). This, together with the demonstration that some of the immunoglobulin G in the spinal fluid in this disease is synthesized within the central nervous system (CNS) (*3*, *4*), suggests that a local immune response directed against an (unidentified) antigen is involved in its pathogenesis. In the absence of definite evidence of a systemic immune response, the reasons just given for supposing that such an antigen exists further suggest that the highest concentration of expressed antigen visible to the immune system occurs in plaques. Because plasma cells may be present in large numbers in perivascular spaces within plaques (*2*, *5*), it has been suggested that these spaces may represent the major site for antigen processing and antibody production within the CNS of patients with MS (*4*–*7*). In the present study, perivascular spaces in old plaques were found to contain organized lymphoid tissue, suggesting the occurrence of persistent antigen expression in such lesions.

Epoxy-embedded CNS tissue, suitably fixed for electron microscopy, was available for study from three patients with MS, a patient with motor neuron disease, and a patient with adrenoleukodystrophy (*8*). In each of these cases, light and electron microscopy revealed that the perivascular spaces in unaffected CNS tissue commonly contained lymphocytes and macrophages. Plasma cells were also present in two MS cases. These three cell types were not randomly distributed within the perivascular spaces; the lymphocytes and macrophages tended to be confined within thin-walled channels (Fig. 1, A and B), whereas any plasma cells that were present were invariably located outside these channels (Fig. 2). The walls of the channels consisted of a single layer of flattened cells that were joined at their edges by desmosomes (Fig. 1C) or separated by gaps of varying width. Each cell had an oval nucleus that was surrounded by scanty cytoplasm containing a few profiles of granular endoplasmic reticulum, several small dense bodies, and modest numbers of microtubules and microfilaments. The thin cytoplasmic processes that formed the channel walls were often less than 0.1 μm thick; they contained numerous smooth and coated pinocytic vesicles; and on their abluminal surface there were numerous hemidesmosomes associated with bundles of fine collagenous fibrils and microfibrils (Figs. 1C and 2B). Cells of the same type were also observed lining the glial and vascular walls of some perivascular spaces, or occurring as isolated cells in the absence of distinct channels.

All of the MS plaques studied were typical old plaques with few fat granule cells present in the demyelinated zone and with only minor perivascular inflammatory cell cuffing apparent in routine histological sections. In these plaques the perivascular spaces surrounding the larger blood vessels in demyelinated tissue revealed similar thin-walled channels containing lymphocytes and macrophages. However, compared to the channels in normal white matter, the channels in this location were more numerous and more irregular in shape, and they were separated by collagenous trabeculae which contained isolated plasma cells or groups of plasma cells clustered around cells of the same type as those that formed the walls of the channels (Fig. 2). Where these two cell types touched, the cell membrane of the plasma cell exhibited a lentiform electron-dense undercoat (Fig. 2B). Intimate contact was also observed between lymphocytes and macrophages inside the thin-walled channels; this specialized contact involved the formation of a number of deep, cylindrical indentations in the macrophage plasma membrane, each indentation enclosing a slender cytoplasmic process extending from the body of the lymphocyte, as reported previously in lymph node sinuses (*9*). Active phagocytosis was also observed occasionally inside the channels (Fig. 2A).

The type of tissue organization just described—with clusters of plasma cells together with free-lying collagen and reticular cells surrounding collagen-free channels containing lymphocytes and macrophages—is similar to the immunoglobulin-secreting medullary region of a lymph node (*10*–*12*). This was confirmed in one MS patient in whom lymph node tissue was available for study. In this patient, a comparison of the fine structure of plaque perivascular spaces and lymph node medulla revealed the same general arrangement of various cell types; the chief differences in the two tissues were the larger number of cells present in the medullary cords, which also contained a specialized vascular endothelium, and the fact that the reticular cells in the lymph node medulla exhibited certain structural differences depending on whether they protruded into the medullary sinuses, formed the walls of the sinuses, or were located among plasma cells outside the walls of the sinuses (*10*, *11*).

The presence of lymphoid tissue of

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