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11 RESEARCH PROPOSAL

1963 - NOW

No.1 pollen extract Global brand

"Research is the key to unlocking new knowledge and advancing our understanding of the world."

POLLITIN
FLOWER POLLEN EXTRACT

STEM CELL SUPPLEMENTS

Pollitin is a high quality natural extract. extracted from rye pollen under the production and research with technology The same standard as the production of drugs according to the requirements of the World Health Organization. therefore has been registered as “NUTRACEUTICAL” or “nutritional therapeutic nutrition” receiving the ORAC standard or the antioxidant concentration and the CAP-e Test or the ability to be absorbed into red blood cells at a very high level

The body receives almost 100% of the nutrients that are extracted from rye grass pollen. Sold to more than 50 countries on 6 continents around the world for more than 50 years, Swedish researchers have found that research studies. extracted from rye pollen contains Substances that are essential for the creation of new life in the plant family and are fundamental in the food chain. It is a natural anabolic steroid.

It has been proven by scientific laboratories that Contains a variety of nutrients including vitamins, minerals, phytosterols, carotenoids, flavonoids, nucleic acids, amino acids, substances necessary for the synthesis of RNA and DNA, antioxidant activity, enzymes, saturated fatty acids, precursors in the synthesis of prostaglandins.

So extracted from rye pollen Therefore, it is the ideal food for use in helping to make the body healthy and perfect holistic. Because there are nutrients that help to relieve fatigue, have antioxidants. The main culprit that causes many serious diseases to humans, contains important substances such as phytosterols that help boost immunity. keep the body healthy until able to cope with various illnesses caused by facing pollution and germs on a daily basis more effectively

IN SCIENCE WE TRUST

CELL REPAIRING

Research has confirmed that there are more than 300 types of nutrients, vitamins, minerals that are essential for the care of the body and cells.

NUTRASCEUTICAL

Contains important substances that have antioxidant properties. Thus helping to slow down aging and help your skin look better.

BODY IMMUNE DEFENCE

Research reports on efficacy that helps to inhibit prostatitis caused by hormones

PHARMACEUTICAL FOOD

Contains nucleic acids and other important substances that stimulates the body to create interferon to stimulate white blood cells to work more efficiently better deal with germs

GUARANTEED WORLD-CLASS PRODUCTION STANDARDS

Natural Choice Nutraceuticals

POLLITIN - EXCLUSIVE STEM CELL SUPPLEMENTS

Our premium natural extracts originate from meticulously selected flower pollen found in "Rye." These extracts undergo a unique proprietary production process crafted by Graminex L.L.C. in Ohio, United States. This exclusive process encompasses every stage, from cultivation and harvesting to the creation of high-quality natural extracts, specifically G60 and G63, derived from GBX flower pollen particles. Graminex holds the sole rights to this process and maintains adherence to strict pharmaceutical production standards in alignment with the World Health Organization's requirements.

Our extracts are renowned for their world-class production standards, boasting ORAC certification for exceptionally high antioxidant concentration and CAP-e Test accreditation, which signifies outstanding absorption into red blood cells. Over more than five decades, we have consistently refined and improved our product's efficacy.

Registered as a "NUTRACEUTICAL" or "nutritional therapy," Pollitin addresses issues at the cellular level, offering antibacterial properties and reinforcing immunity. By delivering essential nutrients tailored to various bodily systems, it equips the body to effectively combat abnormal cells. Our dedication to research is exemplified by over 150 certifications from medical and pharmaceutical institutions.

Moreover, Pollitin is not only a national achievement but a global triumph, available in over 50 countries. Our exclusive patented production process sets us apart as the sole producer of this unique formulation globally, rendering it impossible for anyone else to replicate our success in extracting and utilizing these flower pollen particles.

Pollitin - สารอาหารบำบัดเซลล์อ

สารสกัดธรรมชาติคุณภาพสูง สกัดจากเกสรดอกไม้จาก “ข้าวไรย์” ที่มีสูตรลับเฉพาะของ บริษัท (Graminex L.L.C.) ที่รัฐโอไฮโอ้ ประเทศสหรัฐอเมริกา ในการปลูก เก็บ และผลิตสกัดธรรมชาติคุณภาพสูง G60, G63 จากอณูละอองเกสรดอกไม้ GBX, Graminex® เอกสิทธิ์เฉพาะของบริษัท Graminex เท่านั่นที่ผลิตได้เพียงเจ้าเดียวในโลก อยู่ภายใต้การควบคุมมาตรฐานการผลิตยา ตามข้อกำหนดขององค์การอนามัยโลก

จนเราได้รับการรับรองมาตรฐานการผลิตระดับโลกระดับเดียวกับการผลิตยาเพราะ Pollitin ได้รับรองการทดสอบค่า ORAC หรือ ค่าระดับความเข้มข้นของสารต้านอนุมูลอิสระที่สูงมาก และ CAP-e Test หรือค่าความสามารถในการดูดซึมเข้าสู่เม็ดเลือดแแดงในระดับที่สูงจนได้รับ

การขึ้นทะเบียนเป็น “NUTRACEUTICAL” หรือ “โภชนเภสัช สารอาหารบำบัดระดับเซลล์” ที่สามารถแก้ไขปัญหาฟื้นฟูได้ลึกถึงระดับเซลล์ มีฤทธิ์ฆ่าเชื้อแบคทีเรีย และมีผลเสริมสร้างภูมิต้านทานเมื่อเซลล์ต่างๆ ได้รับสารอาหารที่เหมาะสมตามระบบต่างๆ ในร่างกาย ส่งผลให้ร่างกายสามารถต่อสู้กับ เซลล์ที่ผิดปกติภายในร่างกายได้ถึง 95% และยังได้รับรองมาตรฐานการผลิตและประสิทธิภาพจากองค์กรต่างๆ มากมายระดับโลก รวมไปถึงยังได้รับรางวัลการันตีอีกมากมายจาก เอกสิทธิ์สูตรลับพิเศษเฉพาะของ Graminex ทำให้สินค้ามีคุณภาพและเกิดผลลัพธ์ที่ดีและน่าเชื่อถือ จนได้รับการยอมรับระดับสากลอีกด้วย

ตลอดระยะเวลากว่า 50 ปี เราได้มีการวิจัยพัฒนาประสิทธิภาพอย่างต่อเนื่อง มีการวิจัยจากสถาบันทางการแพทย์และเภสัชกรรมรับรองมากกว่า 150 การวิจัย เรามีความภูมิใจอย่างมากในการเป็นผู้ผลิตหนึ่งเดียวของโลกที่ได้ครอบครอง ถือลิขสิทธิ์เอกสิทธิ์กระบวนการผลิตและสูตรเฉพาะ G60 และ G63 จากละอองเกสรดอกไม้ชนิด GBX ที่ไม่มีใครสามารถทำได้ ส่งผลให้ Pollitin เป็นที่ยอมรับจากคนจำนวนมากใน 6 ทวีป 50 ประเทศทั่วโลก และได้รับผลตอบรับที่ดีจากผู้บริโภคในการซื้อซ้ำสินค้าอย่างต่อเนื่องมากกว่า 50 ปี

"Happy MPM: *The exclusive importer and distributor of Pollitin in Thailand*, Laos, Vietnam, Myanmar, and Malaysia for over two decades. our commitment to *unparalleled reliability has touched the lives of over one billion consumers* worldwide."

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POLLITIN
FLOWER POLLEN EXTRACT

11 งานวิจัย เกสรดอกไม้ต่อโรคภูมิแพ้

www.pollitin.com

Assessment of Sensitizing Potential

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2060 Cernitin T60
2065 Cernitin GBX
November 3, 1992

Introduction

A combination of 2060 CERNITIN T 60 and 2065 CERNITIN GBX was found to be sensitizing in guinea pigs when tested according to the maximization method of Magnusson and Kligman (1). In this test, a 5% suspension of CERNITIN T 60 and CERNITIN GBX (60 + 3) was administered intracutaneously together with complete Freund adjuvant, followed after 7 days by an epidermal application of a 25% suspension of the test substance combination. Two weeks after the induction exposure, the animals were challenged by a further topical application of the same suspension. They revealed a positive reaction.

Thus, the maximization procedure demonstrated an allergenic potential or sensitizing capacity of the test material, without however indicating an actual risk of sensitization in man.

In order to interpret the experimental finding it is important to realize that the guinea pig maximization test is a diagnostic procedure for predicting delayed-type sensitivity to proteins and soluble antigen-antibody complexes on the skin. The cell-mediated reaction produces contact dermatitis and other allergies of the tuberculin-type. However, allergens associated with pollens induce immediate-type reactions which are associated with circulating antibodies of the IgE class. Allergens of this sort are capable of inducing hay fever, bronchial asthma, urticaria, and anaphylactic shock.

It is to be expected that water soluble protein or peptide components of CERNITIN T 60 may induce a delayed type reaction when injected intradermally with complete Freund adjuvant as immune enhancer. The relevance of the laboratory procedure as it was performed is however limited as it does not apply to the practical use conditions of the CERNITIN extracts of pollens. Since the therapeutic use is oral rather than topical, it is more appropriate to rely on information of occupational exposure and of side effects in patients in order to assess the risk of allergic reactions. Atopic patients may be considered to be at particular risk of developing allergic disorders. Such individuals were subjected to immunotherapy with high doses of CERNILTON in order to achieve desensitization upon oral treatment.

Medical Assessment

Occupational Exposure

No symptoms suggestive of pollen allergies have been reported over 5 years in personnel engaged in production of the pollen extracts CERNITIN T 60 and GBX (2).

Adverse Effects during Therapy of Benign Prostatic Hyperplasia

Controlled clinical studies confirmed the good tolerability of CERNILTON N. In a study conducted over 24 weeks, 3 patients out of 92 treated reported gastrointestinal side effects (3). A similar incidence (4%) occurred in an open study which involved 1798 patients treated for 24 weeks (2 tablets 100 mg t.i.d.) (4).

During 1984-1991 (sales volume 145’801’000 tablets CERNILTON/CERNILTON N), post-marketing surveillance in Germany resulted in 113 reports of adverse effects. The large majority (96 cases) consisted of gastrointestinal disturbances, 10 developed a variety of cotaneous symptoms and only 2 developed “allergy” of a non-specified nature (5).

By contrast, no reports on side effects of CERNILTON/ADRENOPROSTAL were received in Switzerland (6), or in Korea of CERNILTON tablets sold since 1975 (7), or in Japan where CERNILTON is marketed since 1969 (8). Likewise, no side effects are reported in Argentina (sales volume > 150 million CERNILTON tablets since 1975) (9), or in Austria (> 8 000'000 CERNILTON/PROSTAFLOR tablets sold since 1983) (10).

Tolerance Study in Patients with Pollen Allergy

Twenty eight patients (18 men and 10 women) suffering from seasonal allergic rhinitis (pollinosis) received 4 daily capsules of STHENOREX (120 mg T60 and 6 mg GBX per capsule) at intervals, including the pollen season. Although the skin test to STHENOREX was positive, no reaction to oral treatment was observed and there was no improvement of the allergic condition. (11).

Immunotherapy

In an open study in Switzerland, 44 patients suffering from seasonal allergic rhinitis were treated for 2 months with daily doses of 840 mg T60 and 42 mg GBX (one FH 84 sachet), or 1680 mg T60 and 84 mg GBX (2 sachets), respectively. This amount is equal to 4.5 to 9 times the usual daily dose of CERNILTON. Apart that the treatment was considered effective in 70% of the patients, there were no untoward allergic reactions, or other side effects (12).

A similar study was carried out in Argentina on a total of 47 patients who received one or two sachets of FH 84, or placebo. Apart from one patient each who experienced transient diarrhea or sinusitis, treatment was uneventful (therapeutic effects due to FH 84 could not be ascertained as other drugs with antiallergic properties were administered) (12).

A double-blind, placebo controlled study was performed in Italy (13). Thirty four pollinosis patients received 2 sachets of FH 84 for 30 days, and 41 matching patients the placebo only. There was no overall significant effect of treatment and no side effects were encountered.

Conclusion

The vast clinical experience indicates that CERNITIN T 60 and CERNITIN GBX are well tolerated. Side effects are rare and generally limited to the gastrointestinal tract. Reactions reminiscent of allergic effects have been reported in single cases only.

It is concluded that the standardized CERNITIN extract of pollen is devoid of allergenic properties when administered by the oral route. This has amply been demonstrated in therapeutic use as well as in special studies involving high dosage in atopic patients.

Robert Hess, M.D.

References

1. Leuschner, F. Examination of a combination consisting of 2060 Cernitin T 60 and 2065 Cernitin GBX (60 + 3) in a skin sensitization test in guinea pigs according to Magnusson and Kligman (Maximization Test). LPT Laboratory of Pharmacology and Toxicology, Report No. 6859/91, May 4, 1992.
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4. Bach, D. and Ebeling, L. Möglichkeiten und Grenzen der Phytotherapie bei BPH: Behandlungsergebnisse mit Cernilton^{R} N im Stadium 1-3 nach Alken (bzw. II-IV nach Vahlensieck). Ibid., pp. 187-194.
5. Pharma Stroschein, Hamburg, Letter Report, Febuary 1, 1992.
6. Gartmann, J. Swiss Drug Monitoring Center SDMC, letter of January 25, 1990.
7. Kim, Y.B. Dong Koo Pharmaceutical Co., Ltd., Seoul, letter of July 25, 1989.
8. Nagaoka, M. Tobishi Pharmaceutical Co., Ltd., Tokyo, letter of September 18, 1992.
9. Mattianich P.H.A. Laboratorios Temis Lostalo, Buenos Aires, letter of October 6, 1992.
10. Schoeller Pharma, Wien, letter of October 22, 1992.
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ALLERGY SUPPORT

GRAMINEX Flower Pollen Extract

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ALLERGY SUPPORT

GRAMINEX Flower Pollen Extract

Double-Blind, Comparative, Clinical Study of the FH 84 and Placebo in Patients with Hay Fever

1989

1. Aim of the study

The aim of the single-centre, double-blind study was to compare the efficacy of a product containing standardized pollen extracts (FH 84) versus a placebo in patients with hay fever.

2. Patients and methods

The double-blind study was carried out in one hospital (Ospedale Maggiore Niguarda, Milan/Italy) under the supervision of Prof. Dr. C. Ortolani.

There have been two patient groups:

- The FH 84 group which received the pollen extracts (34 patients)
- The placebo group which received a non active component (41 patients)

The patients have been randomized to the two groups according a provided randomization list.

The structural homogeneity of the two groups in regard to the concomitant factors (age, sex, weather, wind) was assured.

The pollen extracts as well as the placebo have been given in powder form. The powders were filled in sachets and the patients had to take twice a day one sachet. A sachet with FH 84 contained 840 mg of a water soluble pollen extract (Cernitin T60), 42 mg of a fat soluble pollen extract (Cernitin GBX) and inactive ingredients ad 3000 mg.

A sachet with placebo contained 3000 mg inactive ingredients.

The patients received sachets for 30 days together with a form where they had to report daily their symptoms. The following symptoms were considered for the double-blind study:

- Ocular symptoms (itching, redness, and lacrimation)
- Nasal symptoms (sneezing, running nose and blocked nose)
- Pulmonary symptoms (asthma, dyspnoea and cough)

Every patient had to assess himself the symptoms by means of a valuation scale:
0 = symptoms not present
1 = slight symptoms
2 = moderate symptoms
3 = severe symptoms

The statistical evaluation has been carried out by a simple data description and by the z–test for comparison of the mean values of two very large random samples. In the statistical tests the unilateral alternative hypothesis that the FH 84 treatment acts better than placebo was laid down.

3. Results

For the ocular symptoms, itching, redness and lacrimation, it can be demonstrated that under the treatment with FH 84 the mean intensity was lower than under placebo. The differences ranged from a trend to slight statistical significance ($0.04<p<0.10$). Here, considered globally, a slightly significantly better efficacy of the FH 84 treatment was thus to be observed.

For the nasal symptoms, sneezing, running nose and blocked nose, no better efficacy was observed under the treatment with FH 84 ($p>0.45$).

For the pulmonary symptoms, asthma, dyspnoea and cough, a slight trend can perhaps be recognized for a somewhat better effect with FH 84 than with placebo ($0.05<p<0.15$).

During the whole study no patient of the two groups showed side effects. FH 84 as well as placebo has been very well tolerated.

FH 84 in Allergy Rhinitis
1990

However it seems a statistical evaluation has not been done, that FH 84 had an additive effect when given together with other antiallergic agents.

In Italy a double-blind clinical study has been carried out in 1988. The first group (34 patients) received two sachets with FH 84 daily. The second group (41 patients) received two sachets with a placebo powder daily.

The efficacy of FH 84 and placebo on the following symptoms had to be observed:

- Ocular symptoms (itching, redness and lacrimation)
- Nasal symptoms (sneezing, running nose and blocked nose)
- Pulmonary symptoms (asthma, dyspnoea and cough)

There has been observed a slightly significantly better efficacy of FH 84 concerning the ocular symptoms whereas no better efficacy has been seen for the nasal symptoms. For the pulmonary symptoms a slight trend of a better efficacy with FH 84 than placebo has been found.

FH 84

FH 84 is a product containing standardized pollen extracts. FH 84 is used in the treatment of allergic rhinitis above all against hay fever.

FH 84 is presented in sachets of 3 grams and has the following composition:

- Cernitin T60
  (Water-soluble pollen extract) 840 mg
- Cernitin GBX
  (Fat-soluble pollen extract) 42 mg
- Inactive ingredients ad 3000 mg

Dosage: Twice a day 1 to 2 sachets in half glass water

Side effects and contraindications: Have not been reported up to now.

Clinical studies with FH 84

In Switzerland (Tessin) in 1985 and 1986 44 patients with hay fever have been treated with FH 84. The patients have received 1-2 sachets with FH 84 daily.

A very good efficacy of FH 84 treatment has been observed in 6 patients (13.6%), a good efficacy in 15 patients (34%), a moderate efficacy in 10 patients (22.7%) and an insufficient efficacy in 13 patients (29.6%).

In Argentina a clinical study has been carried out in 1986 with three groups of patients.

The first group (17 patients) received one sachet with FH 84 a day. The second group (10 patients) received two sachets with FH 84 a day and to the third group (20 patients) was given daily one sachet with placebo.

Most of the patients of all three groups were treated besides the test substances (FH 84 or placebo) with other antiallergic agents. For this reason it must be said that the mostly good effects of the treatment have not been exclusively the result of FH 84 treatment.

Physicochemical and immunochemical characterization of allergenic proteins from rye-grass (*Lolium perenne*) pollen prepared by a rapid and efficient purification method

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Three fractions of rye-grass (Lolium perenne) pollen extract have been isolated by preparative isoelectric focusing (i.e.f.) and characterized in terms of physicochemical and immunochemical properties. The purified components were designated 'R7' and 'R14' on the basis of their positions in relation to other rye-grass pollen extract components on SDS/polyacrylamide-gel electrophoresis and their apparent molecular masses were assessed as 31 and 11 kDa respectively. On i.e.f., R14 split into two components, one acidic (pI 5.0) and one basic (pI 9.0), termed 'R14a' and 'R14b' respectively, and R7 focused at pI 5.8. R7 and R14a were shown to be allergenic by skin-prisk test and all three components were recognized by rye-grass-pollen-specific human IgE. On SDS/polyacrylamide-gel electrophoresis and i.e.f., R7 behaved in a manner identical with that shown by an authentic sample of Rye I and gave an amino acid analysis similar to published data [Johnson & Marsh (1966) Immunochemistry 3, 91-100] for Rye group-I isoallergens; the amino acid sequence of the first 27 N-terminal amino acids was also determined. Physicochemical analysis revealed that R14a was equivalent to Rye II and 14b to Rye III. Preparative i.e.f. followed by gel-permeation chromatography proved to be a rapid and efficient method for purifying the allergenic components of Rye I (R7), Rye II (R14a) and Rye III (R14b) from rye-grass pollen extract.

Introduction

Extracts of pollen aeroallergens are usually composed of heterogeneous mixtures of protein and glycoprotein components. Such complexity has compromised both the investigation of the immunological events underlying the clinical manifestations of pollenosis and the standardization of pollen extracts for diagnostic and therapeutic use (Randolf, 1981; Yunginger, 1983). For progress to be made in these areas, therefore, it is evident that the availability of rapid, reproducible and efficient fractionation procedures allowing for the isolation and preparation of major allergen components is highly desirable.

Early fractionations of pollen extracts utilized conventional protein-chemistry separation procedures such as salt precipitation, and ion-exchange and gel-permeation chromatography (see, e.g. Johnson & Marsh, 1965). These multi-step methods, although often yielding relatively homogeneous materials, are commonly limited in usefulness by protracted preparation times and low yields. Preparative i.e.f. using either polyacrylamide gels or granulated gel beds now offers the potential of preparing components of

preparing components of high quality in good yield (Topping *et al.,* 1978; Ekramaddoullah *et al.,* 1981; Chakrabarty *et al.,* 1981).

The present paper describes the use of such methodology in the fractionation of rye-grass (*Lolium perenne)* pollen extract, the characterization of three major allergenic components, and their relationship to the earlier classification of Johnson & Marsh (1965).

MATERIALS AND METHODS

Materials

Rye grass (*Lolium perenne)* pollen was supplied by Bencard (Worthing, Sussex, U.K.). Authentic samples of rye groups I, II, and III were obtained from the Bureau of Biologics (Bethesda, MD, U.S.A.). Sephadex G-75 (superfine) and i.e.f. standards were obtained from Pharmacia AB; Ampholines, PAG-Plates for analytical i.e.f. and TSK 2000 SW h.p.l.c. columns were from LKB-Produkter AB; cellulose discs (size 0.6 cm; lot no. 541) were from Whatman. Diafiltration membranes were purchased from Amicon U.K. and membrane filters from Millipore Corp. $Na^{125}I$ was obtained from Amersham International. Other chemical reagents were from Sigma Chemical Co. or BDH.

Amino acid analysis

Protein samples that had been hydrolysed with 6 M-HCl for 24 h *in vacuo* were separated by cation-exchange chromatography, using a citrate/borate buffer (pH gradient of 2-11.5), on a Chromospek analyser. Amino acids were detected by post-column derivatization with ninhydrin.

Analytical gel-permeation h.p.l.c.

This was performed on a TSK 2000 SW column with either 0.3 M-sodium phosphate, pH 6.9, or 0.08 M-sodium phosphate/0.32 M-NaCl/20% (v/v) ethanol buffer, pH 7.0, as eluent.

Automatic *N*-terminal sequence analysis

This was performed by Mr. B. Dunbar and Professor J.E. Fothergill (Department of Biochemistry, University of Aberdeen, Aberdeen, Scotland, U.K.) using a Beckman 890c sequencer in conjunction with a Waters 5μ spherical C18 reverse-phase h.p.l.c. column. The methodology was described by Smith *et al.* (1982) and Carter *et al.* (1983).

Carbohydrate analysis

The phenol/H_2SO_4 method of Dubois *et al.* (1956) was used to determine the total carbohydrate content relative to a glucose standard.

Detection of contaminating Ampholines

T.l.c. was performed on protein samples with 10% (w/v) trichloroacetic acid as the solvent. Under these conditions the protein was precipitated at the origin, whereas any Ampholines present migrated with the solvent front and could be detected with ninhydrin.

Electrophoretic analysis

Polyacrylamide-gel electrophoresis in the presence of SDS was performed on a vertical slab-gel apparatus (Bio-Rad Laboratories) by the method of Laemmli (1970). The apparent molecular masses of the allergens were estimated by using the following marker proteins: bovine serum albumin (68 kDa), H-chain (50 kDa) and L-chain (23.5 kDa) of human IgG, ovalbumin (43 kDa), myoglobin (17.2 kDa) and cytochrome *c* (11.7 kDa) or prestained protein standards (3-43 kDa) from Bethesda Research Laboratories.

Analytical i.e.f. was performed on PAG-Plates, pH range 3.5-10, according to the manufacturer's instructions.

Preparation of rye-grass pollen extract

Rye-grass pollen (100 g) was defatted with sodium-metal-dried diethyl ether (2x1 liter) and extracted for 24 h with 10 mM-NH_4HCO_3 (litre, pH 7.0). Clarification of the solution was

achieved by centrifugation (10000 *g* for 30 min) and by sequential filtration through 1.2 µm-down to 0.22 µm-pore-size membrane filters. The resulting aq. 1% (w/v) extract was either stored at –20 °C before further purification, or dialyzed against 10 mM- NH_4HCO_3 and freeze-dried. This latter material is referred to as 'rye-grass pollen extract' in the text.

Purification of rye fractions

The aq. 1% (w/v) rye-grass pollen extract (100 ml) was diafiltered against distilled water over an Amicon PM10 membrane (10000-M_r nominal cut off). Pre-washed and dried Sephadex G-75 SF (5.5g) was suspended in the dialysis residue together with a 40% Ampholine buffer solution (5.5 ml), pH range 3.5-10. Preparative i.e.f. was performed on an LKB 2117 Multiphor apparatus as described by Winter *et al.* (1975). Subsequently, the gel bed was sectioned, fractions were eluted with distilled water (10 ml), and freeze-dried before characterization.

Fractions from the preparative i.e.f. run were further purified by gel-permeation chromatography on Sephadex G-75 SF in 50 mM-NH_4HCO_3, pH 8.0. Although a single passage of the three fractions yielded essentially homogeneous materials, as assessed by SDS/polyacrylamide-gel electrophoresis, a second chromatographic elution was routinely employed to ensure the removal of carrier ampholytes. The absence of contaminating ampholytes in the three purified fractions was confirmed by t.l.c., amino acid analysis and SDS/polyacrylamide-gel electrophoresis.

There appeared to be no further advantage in subjecting R7, R14a and R14b to ion-exchange chromatography after the gel-filtration step.

Determination of allergenic activity

Skin testing was performed, with informed consent, on the forearm of grass-pollen-sensitive human volunteers as described by Marsh *et al.* (1966), a range of concentrations of the starting rye-grass pollen extract and the purified components being used.

Radioallergosorbent test (RAST) inhibition assays were performed by incubating various concentrations of inhibitor (grass pollen extract and purified fractions) with a serum pool obtained from five grass-pollen-sensitive individuals (25 µl at 1:5 dilution) for 4 h at ambient temperature, before carrying out the RAST assays described by Ceska *et al.* (1972). Bound IgE was detected with ^{125}I-labelled rabbit anit-human IgE, raised against the YUIgE myeloma, purified and iodinated as described by Johansoon *et al.* (1971). Assay methodology used and calculations of the results have been described in detail by Gleich *et al.* (1974) and Chakrabarty *et al.* (1981).

Determination of antigenic activity

This was performed by a modification of the micro immunoassay procedure described by Moran *et al.* (1978) to measure allergen-specific IgG. A serum pool obtained from 23 grass-pollen-sensitive humans and known to contain rye-specific IgG antibodies was pre-incubated with various dilutions of rye or purified rye components before the addition of purified ^{125}I-R7. After incubation, the radiolabelled R7 bound to IgG antibody was immunoprecipitated with Protein A-Sepharose, and the radioactivity present on the solid phase counted in a counter. All reagents were prepared and used in the same proportions as described previously (Moron *et al.,* 1978).

RESULTS AND DISCUSSION

Physical and chemical characterization of purified components

Rye-grass pollen extract is composed of a mixture of protein-staining components with M_r (by SDS/polyacrylamide-gel electrophoresis) values between 11000 and 88000, which have been numbered sequentially R1-R14 in order of decreasing M_r (Moran *et al.,* 1982).

Preparative i.e.f. of rye-grass pollen extract was carried out over the pI range 3-10, since whole extract exhibited protein-staining components on analytical i.e.f. of pI values over the range 3.5-9.3. This yielded at least seven discrete fractions, as shown by SDS/polyacrylamide-gel electrophoresis. The major fraction, focusing around pH 5.8, was designated 'R7', by comparison of its SDS/polyacrylamide-gel electrophoresis profile with that of whole pollen extract (approx. M_r 31000). Two other main components, both with apparent M_r values of 11000, were recovered, one in the acidic region (approx. pI 5.0), and one in the basic region (approx. pI 9.0). These components, designated 'R14a' and 'R14b' respectively, are the main constituents of the R14 band of whole pollen extract. Electrophoretic analysis of other fractions obtained from preparative i.e.f. revealed the partial separation of other components of rye-grass pollen extract, including R4 (M_r 64000), R8 (M_r 27000), R9 (M_r 25000) and a basic red material of apparent M_r 10000. No attempt was made to purify or characterize these latter components further.

The preparative i.e.f. fractions containing R7 and the two R14 components were subjected to gel-permeation chromatography on Sephadex G-75 SF to remove contaminating proteins. The R7, R14a and R14b thus prepared had electrophoretic mobilities identical with those of authentic samples of Rye groups I, II, and III respectively (Fig. 1).

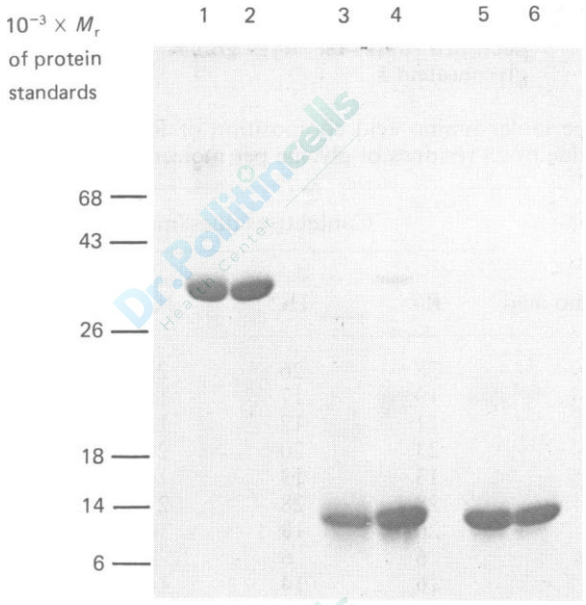

Fig. 1. Analysis of the purified components of rye-grass pollen extract by SDS/polyacrylamide-gel electrophoresis and protein staining
Rye I (1), R7 (2), Rye 11 (3), Rl14a (4), Rye III (5) and Rll4b (6) were electrophoresed on a 12.5% (w/v) polyacrylamide gel under reducing conditions. A 5 1ag sample of protein was loaded per track.

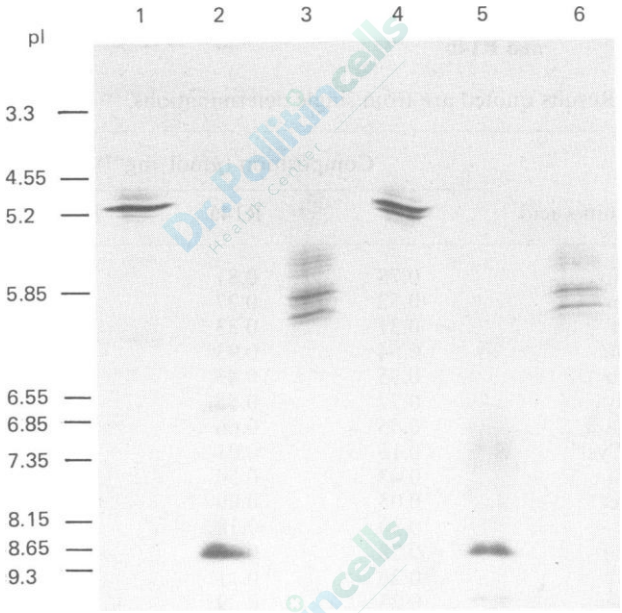

Fig. 2. Analytical i.e.f. of purified rye components
Rl4a (1), Rl4b (2), R7 (3), Rye 11 (4) Rye III (5) and Rye 1 (6) were subjected to i.e.f. on PAG-Plates (LKB-Produkter AR), pH range 3.5-10; 7 /sg of each protein was used. The pl values for the protein standards (Pharmacia AR) are indicated.

The contamination of these components by other rye proteins was estimated at less than 2% (w/w) by consideration of the limits of sensitivity of Coomassie Blue staining.

The average yield of purified R7 from freeze-dried dialyzed rye-grass pollen extract was found to be 8% (w/w); its M_r of 11000; the R14b component, recovered in 2% yield, had an M_r of 11000, but migrated very slightly in front of R14a (Fig. 1). The yields reported for fractions of rye-grass pollen extracts of 1.3% for rye group 1B (Johnson & Marsh, 1965) and 2.7% for glycoprotein 1 (Howlett & Clarke, 1981) compare favorably with these.

All three purified rye components chromatographed as single entities on a pre-calibrated TSK 2000 SW gel-permeation h.p.l.c. column. Both R14a and R14b were eluted with 0.3м-phosphate buffer, pH 6.9, and found to have a M_r of 12000. R7 aggregated in this buffer, but was successfully eluted in 0.08м-phosphate/0.32м-NaCl/20% ethanol, pH 7.0, with an M_r of 32500.

Analytical i.e.f. revealed microheterogeneity of R7 and R14a, arising from minor charge variations (Fig. 2). Fraction R7 had a pI over the range 5.5—6.0, R14a focused over the pI range 4.6—5.2 and R14b appeared as a diffuse band at a pI of 9.0. In agreement with the SDS/polyacrylamide-gel electrophoresis data, R7, R14a and R14b were found to correspond to Rye groups I, II and III respectively (Fig. 2). The charge inhomogeneity for R7 and Rye group I shown on i.e.f. is consistent with previously reported date for both Rye group I (Johnson & Marsh, 1966) and glycoprotein I (Howlett & Clarke, 1981). This latter material is reported to show poor affinity for the lectin concanavalin A; R7, also glycosylated, behaved similarly (R. Standring, unpublished work).

Typical amino acid contents of R7, R14a and R14b are shown in Table 1. When the amino acid composition of R7 was compared with published data (Table 2) for rye groups 1B and 1C (Johnson & Marsh, 1966) and for glycoprotein I (Howlett & Clarke, 1981), a good correlation was found that was consistent with these proteins being equivalent.

The carbohydrate contents of the three fractions R7, R14a and R14b, determined against a glucose standard, were 4, 2, and 2% respectively. The low carbohydrate content of R14b is especially surprising in the light of the low recovery in the amino acid analysis and the apparent absence of any salt contamination as determined by chromatographic analysis. A similar low – M_r highly basic fraction isolated Kentucky-blue-grass pollen extract (allergen C) (Chakrabarty *et al.,* 1981) had a carbohydrate content of up to 500 µg/mg of protein, as assayed by a similar phenol/ H_2SO_4 method.

Partial primary sequence data for fraction R7 were also obtained (Fig. 3). Despite the microheterogeneity in R7, indicated by charge variations evident in the i.e.f. pattern, 27 of the first 30 *N*-terminal amino acids were readily identified. The unique *N*-terminus was identified as isoleucine and was in accord with that reported for rye group I (Johnson & Marsh, 1966). Of the three unidentified residues, positions 5 and 8 were tentatively assigned as cysteine (R7 was not submitted for sequence analysis as the carboxymethylated form) and position 9 was possibly glycosylated. Sequence data were not obtained for fractions R14a and R14b. The ability to assign the partial sequence of R7 positively, despite the obvious inhomogeneity as seen on i.e.f., supports the view that the charge variations seen in the rye group-I isoallergens arise from minor differences in glycosylation or amidation rather than major differences in the primary structure.

Table 1. Typical amino acid composition of fractions R7, R14a and R14b

Results quoted are from single determinations.

Amino acid	Composition (μmol• mg⁻¹) R7	R14a	R14b
Asp	0.78	0.81	038
Thr	0.52	0.27	0.41
Ser	0.31	0.33	0.16
Glu	0.64	0.93	0.44
Pro	0.35	0.43	0.18
Gly	0.77	0.55	0.34
Ala	0.55	0.66	0.15
½-Cys	0.16	0.03	
Val	0.43	0.50	0.30
Met	0.05	0.09	0.10
Ile	0.30	0.18	0.07
Leu	0.28	0.36	0.31
Tyr	0.26	0.11	0.11
Phe	0.23	0.29	0.16
His	0.14	0.13	0.06
Lys	0.72	0.78	0.43
Arg	0.21	0.22	0.13
Total recovery (μ• mg⁻¹)…	840	842	480

Table 2. Comparison of the amino acid content of R7 with published date on Rye groups 1B and 1C and glycoprotein 1

The molar amino acid composition of R7 is based on a value of 28 residues of glycine per molecule of R7.

Amino acid	Content (residues/ molecule) R7	1B*	1C*	Glyco-protein 1 †
Asp	28	26	26	26
Thr	19	17	16	19
Ser	11	12	11	14
Glu	23	20	20	22
Pro	13	13	14	14
Gly	28	28	27	28
Ala	20	18	18	24
½-Cys	6	6	6	4
Val	16	14	14	15
Met	2	2	2	2
Ile	11	10	10	11
Leu	10	9	11	11
Tyr	9	9	9	8
Phe	8	8	8	9
His	5	3	3	3
Lys	26	26	26	25
Arg	8	6	6	6

* Data from Johnson & Marsh (1966).
† Data from Howlett & Clarke (1981).

Immunochemical/biological characterization

Since R7, R14a and R14b were found to resemble major rye-grass allergens Rye I, II and III with regard to their physicochemical properties, their interaction with human IgE (allergenicity) was investigated. Two methods were employed: skin testing (components R7 and R14a only) and radioallergosorbent test (RAST) inhibition. Table 3 shows the minimum concentration of allergen required to elicit a weal of area greater than 20 mm² in the skin-prick test performed on six grass-pollen-sensitive individuals. Overall the R7 component elicited a response at lower concentrations than that required for the whole rye-grass pollen extract; in three of six subjects studied 10-fold lower concentrations of R7 than whole extract were required. The R14a component, however, although matching these responses in four volunteers, did not elicit any significant reaction in two individuals (C and F, Table 3) up to a concentration of 10 μg•ml⁻¹. It is noteworthy that, on ummunoprecipitation of ^{125}I-rye-protein-IgG complexes from the sera of these two individuals followed by SDS/polyacrylamide-gel electrophoresis and autoradiography [by the methodology described previously (Moran *et al.,* 1982)], there was no apparent serological response to the R14a/R14b band at 11000 M_r.

Typical RAST inhibition data is shown in Fig. 4. In general, although there were slight batch-to-batch variations in the actual inhibition curves, the three fractions R7, R14a and R14b showed a loss in inhibitory activity when compared with whole rye-grass pollen extract. Even at high concentrations (1 mg • ml⁻¹), the purified components did not completely inhibit the uptake of the grass-pollen-specific IgE on to the rye-protein coated discs. The human serum pool

employed in the assay was shown to contain IgE antibodies against the R7, R14a and R14b components by using the Western-blotting method, as described by Sutton *et al.* (1982). This confirmed that the loss of ability to inhibit 100% of the IgE-reactive property of the whole extract coupled to the disc was due to removal of allergenic specificities on purification.

From the electrophoretic analysis (Fig. 1) and analytical h.p.l.c. results, any cross-contamination between R7 and R14a appeared to be at low levels. Further confirmation of this and any possible antigenic cross-reactivity between these two components was obtained by a ^{125}I-R7 inhibition assay (Fig. 5). The uptake of ^{125}I-R7 on to human IgE from grass-pollen-sensitive individuals was inhibited by R7 than whole rye-grass pollen extract, whereas R14a was a much less effective inhibitor. From these results, it could be calculated that rye-grass pollen extract contained approx. 18% (w/w) of R7 antigenic activity and R14a contained no more than 1% (w/w) of R7 activity.

```
Ile – Ala – Lys – Val – * – Pro – Gly – * – † – Ile –
 1                       5                      10

Thr – Ala – Glu – Tyr – Gly – Asp – Lys – Trp – Leu – Asp
11                      15                      20

Ala – Lys – Ser – Thr – Trp – Tyr – Gly – Lys – Pro – Thr
21                      25                      30
```

Fig. 3. Automatic *N*-terminal sequence analysis of R7

*****, Residures 5 and 8 tentatively assigned as Cysteine; †, residue 9 is possibly glycosylated.

Table 3. Comparison of the allergenicity of Rye, R7 and R14a by prick test in human volunteers

Minimum concentration for weal 20 mm² (μg • ml⁻¹)

	(a)		(b)	
Volunteer	R7	Rye	R14a	Rye
A	2.5	3.1	1.25	3.1
B	1.25	12.5	0.63	6.3
C	1.25	50	No response at 10	12.5
D	10	25	0.63	6.3
E	2.5	3.1	0.63	1.6
F	1.25	25	10	6.25

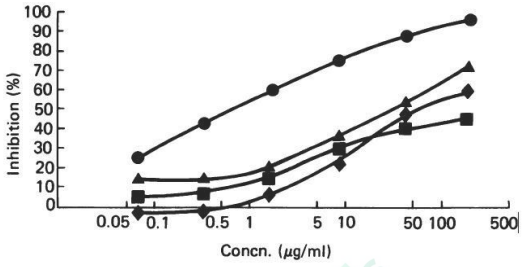

Fig. 4. Allergenicity of purified rye components relative to whole rye-grass pollen extract assessed by the RAST inhibition assay

The uptake of rye-pollen-extract-specific IgE from a human serum pool on to whole-extract-coated cellulose discs is inhibited by whole rye-grass pollen extract (●), R7 (▲), R14a (■) and R14b (♦).

In agreement with results obtained for rye groups I and II (Marsh *et al.*, 1966), both R7 and R14a were shown to have potent allergenic activities as assessed by skin test, RAST inhibition and direct IgE binding. The R14b component was tested only by 'in vitro' techniques, but was shown to be recognized by human IgE on Western blotting and RAST inhibition. The apparent lower activity of all three components relative to whole rye-grass pollen extract by RAST

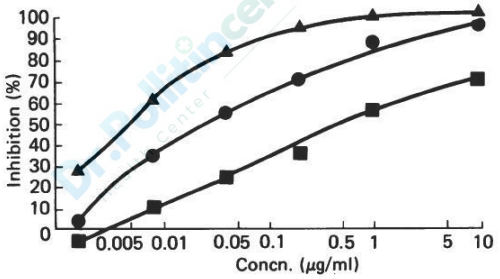

Fig. 5. Inhibition of the uptake of ^{125}I-R7 binding to human IgG from a pool of serum containing antibodies directed against antigenic components of whole rye-grass pollen extract. Inhibitors were whole-rye-grass pollen extract (●), R7 (▲), and R14a (■).

inhibition suggested that a significant part of the response to rye-grass pollen is directed against components other than R7, R14a or R14b. However, no attempt was made to confirm this point with a mixture of all three components. Partial denaturation of the purified fractions during the extractive procedures could not be excluded as an explanation for this effect. Nevertheless, similarly reduced RAST inhibitory activity was apparent with authentic Rye groups I, II and III, obtained from the Bureau of Biologics. The poor skin-test response of two individuals to R14a (Table 3) could be explained by variations in the response of humans to individual allergenic components in rye-grass pollen and accords with the findings of Marsh *et al.* (1970), who showed that only 70% of grass-pollen-sensitive individuals responded to rye group II (R14a).

Conclusions

Three fractions of rye-grass pollen extract were readily purified from whole extract by a combination of preparative i.e.f. and gel-permeation chromatography. The purities of the fractions were formally assessed as being not less than 98%, with no contaminating ampholytes being detected. The three fractions R7, R14a and R14b were identified with the previously described rye groups I, II and III respectively, on the basis of physicochemical and immunological properties. This rapid and reproducible procedure for obtaining relatively large quantities of purified major allergens has obvious applications in the development of improved diagnostic and therapeutic reagents.

We thank Mr. B. Dunbar and Professor J.E. Fothergill, Department of Biochemistry, University of Aberdeen, for carrying out the sequence analysis and Miss S. J. Porter and Mr. Y.K. Davé for their excellent technical assistance.

References

Carter, P.E., Dunbar, B. & Fothergill, J.E. (1983) Biochem. J. 215, 565-571

Ceska, M., Eriksson, R. & Varga, J.M. (1972) J. Alergy Clin. Immunol. 49, 1-9

Chakrabarty, S., Ekramaddoullah, A.K.M., Kisil, F.T. & Sehon, A.H. (1981) Int. Arch. Allergy Appl. Immunol. 65, 377-389

Dubois, M., Gilles, K.A. Hamilton, J.K., Rebers, P.A. & Smith, F. (1956) Anal. Chem. 28, 350-356

Ekramaddoullah, A.K.M., Kisil, F.T. & Sehon, A.H. (1981) Int. Arch. Allergy Appl. Immunol. 65, 367-376

Gleich, G.J., Larson, J.B., Jones, R.T. & Baer, H. (1974) J. Allergy Clin. Immunol. 53, 158-169

Howlett, B.J. & Clarke, A.E. (1981) Biochem. J. 197, 695-706

Johansson, S.G.O., Bennich, H. & Berg, T. (1971) Int. Arch. Allergy Appl. Immunol. 41, 443-451

Jognson, P & Marsh, D.G. (1965) Eur. Polymer J. 1, 63-77

Johnson, P. & Marsh, D.G. (1966) Immunochemistry 3, 91-100

Laemmli, U.K. (1970) Nature (London) 227, 680-685

Marsh, D.G., Milner, F.H. & Johnson, P. (1966) Int. Arch. Allergy Appl. Immunol. 29, 521-535

Marsh, D.G. Haddad, Z.H. & Campbell, D.H. (1970) J. Allergy 46, 107-121

Moran, D.M., Dupe, B.E. & Guantlett, S. (1978) J. Immunol. Methods 24, 183-191

Moran, D.M., Strandring, R. & Henderson, D.C. (1982) Int. Arch. Allergy Appl. Immunol. 69, 120-126

Randolf, W.F. (1981) Fed. Regist. 46, 39135-39136

Smith, M.A., Gerrie, L.M., Dunbar, B. & Fothergill, J.E. (1982) Biochem. J. 207, 253-260

Sutton, R., Wrigley, C.W. & Baldo, B.A. (1982) J. Immunol. Methods 52, 183-194

Topping, M.D., Brighton, W.D., Stokell, M. & Patterson, J.M. (1978) J. Immunol. Methods 19, 61-67

Winter, A., Perlmutter, H. & Davies, H. (1975) LKB Application Note 198, LKB-Produkter A.b>, Stockholm

Yunginger, J.W. (1983) Symp. Pediat. Allergy: Pediat. Clin. North Am. 30, 225-23

ALLERGY SUPPORT

GRAMINEX Flower Pollen Extract

Study of Tolerance of the Stheborex in Patients with Pollen Allergy

Dr. GARCELON

The term pollen allergy covers the totality of pathological processes that occur when pollen grains come into contact with the conjunctival and respiratory mucosa of specifically sensitized individuals. But what happens if the contact takes place with a different mucosal surface, such as that of the digestive tract?

This is the question that one is entitled to ask in relation to the drug STHENOREX, an appetite-stimulant drug composed of water-soluble and lipid-soluble extracts of pollens, comprising:

- 2 species of tree pollen: pine and alder.
- 4 species of grass pollen, viz:
  - 2 cerals: rye and maize, and
  - 2 species of hay-grasses, timothy and cocksfoot.

These extracts are contained in a ‘gelule’ which only releases the active compounds contained in it in the presence of gastric juice.

Research carried out several years ago by Madame VAN CAMPO, Director of Research at CARS, demonstrated the presence of numerous pollens in ordinary white bread and rye bread.

Thus:

- in 18g of ordinary white bread she found 364 grains of all kinds of pollens, representing, 20 grains of pollen per gram of bread, of which 17 were grains of cereal pollen (table A);
- in 10g of crumb of rye bread, she found 701 grains of pollen, or 70 grains per gram, of which 25 were grains of cereal pollen (table B).

Now individuals who suffer from typical pollen allergy eat bread without thereby aggravating their symptoms.

It is therefore justifiable to expect that pollen that is ingested and therefore digested, undergoes such a degree of chemical breakdown that it loses all capacity of provoking allergic reactions on the digestive mucosa.

This hypothesis, in the particular case of STHENOREX, has been completely confirmed by the clinical trial carried out by Dr. Garcelon.

We have made a search for clinical sensitivity to STHENOREX in patients consulting us for spasmodic coryza, conjunctivitis or seasonal asthma (in May, June or July), provoked by allergy to a variety of pollens.

These symptoms were present individually or in various combinations, in a total of 28 patients.

A gelule of STHENOREX contains:

- Water-soluble pollen extract 120mg
- Lipid-soluble pollen extract 6mg
- Base: Q.S.P. one gelule
- Sulphurous anhydride 1g p. 1000

The composition of pollens contained in STHENOREX is as follows:

- PINE (Pinus montana)
- ALDER (Alnus glutinosa)
- RYE (Secale oereale)
- MAIZE (Zea mais)
- TIMOTHY (Phleum pratense)
- COCKSFOOT (Dactylis glomerata)

The 28 patients studied were distributed as follows:

- 18 males, mean age 26 (range 9 to 51),
- 10 females, mean age 25 years (range 9 to 40).

This confirms that pollen allergy is most commonly found amongst young people.

Pollen allergy can be objectively demonstrated by skin tests carried out with a control solution and concentrated extracts prepared by the Stallergenes laboratory:

- Trees (particularly group II).
- Grasses (12 fodder grasses and 3 cereals),
- Weeds.

A number of observations were carried out using a test based on a concentrated rye-pollen extract prepared by the Pasteur Institute. In addition, one test was systematically carried out using STHENOREX powder diluted in one drop of 0.1 N sodium bicarbonate.

EXPERIMENTAL PROTOCOL

Once the diagnosis of pollen allergy had been made and skin sensitivity to one or more groups of pollens (including the dry extract of STHENOREX) had been demonstrated, the first stage of the clinical trial comprised the oral administration of one gelule of STHENOREX. The patient remained under medical supervision for three hours, so that any immediate-type allergic reaction could be demonstrated.

Once this stage had been passed uneventfully, the patient took a further four gelules daily for one week, this being the usual dosage of the drug. If no reaction was noted, treatment was re-started 15 or 30 days later, at the same dosage, so as to investigate any possible antigenicity of the product.

Finally, when the preceding stages of the trial had passed without incident, STHENOREX was administered to sensitized subjects during the pollen season.

RESULTS

In 20 subjects tested, we made the following observations:

POSITIVE TESTS:

Fodder grasses: 27
(One subject being sensitized only to rye pollens),
Trees……..9
Cereals…….24
STHENOREX…...9

In 20 subjects who ingested STHENOREX as described above, no reaction was seen. Treatment was perfectly tolerated, even during the pollen season (June). However, patients who had been prescribed the drug for therapeutic purposes during this period (there were 5 of these) showed no improvement in their allergic symptoms from its use.

DISCUSSION

Apart from the sensitivity to rye pollens alone, seen in one of the subjects we studied, it is not surprising to note that allergy to grass pollens, which is a feature of most pollen allergy in the Paris region, was the predominant pattern, and was most commonly accompanied by sensitivity to cereal pollens, while the importance of tree pollens, though not negligible, was of minor degree.

The fact that one third of tests with STHENOREX powder gave a positive result demonstrates that despite the various modifications under-gone by the product in the course of manufacture (during which the allergenic polypeptide fractions are broken down to amino acids), the product retains its specific antigenic properties.

The degree of hypersensitivity varies from one individual to another, and it is worthy of note that seven of the eight patients who reacted to STHENOREX were those with the greatest number of positive reactions to the various groups of pollens studied.

Finally, even though cases of 'ultra-specificity' may be rare, (1 out of 28), certain patients may be sensitized to a single specific pollen, e.g. rye pollen, which is in fact contained in STHENOREX.

Other clinical and immunobiological investigations carried out in various hospitals have also shown analogous instances of cross-antigenicity between STHENOREX and various types of pollen.

CONCLUSION

At all events, clinical tolerance of STHENOREX is excellent. Its oral administration to a group of patients with pollen allergy did not give rise to any allergic reactions. The product is not itself a sensitizer, and while it contains amino acids of vegetable origin that are capable of giving rise to positive skin tests in certain subjects, it is likely that it rapidly loses all antigenic specificity during its absorption by the digestive tract.

Dr. M. GARCELON
July 1975

Hay Fever and Pollen Tablets

By Einar Helander

Pollen preparations have been marketed since 1952. Until recently, they have not been used for medical purposes, but have been sold without restrictions as a commercial article. In a paper in this journal, Ask-Upmark (1960) has, however, suggested that pollen tablets should be used in the treatment of patients with prostatitis. It is still too early to state whether such therapy is rational, since a report is given of only a few patients. On the other hand, it can be expected that pollen preparations will be tested in the near future. The object of the present paper is therefore to clarify a problem of importance in this connection—one also raised by Ask-Upmark—i.e., can pollen preparations be administered to patients with pollen allergy without causing side-effects?

This question is important, since pollen allergy—usually in the form of hay fever—has been calculated to occur in 0.5-1% of all persons in Sweden. A number of different pollen types are responsible for these allergies. During a 10-year period (Sept. 1949 to Sept. 1959), 10.509 skin tests were made at the Allergy Department, Gothenburg, on patients who attended for various allergies. Routine tests were made for pollen grains of the following species: timothy, oxeye daisy, mugwort (*Artemisia vulgaris*), birch, alder, hazel and aspen. In a few cases additional tests were, for certain reasons, made with other pollen extracts. On the basis of these skin tests, provocation tests and data given by the patients, 2072 pollen allergies were diagnosed and subsequently treated.

The distribution of these allergies can be inferred from Tab. 1 (cf. Arnoldsson 1955). Since about one-third of the patients tested were allergic to more than one pollen species, the number treated is only just over half the total figure. The figures in this table can be regarded as representative of the Gothenburg region, whereas the distribution differs slightly in other parts of Sweden (Arner 1959).

The pollen preparations on the market (Cernelle, Cernident, Cernitin, Cernitol, Cerniton, Polloton, and Pollisan) contain both pollen husks and pollen extract. The husks are separated mechanically, and then heated with a view to decreasing the risk of allergy. The pollen extracts are obtained with water and organic solvents. In the different extraction procedures, up to 82% of the total nitrogen content of the pollen grains has been recovered. The various fractions are evaporated, and combined into a substance denoted as Cernitin.

According to statements from the manufacturers, the following pollen species are used in the preparation.

1. Timothy 26%
2. Maize 26%
3. Rye 19%
4. Hazel 6%
5. Sallow 6%
6. Aspen 6%
7. Oxeye daisy 6%
8. Pine 5%

It is already mentioned that allergies to timothy, oxeye daisy, hazel and aspen are common in the Gothenburg region. Allergy to sallow is not uncommon in other parts of Sweden in which it grows more extensively (Arner 1959). Allergy to rye pollen is relatively rare, and that to pine pollen still more rare. Allergy to maize pollen is unknown in Sweden, but occurs in the U.S.A. (Urbach & Gottlieb 1946).

Present Investigation[1]

The composition of the pollen preparations gives good reason to investigate whether they can produce allergic symptoms. Several allergens cause allergic symptoms on oral administration, but the literature contains no data no whether this applies to pollen or preparations of it.

The tests were made on 25 patients who were allergic to pollen, but were healthy in other respects (see Tab. 2). The pollens to which these patients were allergic can be inferred from the table. The results of the tests were graded as follows. Histamine (1:10,000) was used as the positive control and 0.9% NaCl as the negative, the results being given in proportion to the area of the wheal produced by histamine, which is denoted as 3. Thus, 1 = a wheal with an area $^1/_3$ as large, 2 = $^2/_3$ of the area, 6 = twice as large, etc.

[1] The pollen preparations were kindly placed at my disposal by AB Cernelle, Vegeholm.

Tab. 1. Pollen allergies diagnosed at the Allergy Department, Gothenburg, 1949-1959.

Timothy and related grasses	913
Birch	358
Oxeye daisy and related plants	330
Alder	164
Hazel	150
Aspen	143
Rye	11
Fir	2
Reeds	1

1. Skin Tests with Extract of Pollen Tablets and Cernitin

After removing the sugar-coating, the Cernelle tablets were broken up and extracted with 5 parts of 0.9% NaCl, during vigorous shaking, for 2 hours on each of two consecutive days. This solution was sterile-filtered and then used for the tests. So-called cernitin, diluted to 1:10 with 0.9% NaCl, was used in the same way. Here as well, histamine was used as the positive control and 0.9% NaCl as the negative. The results were graded as described above. The extracts were tested on non-allergic subjects with negative results.

2. Demonstration of Antigen According to Praussnitz & Küstner

Venous blood was drawn from the 25 patients in question. After centrifugation, 0.1 ml of serum from each patient was injected intradermally into at least two healthy, non-allergic subjects. The latter had been given 5-25 Cernilton tablets on an empty stomach 60-90 minutes before the experiments. The results were graded as already started (Tab. 2).

3. Direct Administration of Pollen Tablets to Patients with Pollen Allergy

Each of the 25 patients with pollen allergy was given a test dose of one tablet of Cerniton. After one hour, a further four tablets were given, and somewhat later on the same day an additional 20 tablets on an empty stomach.

Tab. 2.

		Age	Skin Test for Pollen								Skin Test		Inverse Prauss.-Küst.			Reaction on oral adminis. No. of Tablets		
No.	Sex	Yrs	Ph	Be	Pr	Al	Co	Po	Ar	Se	Pt	Pe	5	15	25	1	5	25
1	F	19	–	–	–	2	3	–	–	–	2	2	–	–	(1)	–	–	–
2	M	38	–	7	–	–	–	–	–	–	2	4	–	(1)	1	–	–	–
3	M	21	3	6	–	–	–	–	–	–	2	3	–	–	–	–	–	–
4	F	24	–	3	5	–	–	–	–	–	3	3	–	(1)	1	–	–	–[a]
5	M	15	–	4	3	–	–	–	–	–	2	1	–	–	(1)	–	–	–
6	F	45	–	–	4	–	–	–	–	–	2	1	–	–	(1)	–	–	–
7	M	34	4	–	3	–		–	–	–	3	4	–	–	–	–	–	–
8	M	53	5	–	3	–	–	–	–	–	1	3	–	–	–	–	–	–[b]
9	M	23	4	–	3	–	–	–	–	4	5	7	–	–	–	–	–	–
10	M	25	4	–	–	–	–	–	–	–	2	4	–	–	(1)	–	–	–
11	M	35	5	5	–	–	–	–	–	–	3	5	–	–	–	–	–	–
12	M	57	7	4	–	–	–	–	–	–	3	5			–	–	–	–
13	F	18	5	5	6	4	3	4	–	–	3	4	–	–	–	–	–	–
14	M	39	5	–	–	–	–	–	–	–	4	5	–	–	–	–	–	–
15	F	39	–	–	3	–	–	–	–	–	1	1	–	–	(1)	–	–	–
16	M	28	–	–	6	–	–	6	–	–	2	3	–	–	–	–	–	–
17	M	23	5	3	–	–	–	–	–	–	3	5	–	–	1	–	–	–
18	M	17	4	8	–	–	–	–	–	–	3	5	–	–	(1)	–	–	–
19	F	26	–	–	4	–	–	5	–	–	2	3	–	–	–	–	–	–
20	F	22	6	–	–	–	–	–	–	–	3	5	–	–	–	–	–	–
21	M	17	4	–	–	–	–	–	–	–	2	3	–	–	–	–	–	–
22	F	52	–	–	5	–	–	–	3	–	2	4	–	–	–	–	–	–
23	M	14	7	–	–	–	–	–	–	–	4	5	–	–	(1)	–	–	–
24	M	31	5	5	–	3	1	–	–	–	2	4	–	–	(1)	–	–	–
25	F	48	6	4	–	–	–	–	–	–	4	5	–	–	–	–	–	–

Ph = timothy; Be = birch; Pr = oxeye daisy; Al = alder; Co = hazel; Po = aspen; Ar = mugwort (*Artemisia vulgaris*); Se = rye; Pt = extract of pollen tablets; Pe = Cernitin. For grading of cutaneous reactions: see text.

a Inapp. incr. coryza.
b Flatulence.

Results

The results of the experiments are recorded in Tab 2.

The skin tests, both will extract of pollen tablets and cernitin, showed that the preparations contain extremely potent allergens. In most cases, a very large wheal appeared. It can be mentioned that a patient who was allergic to birch only (pollen preparations do not contain birch pollen) also had positive reactions. I have been unable to find any explanation of this cutaneous reaction.

When the pollen preparation was administered orally the so-called inverse Praussnitz-Küstner test showed that a small but sufficiently large quantity of antigen was absorbed in some cases. The reactions were, however, inappreciable in the large majority of cases, and a definite reaction occurred in three patients only. The reactions denoted as (1) may have been unspecific.

In the cases with a definite reaction occurred, the reaction was nevertheless slight. Thus, despite the large quantity of tablets ingested, only small amounts of pollen antigen were absorbed. For this reason, the preparation might possibly be used in attempting oral desensitization in hay fever.

The tests with administration of pollen tablets showed that the incidence of side-effects was low, even with large doses. One patient stated that the large number of tablets produced flatulence, and another that he seemed to feel some increased coryza, persisting for about 12 hours. The patient's complaints were regarded as so negligible that no therapy was indicated.

Unfortunately, the preparation could not be tested in patients with allergy to maize, pine, or sallow pollen. However, in Sweden these allergies play an insignificant or no role. Moreover, there is no reason to believe that they involve any essential differences.

To sum up, it can be stated that the experiments have shown the following:

1. The pollen tablets contain highly concentrated pollen antigens, which are not inactivated by the technical procedure used in their preparation.
2. On oral administration of pollen preparations, the antigen or components of it may be absorbed.
3. Absorption is, however, so slight that no risk of serious complications seems to exist, even if large doses are taken by subjects with pollen allergies. Consequently, hay fever is not a contraindication in those cases in which it is desired to test the effect of pollen preparations in, e.g., prostatitis.

References

1. ARNER, B.: Allergisk snuvn. Recip inform. 1959.
2. ARNOLDSSON, H.: Nord. Med. 54: 1885, 1955.
3. ASK-UPMARK, E.: Grana palynol. 2: 115, 1960; see also Sv. Läkartidn. 56: 1840, 1959.
4. URBACII, E. & GOTTLIEB, P. M.: Allergy 2nd. Ed. Heineman, London, 1946.

Physicochemical and immunochemical characterization of allergenic proteins from rye-grass (Lolium perenne) pollen prepared by a rapid and efficient purification method

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Three fractions of rye-grass (Lolium perenne) pollen extract have been isolated by preparative isoelectric focusing (i.e.f.) and characterized in terms of physicochemical and immunochemical properties. The purified components were designated 'R7' and 'R14' on the basis of their positions in relation to other rye-grass pollen extract components on SDS/polyacrylamide-gel electrophoresis and their apparent molecular masses were assessed as 31 and 11 kDa respectively. On i.e.f., R14 split into two components, one acidic (pI 5.0) and one basic (pI 9.0), termed 'R14a' and 'R14b' respectively, and R7 focused at pI 5.8. R7 and R14a were shown to be allergenic by skin-prisk test and all three components were recognized by rye-grass-pollen-specific human IgE. On SDS/polyacrylamide-gel electrophoresis and i.e.f., R7 behaved in a manner identical with that shown by an authentic sample of Rye I and gave an amino acid analysis similar to published data [Johnson & Marsh (1966) Immunochemistry 3, 91-100] for Rye group-I isoallergens; the amino acid sequence of the first 27 N-terminal amino acids was also determined. Physicochemical analysis revealed that R14a was equivalent to Rye II and 14b to Rye III. Preparative i.e.f. followed by gel-permeation chromatography proved to be a rapid and efficient method for purifying the allergenic components of Rye I (R7), Rye II (R14a) and Rye III (R14b) from rye-grass pollen extract.

Introduction

Extracts of pollen aeroallergens are usually composed of heterogeneous mixtures of protein and glycoprotein components. Such complexity has compromised both the investigation of the immunological events underlying the clinical manifestations of pollenosis and the standardization of pollen extracts for diagnostic and therapeutic use (Randolf, 1981; Yunginger, 1983). For progress to be made in these areas, therefore, it is evident that the availability of rapid, reproducible and efficient fractionation procedures allowing for the isolation and preparation of major allergen components is highly desirable.

Early fractionations of pollen extracts utilized conventional protein-chemistry separation procedures such as salt precipitation, and ion-exchange and gel-permeation chromatography (see, e.g. Johnson & Marsh, 1965). These multi-step methods, although often yielding relatively homogeneous materials, are commonly limited in usefulness by protracted preparation times and low yields. Preparative i.e.f. using either polyacrylamide gels or granulated gel beds now offers the potential of preparing components of

high quality in good yield (Topping *et al.,* 1978; Ekramaddoullah *et al.,* 1981; Chakrabarty *et al.,* 1981).

The present paper describes the use of such methodology in the fractionation of rye-grass (*Lolium perenne)* pollen extract, the characterization of three major allergenic components, and their relationship to the earlier classification of Johnson & Marsh (1965).

MATERIALS AND METHODS

Materials

Rye grass (*Lolium perenne)* pollen was supplied by Bencard (Worthing, Sussex, U.K.). Authentic samples of rye groups I, II, and III were obtained from the Bureau of Biologics (Bethesda, MD, U.S.A.). Sephadex G-75 (superfine) and i.e.f. standards were obtained from Pharmacia AB; Ampholines, PAG-Plates for analytical i.e.f. and TSK 2000 SW h.p.l.c. columns were from LKB-Produkter AB; cellulose discs (size 0.6 cm; lot no. 541) were from Whatman. Diafiltration membranes were purchased from Amicon U.K. and membrane filters from Millipore Corp. $Na^{125}I$ was obtained from Amersham International. Other chemical reagents were from Sigma Chemical Co. or BDH.

Amino acid analysis

Protein samples that had been hydrolysed with 6 M-HCl for 24 h *in vacuo* were separated by cation-exchange chromatography, using a citrate/borate buffer (pH gradient of 2-11.5), on a Chromospek analyser. Amino acids were detected by post-column derivatization with ninhydrin.

Analytical gel-permeation h.p.l.c.

This was performed on a TSK 2000 SW column with either 0.3 M-sodium phosphate, pH 6.9, or 0.08 M-sodium phosphate/0.32 M-NaCl/20% (v/v) ethanol buffer, pH 7.0, as eluent.

Automatic *N*-terminal sequence analysis

This was performed by Mr. B. Dunbar and Professor J.E. Fothergill (Department of Biochemistry, University of Aberdeen, Aberdeen, Scotland, U.K.) using a Beckman 890c sequencer in conjunction with a Waters 5μ spherical C18 reverse-phase h.p.l.c. column. The methodology was described by Smith *et al.* (1982) and Carter *et al.* (1983).

Carbohydrate analysis

The phenol/H_2SO_4 method of Dubois *et al.* (1956) was used to determine the total carbohydrate content relative to a glucose standard.

Detection of contaminating Ampholines

T.l.c. was performed on protein samples with 10% (w/v) trichloroacetic acid as the solvent. Under these conditions the protein was precipitated at the origin, whereas any Ampholines present migrated with the solvent front and could be detected with ninhydrin.

Electrophoretic analysis

Polyacrylamide-gel electrophoresis in the presence of SDS was performed on a vertical slab-gel apparatus (Bio-Rad Laboratories) by the method of Laemmli (1970). The apparent molecular masses of the allergens were estimated by using the following marker proteins: bovine serum albumin (68 kDa), H-chain (50 kDa) and L-chain (23.5 kDa) of human IgG, ovalbumin (43 kDa), myoglobin (17.2 kDa) and cytochrome *c* (11.7 kDa) or prestained protein standards (3-43 kDa) from Bethesda Research Laboratories.

Analytical i.e.f. was performed on PAG-Plates, pH range 3.5-10, according to the manufacturer's instructions.

Preparation of rye-grass pollen extract

Rye-grass pollen (100 g) was defatted with sodium-metal-dried diethyl ether (2x1 liter) and extracted for 24 h with 10 mM-NH_4HCO_3 (litre, pH 7.0). Clarification of the solution was

achieved by centrifugation (10000 *g* for 30 min) and by sequential filtration through 1.2 µm-down to 0.22 µm-pore-size membrane filters. The resulting aq. 1% (w/v) extract was either stored at –20 °C before further purification, or dialyzed against 10 mM- NH_4HCO_3 and freeze-dried. This latter material is referred to as 'rye-grass pollen extract' in the text.

Purification of rye fractions

The aq. 1% (w/v) rye-grass pollen extract (100 ml) was diafiltered against distilled water over an Amicon PM10 membrane (10000-M_r nominal cut off). Pre-washed and dried Sephadex G-75 SF (5.5g) was suspended in the dialysis residue together with a 40% Ampholine buffer solution (5.5 ml), pH range 3.5-10. Preparative i.e.f. was performed on an LKB 2117 Multiphor apparatus as described by Winter *et al.* (1975). Subsequently, the gel bed was sectioned, fractions were eluted with distilled water (10 ml), and freeze-dried before characterization.

Fractions from the preparative i.e.f. run were further purified by gel-permeation chromatography on Sephadex G-75 SF in 50 mM-NH_4HCO_3, pH 8.0. Although a single passage of the three fractions yielded essentially homogeneous materials, as assessed by SDS/polyacrylamide-gel electrophoresis, a second chromatographic elution was routinely employed to ensure the removal of carrier ampholytes. The absence of contaminating ampholytes in the three purified fractions was confirmed by t.l.c., amino acid analysis and SDS/polyacrylamide-gel electrophoresis.

There appeared to be no further advantage in subjecting R7, R14a and R14b to ion-exchange chromatography after the gel-filtration step.

Determination of allergenic activity

Skin testing was performed, with informed consent, on the forearm of grass-pollen-sensitive human volunteers as described by Marsh *et al.* (1966), a range of concentrations of the starting rye-grass pollen extract and the purified components being used.

Radioallergosorbent test (RAST) inhibition assays were performed by incubating various concentrations of inhibitor (grass pollen extract and purified fractions) with a serum pool obtained from five grass-pollen-sensitive individuals (25 µl at 1:5 dilution) for 4 h at ambient temperature, before carrying out the RAST assays described by Ceska *et al.* (1972). Bound IgE was detected with ^{125}I-labelled rabbit anit-human IgE, raised against the YUIgE myeloma, purified and iodinated as described by Johansoon *et al.* (1971). Assay methodology used and calculations of the results have been described in detail by Gleich *et al.* (1974) and Chakrabarty *et al.* (1981).

Determination of antigenic activity

This was performed by a modification of the micro immunoassay procedure described by Moran *et al.* (1978) to measure allergen-specific IgG. A serum pool obtained from 23 grass-pollen-sensitive humans and known to contain rye-specific IgG antibodies was pre-incubated with various dilutions of rye or purified rye components before the addition of purified ^{125}I-R7. After incubation, the radiolabelled R7 bound to IgG antibody was immunoprecipitated with Protein A-Sepharose, and the radioactivity present on the solid phase counted in a counter. All reagents were prepared and used in the same proportions as described previously (Moron *et al.,* 1978).

RESULTS AND DISCUSSION

Physical and chemical characterization of purified components

Rye-grass pollen extract is composed of a mixture of protein-staining components with M_r (by SDS/polyacrylamide-gel electrophoresis) values between 11000 and 88000, which have been numbered sequentially R1-R14 in order of decreasing M_r (Moran *et al.,* 1982).

Preparative i.e.f. of rye-grass pollen extract was carried out over the pI range 3-10, since whole extract exhibited protein-staining components on analytical i.e.f. of pI values over the range 3.5-9.3. This yielded at least seven discrete fractions, as shown by SDS/polyacrylamide-gel electrophoresis. The major fraction, focusing around pH 5.8, was designated 'R7', by comparison of its SDS/polyacrylamide-gel electrophoresis profile with that of whole pollen extract (approx. M_r 31000). Two other main components, both with apparent M_r values of 11000, were recovered, one in the acidic region (approx. pI 5.0), and one in the basic region (approx. pI 9.0). These components, designated 'R14a' and 'R14b' respectively, are the main constituents of the R14 band of whole pollen extract. Electrophoretic analysis of other fractions obtained from preparative i.e.f. revealed the partial separation of other components of rye-grass pollen extract, including R4 (M_r 64000), R8 (M_r 27000), R9 (M_r 25000) and a basic red material of apparent M_r 10000. No attempt was made to purify or characterize these latter components further.

The preparative i.e.f. fractions containing R7 and the two R14 components were subjected to gel-permeation chromatography on Sephadex G-75 SF to remove contaminating proteins. The R7, R14a and R14b thus prepared had electrophoretic mobilities identical with those of authentic samples of Rye groups I, II, and III respectively (Fig. 1).

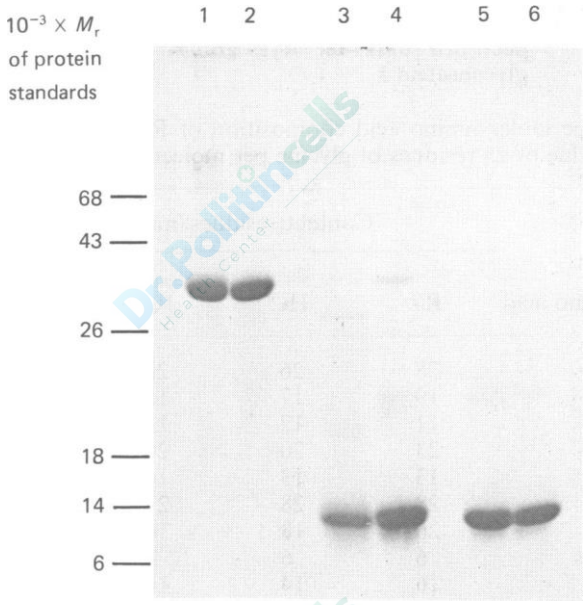

Fig. 1. Analysis of the purified components of rye-grass pollen extract by SDS/polyacrylamide-gel electrophoresis and protein staining
Rye I (1), R7 (2), Rye 11 (3), Rl14a (4), Rye III (5) and Rll4b (6) were electrophoresed on a 12.5% (w/v) polyacrylamide gel under reducing conditions. A 5 1ag sample of protein was loaded per track.

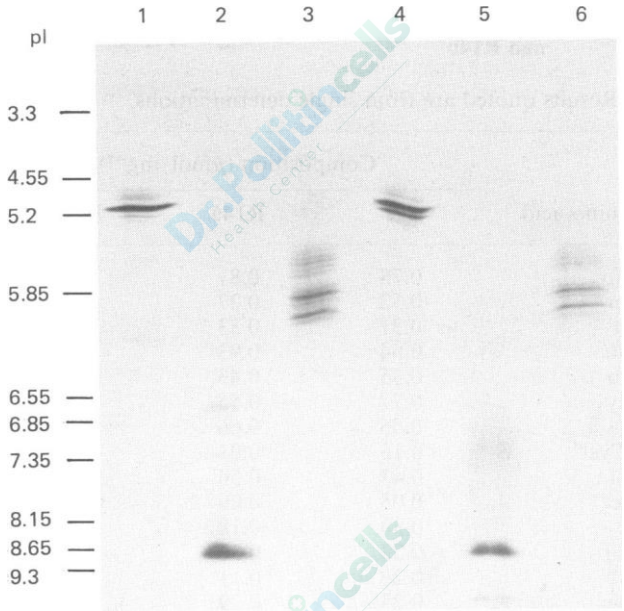

Fig. 2. Analytical i.e.f. of purified rye components
Rl4a (1), Rl4b (2), R7 (3), Rye 11 (4) Rye III (5) and Rye 1 (6) were subjected to i.e.f. on PAG-Plates (LKB-Produkter AR), pH range 3.5-10; 7 /sg of each protein was used. The pl values for the protein standards (Pharmacia AR) are indicated.

The contamination of these components by other rye proteins was estimated at less than 2% (w/w) by consideration of the limits of sensitivity of Coomassie Blue staining.

The average yield of purified R7 from freeze-dried dialyzed rye-grass pollen extract was found to be 8% (w/w); its M_r of 11000; the R14b component, recovered in 2% yield, had an M_r of 11000, but migrated very slightly in front of R14a (Fig. 1). The yields reported for fractions of rye-grass pollen extracts of 1.3% for rye group 1B (Johnson & Marsh, 1965) and 2.7% for glycoprotein 1 (Howlett & Clarke, 1981) compare favorably with these.

All three purified rye components chromatographed as single entities on a pre-calibrated TSK 2000 SW gel-permeation h.p.l.c. column. Both R14a and R14b were eluted with 0.3м-phosphate buffer, pH 6.9, and found to have a M_r of 12000. R7 aggregated in this buffer, but was successfully eluted in 0.08м-phosphate/0.32м-NaCl/20% ethanol, pH 7.0, with an M_r of 32500.

Analytical i.e.f. revealed microheterogeneity of R7 and R14a, arising from minor charge variations (Fig. 2). Fraction R7 had a pI over the range 5.5—6.0, R14a focused over the pI range 4.6—5.2 and R14b appeared as a diffuse band at a pI of 9.0. In agreement with the SDS/polyacrylamide-gel electrophoresis data, R7, R14a and R14b were found to correspond to Rye groups I, II and III respectively (Fig. 2). The charge inhomogeneity for R7 and Rye group I shown on i.e.f. is consistent with previously reported date for both Rye group I (Johnson & Marsh, 1966) and glycoprotein I (Howlett & Clarke, 1981). This latter material is reported to show poor affinity for the lectin concanavalin A; R7, also glycosylated, behaved similarly (R. Standring, unpublished work).

Typical amino acid contents of R7, R14a and R14b are shown in Table 1. When the amino acid composition of R7 was compared with published data (Table 2) for rye groups 1B and 1C (Johnson & Marsh, 1966) and for glycoprotein I (Howlett & Clarke, 1981), a good correlation was found that was consistent with these proteins being equivalent.

The carbohydrate contents of the three fractions R7, R14a and R14b, determined against a glucose standard, were 4, 2, and 2% respectively. The low carbohydrate content of R14b is especially surprising in the light of the low recovery in the amino acid analysis and the apparent absence of any salt contamination as determined by chromatographic analysis. A similar low – M_r highly basic fraction isolated Kentucky-blue-grass pollen extract (allergen C) (Chakrabarty *et al.,* 1981) had a carbohydrate content of up to 500 µg/mg of protein, as assayed by a similar phenol/ H_2SO_4 method.

Partial primary sequence data for fraction R7 were also obtained (Fig. 3). Despite the microheterogeneity in R7, indicated by charge variations evident in the i.e.f. pattern, 27 of the first 30 *N*-terminal amino acids were readily identified. The unique *N*-terminus was identified as isoleucine and was in accord with that reported for rye group I (Johnson & Marsh, 1966). Of the three unidentified residues, positions 5 and 8 were tentatively assigned as cysteine (R7 was not submitted for sequence analysis as the carboxymethylated form) and position 9 was possibly glycosylated. Sequence data were not obtained for fractions R14a and R14b. The ability to assign the partial sequence of R7 positively, despite the obvious inhomogeneity as seen on i.e.f., supports the view that the charge variations seen in the rye group-I isoallergens arise from minor differences in glycosylation or amidation rather than major differences in the primary structure.

Table 1. Typical amino acid composition of fractions R7, R14a and R14b

Results quoted are from single determinations.

Amino acid	Composition (μmol• mg⁻¹) R7	R14a	R14b
Asp	0.78	0.81	038
Thr	0.52	0.27	0.41
Ser	0.31	0.33	0.16
Glu	0.64	0.93	0.44
Pro	0.35	0.43	0.18
Gly	0.77	0.55	0.34
Ala	0.55	0.66	0.15
½-Cys	0.16	0.03	–
Val	0.43	0.50	0.30
Met	0.05	0.09	0.10
Ile	0.30	0.18	0.07
Leu	0.28	0.36	0.31
Tyr	0.26	0.11	0.11
Phe	0.23	0.29	0.16
His	0.14	0.13	0.06
Lys	0.72	0.78	0.43
Arg	0.21	0.22	0.13
Total recovery (μ• mg⁻¹)…	840	842	480

Immunochemical/biological characterization

Since R7, R14a and R14b were found to resemble major rye-grass allergens Rye I, II and III with regard to their physicochemical properties, their interaction with human IgE (allergenicity) was investigated. Two methods were employed: skin testing (components R7 and R14a only) and radioallergosorbent test (RAST) inhibition. Table 3 shows the minimum concentration of allergen required to elicit a weal of area greater than 20 mm² in the skin-prick test performed on six grass-pollen-sensitive individuals. Overall the R7 component elicited a response at lower concentrations than that required for the whole rye-grass pollen extract; in three of six subjects studied 10-fold lower concentrations of R7 than whole extract were required. The R14a component, however, although matching these responses in four volunteers, did not elicit any significant reaction

Table 2. Comparison of the amino acid content of R7 with published date on Rye groups 1B and 1C and glycoprotein 1

The molar amino acid composition of R7 is based on a value of 28 residues of glycine per molecule of R7.

Amino acid	Content (residues/ molecule) R7	1B*	1C*	Glyco-protein 1 †
Asp	28	26	26	26
Thr	19	17	16	19
Ser	11	12	11	14
Glu	23	20	20	22
Pro	13	13	14	14
Gly	28	28	27	28
Ala	20	18	18	24
½-Cys	6	6	6	4
Val	16	14	14	15
Met	2	2	2	2
Ile	11	10	10	11
Leu	10	9	11	11
Tyr	9	9	9	8
Phe	8	8	8	9
His	5	3	3	3
Lys	26	26	26	25
Arg	8	6	6	6

* Data from Johnson & Marsh (1966).
† Data from Howlett & Clarke (1981).

in two individuals (C and F, Table 3) up to a concentration of 10 μg•ml⁻¹. It is noteworthy that, on ummunoprecipitation of ^{125}I-rye-protein-IgG complexes from the sera of these two individuals followed by SDS/polyacrylamide-gel electrophoresis and autoradiography [by the methodology described previously (Moran *et al.,* 1982)], there was no apparent serological response to the R14a/R14b band at 11000 M_r.

Typical RAST inhibition data is shown in Fig. 4. In general, although there were slight batch-to-batch variations in the actual inhibition curves, the three fractions R7, R14a and R14b showed a loss in inhibitory activity when compared with whole rye-grass pollen extract. Even at high concentrations (1 mg • ml⁻¹), the purified

components did not completely inhibit the uptake of the grass-pollen-specific IgE on to the rye-protein coated discs. The human serum pool employed in the assay was shown to contain IgE antibodies against the R7, R14a and R14b components by using the Western-blotting method, as described by Sutton *et al.* (1982). This confirmed that the loss of ability to inhibit 100% of the IgE-reactive property of the whole extract coupled to the disc was due to removal of allergenic specificities on purification.

From the electrophoretic analysis (Fig. 1) and analytical h.p.l.c. results, any cross-contamination between R7 and R14a appeared to be at low levels. Further confirmation of this and any possible antigenic cross-reactivity between these two components was obtained by a ^{125}I-R7 inhibition assay (Fig. 5). The uptake of ^{125}I-R7 on to human IgE from grass-pollen-sensitive individuals was inhibited by R7 than whole rye-grass pollen extract, whereas R14a was a much less effective inhibitor. From these results, it could be calculated that rye-grass pollen extract contained approx. 18% (w/w) of R7 antigenic activity and R14a contained no more than 1% (w/w) of R7 activity.

```
Ile – Ala – Lys – Val – * – Pro – Gly – * – † – Ile –
 1                        5                       10

Thr – Ala – Glu – Tyr – Gly – Asp – Lys – Trp – Leu – Asp
11                       15                       20

Ala – Lys – Ser – Thr – Trp – Tyr – Gly – Lys – Pro – Thr
21                       25                       30
```

Fig. 3. Automatic *N*-terminal sequence analysis of R7

*****, Residures 5 and 8 tentatively assigned as Cysteine; †, residue 9 is possibly glycosylated.

Table 3. Comparison of the allergenicity of Rye, R7 and R14a by prick test in human volunteers

Minimum concentration for weal 20 mm² (μg • ml⁻¹)

	(a)		(b)	
Volunteer	R7	Rye	R14a	Rye
A	2.5	3.1	1.25	3.1
B	1.25	12.5	0.63	6.3
C	1.25	50	No response at 10	12.5
D	10	25	0.63	6.3
E	2.5	3.1	0.63	1.6
F	1.25	25	10	6.25

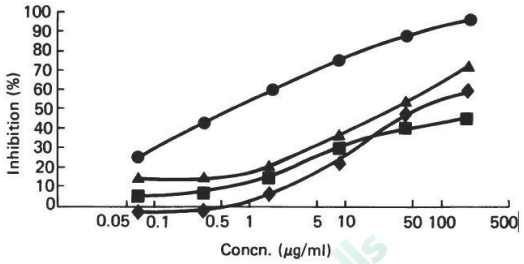

Fig. 4. Allergenicity of purified rye components relative to whole rye-grass pollen extract assessed by the RAST inhibition assay

The uptake of rye-pollen-extract-specific IgE from a human serum pool on to whole-extract-coated cellulose discs is inhibited by whole rye-grass pollen extract (●), R7 (▲), R14a (■) and R14b (♦).

In agreement with results obtained for rye groups I and II (Marsh *et al.*, 1966), both R7 and R14a were shown to have potent allergenic activities as assessed by skin test, RAST inhibition and direct IgE binding. The R14b component was tested only by 'in vitro' techniques, but was shown to be recognized by human IgE on Western blotting and RAST inhibition. The apparent lower activity of all three components relative to whole rye-grass pollen extract by RAST

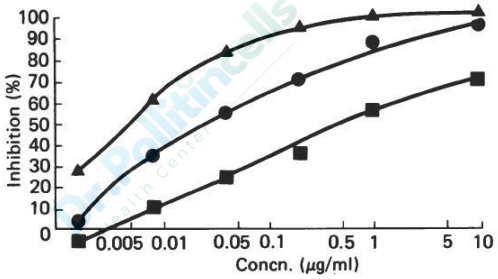

Fig. 5. Inhibition of the uptake of ^{125}I-R7 binding to human IgG from a pool of serum containing antibodies directed against antigenic components of whole rye-grass pollen extract. Inhibitors were whole-rye-grass pollen extract (●), R7 (▲), and R14a (■).

inhibition suggested that a significant part of the response to rye-grass pollen is directed against components other than R7, R14a or R14b. However, no attempt was made to confirm this point with a mixture of all three components. Partial denaturation of the purified fractions during the extractive procedures could not be excluded as an explanation for this effect. Nevertheless, similarly reduced RAST inhibitory activity was apparent with authentic Rye groups I, II and III, obtained from the Bureau of Biologics. The poor skin-test response of two individuals to R14a (Table 3) could be explained by variations in the response of humans to individual allergenic components in rye-grass pollen and accords with the findings of Marsh *et al.* (1970), who showed that only 70% of grass-pollen-sensitive individuals responded to rye group II (R14a).

Conclusions

Three fractions of rye-grass pollen extract were readily purified from whole extract by a combination of preparative i.e.f. and gel-permeation chromatography. The purities of the fractions were formally assessed as being not less than 98%, with no contaminating ampholytes being detected. The three fractions R7, R14a and R14b were identified with the previously described rye groups I, II and III respectively, on the basis of physicochemical and immunological properties. This rapid and reproducible procedure for obtaining relatively large quantities of purified major allergens has obvious applications in the development of improved diagnostic and therapeutic reagents.

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References

Carter, P.E., Dunbar, B. & Fothergill, J.E. (1983) Biochem. J. 215, 565-571

Ceska, M., Eriksson, R. & Varga, J.M. (1972) J. Alergy Clin. Immunol. 49, 1-9

Chakrabarty, S., Ekramaddoullah, A.K.M., Kisil, F.T. & Sehon, A.H. (1981) Int. Arch. Allergy Appl. Immunol. 65, 377-389

Dubois, M., Gilles, K.A. Hamilton, J.K., Rebers, P.A. & Smith, F. (1956) Anal. Chem. 28, 350-356

Ekramaddoullah, A.K.M., Kisil, F.T. & Sehon, A.H. (1981) Int. Arch. Allergy Appl. Immunol. 65, 367-376

Gleich, G.J., Larson, J.B., Jones, R.T. & Baer, H. (1974) J. Allergy Clin. Immunol. 53, 158-169

Howlett, B.J. & Clarke, A.E. (1981) Biochem. J. 197, 695-706

Johansson, S.G.O., Bennich, H. & Berg, T. (1971) Int. Arch. Allergy Appl. Immunol. 41, 443-451

Jognson, P & Marsh, D.G. (1965) Eur. Polymer J. 1, 63-77

Johnson, P. & Marsh, D.G. (1966) Immunochemistry 3, 91-100

Laemmli, U.K. (1970) Nature (London) 227, 680-685

Marsh, D.G., Milner, F.H. & Johnson, P. (1966) Int. Arch. Allergy Appl. Immunol. 29, 521-535

Marsh, D.G. Haddad, Z.H. & Campbell, D.H. (1970) J. Allergy 46, 107-121

Moran, D.M., Dupe, B.E. & Guantlett, S. (1978) J. Immunol. Methods 24, 183-191

Moran, D.M., Strandring, R. & Henderson, D.C. (1982) Int. Arch. Allergy Appl. Immunol. 69, 120-126

Randolf, W.F. (1981) Fed. Regist. 46, 39135-39136

Smith, M.A., Gerrie, L.M., Dunbar, B. & Fothergill, J.E. (1982) Biochem. J. 207, 253-260

Sutton, R., Wrigley, C.W. & Baldo, B.A. (1982) J. Immunol. Methods 52, 183-194

Topping, M.D., Brighton, W.D., Stokell, M. & Patterson, J.M. (1978) J. Immunol. Methods 19, 61-67

Winter, A., Perlmutter, H. & Davies, H. (1975) LKB Application Note 198, LKB-Produkter A.b>, Stockholm

Yunginger, J.W. (1983) Symp. Pediat. Allergy: Pediat. Clin. North Am. 30, 225-23

COLD SYMPTOM SUPPORT:

GRAMINEX Flower Pollen Extract

Pollen as a Prophylactic against the Common Cold

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Pollen extract has been employed to a considerable extent, since 1955, in the treatment of prostate problems of various kinds (1-5, 8-11).

There would appear to be a widespread opinion that pollen extract also possesses a certain value as a roborant and cold-preventative. This effect has been referred to by Noyes [12] on the basis of a small amount of research material. The roborant effect has also been studied by Glömme [6] in comprehensive experiments on animals.

Critical epidemiological investigations on a large scale have not, however, been carried out. Against this background it seemed desirable to conduct a major field study of the effect of pollen extract on those liable to military service, in connection both with prevention of colds and with general roborant properties.

MATERIALS AND METHODS

The investigation was initiated by the Defense Department Research Institute, and carried out with the consent of the Military Governor in the Sixth Military Area (Upper Norrland), the Chief Physician to the Army, and the State Pharmaceutical Laboratory. The study was carried out on three separate occasions on a total of 775 conscripts in the Sixth Military Region. The designation and size of the groups studied are shown in Table 1.

Table 1. Group division and number of experimental persons

Group	Unit	Number of experimental persons
A	Eng. 3	224
B	Eng. 3	116
C	A 8	99
D	A 8	140
E	Eng. 3 rep-unit	44
F	Eng. 3	152

Group A consisted of newly enrolled conscripts, who were confined to barracks during the whole test period. The object of this was to test the problem during a period in which conscripts, who often come from different environments and different infective situations, are known from experience to be affected by a large number of mixed infections. With regard to Groups B-F, the experiments were carried out in connection with winter field-exercises, under conditions where troops are often exposed to major physical and psychical strains in a period when the danger of infection is great. In other particulars the experiments were carried out on all the field-service groups under substantially identical conditions.

The preparation to be tested, Cernilton, was made available by the manufacturers, AB Cernelle of Vegeholm. The dosage in group A, B and C was one tablet three times daily for 14 days. Two tablets were administered once daily for the same period to subjects of group E and F. The specifications of the preparations tested are shown in Table 2.

Table 2. The specifications of the preparations tested

Specification	Groups	
	A - C	E - F
Cernitin T60 sec. (Extr. pollinis aquos sec.)	60 mg	200 mg
Cernitin GBX$_t$ (Extr. pollinis oleos.)	3 mg	10 mg
Constituentiae et coloris	q. s.	q. s.
M.F. tabl. No. 1		

The experimental model was of the so-called double-blind type. Each unit was divided up into more-or-less equal ,,primar'' research units of 10 - 15 men, generally consisting of personel belonging to the same barrack-room, of smaller working group, with high individual working frequency. With the change distribution of the tablets it was ensured that every ,,primary unit'' was represented by more-or-less an equal number of experimental persons, with Cernilton or placebo-medication. This arrangement was made in order to balance any effect which might arise between the experimental persons within the various ,,primary units'' (infected). The blind tablets and Cernilton tablets had exactly the same taste and appearance.

A leader was selected for each group, whose responsibility it was to see to it that the tablets were taken in the way arranged. The experimental persons were asked to make notes on a special diary card during the whole experimental period concerning their state of health, with special attention to certain subjective symptoms, visits to the doctor, and sickness certification. The group leader was responsible for seeing that this was thoroughly carried out. No doctor participated in this part of the experiment.

RESULTS

The possible prophylactic effect of a preparation against symptoms of the common cold can obviously only be evaluated on the basis of material where there is ,,normally'' a rather high incidence of sickness. Of the six units tested during the relevant experimental periods, symptoms indicative of infection of the upper air passage occurred as indicated in Table 3. The table shows that the frequency of colds was low or very low in groups B and E. These groups have therefore been excluded from following discussion. The incidence of certain symptoms of infection of the upper air passage, divided up in accordance with the investigation group and type of tablet, is shown in Table 4.

Table 4. Incidence of sore throat, coughing, hoarseness, and nasal catarrh within the experimental groups

Symptom	Experimental groups							
	A		C		D		F	
	P	C	P	C	P	C	P	C
Sore throat	21,4	18,8	23,6	12,5	17,3	9,8	17,9	21,2
Coughing	28,0	30,7	35,3	29,2	18,8	11,2	31,3	21,2
Hoarseness	11,1	14,5	11,8	20,9	13,0	7,0	13,5	10,6
Nasal catarrh	37,5	35,0	31,4	29,3	24,6	28,2	32,9	31,7
Basic number	107	117	51	48	69	71	67	85

P – placebo (%), C – Cernilton (%)

The table shows a clear distinction between Cernilton and placebo treated experimental persons in investigation groups C and D in relation to sore throat. The differences are in favour of the preparation, and are significant at the 10% level, Khisquare analysis with correlation for continuity in the present case. Coughing also tends to occur rather less frequently with the Cernilton-treated groups (C, D, and F), though it is only within group F that the results are significant at the 10% level. The figures shown in the table for hoarseness and nasal catarrh symptoms can not be regarded as showing any effect: the difference between Cernilton-series and placebo-series are not significantly different from zero. Symptoms of influenza occurred only to a slight extent, and could not be used to evaluate any possible prophylactic effect.

The relative numbers of persons during the observation period who visited the doctor or were certified sick are shown in Table 5.
Visits to the doctor and sick-certification occurred practically only in groups D and F. There was a clear distinction favourable to the preparation between the Cernilton and placebo treated experimental persons, particularly in group D, but also to some extent in group F. The distinction for group D is significant at the 5% level with respect to visits to the doctor, and at the 1% level with respect to sick-certification.

Table 5. Visits to the doctor and sickness certification within the experimental groups

Visits	Experimental groups							
	A		C		D		F	
	P	C	P	C	P	C	P	C
Visited doctor	0,9	0,0	3,9	4,2	13,0	2,8	7,5	4,7
Certified sick	2,8	0,0	0,0	0,0	17,3	2,8	16,5	10,7
Basic number	107	117	51	48	69	71	67	85

P – placebo (%), C – Cernilton (%)

With respect to all the symptoms discussed here, and also to sick-certification, the experimental persons were asked to indicate for how long the symptoms or the certification had lasted. There was no clear distinction between Cernilton-treated and placebo-treated individuals, although there was a certain non-significant tendency for shorter times observed in the case of the Cernilton groups.

The experimental persons were also asked to give a general opinion about their condition during the experimental period, in particular as to whether they felt more tired or more alertt than usual. The alternative answers were formulated differently in the 1965 and 1966 investigations. In 1965 only the two alternatives ,,more tired than usua'' and ,,more alert than usual'' were given, with the result that the experimental persons were,,compelle'' to choose one alternative or the other, or to leave the question unanswered. In the 1966 investigations a further alternative ,,unchange'' was allowed.

Comparison shows that the experimental persons treated with Cernilton in groups C and D show a higher percent of ,,more tired'' than those with placebo-treatment The frequency ,,more tired" is higher throughout for the placebo-treated persons in all four groups. A summing-up of all the experimental groups gives significance at the 10% level.

Finally, it should be said that only individuals with common cold symptoms in the four groups have been considered. The frequencies of ,,more alert'' and ,,more tired'' amongst the persons showing symptoms of colds are given. The tendency is thus amplified and the effects of Cernilton summed up over the groups then reaches the significance-level of 2.5%.

DISCUSSION

The field experiment carried out has not given an unquivocal result in relation to the prophylactic effect of the preparations used against the common cold. It has been shown that under certain conditions it is effective against some symptoms, that is, sore throat and coughing, in groups C and D. That the corresponding effects could not be deduced from groups A and F indicates the need for great caution in generalizing the results. It lies in the nature of the experiment that the Cernilton-treated and placebo-treated experimental persons are fully comparable within the units because of the,,blind'' randomizing. On the other hand, the four main groups themselves are not comparable on the same basis because of the different risks of being infected by the common cold, or of the type of infection experienced. Thus, fur example, group A consisted of a depot unit, which differs from the exercise units with relation both to the incidence of infection and the extent of strain experienced.

The frequency of visits to the doctor and sick-certification indicate that group D and F may have experienced heavier burdens than the two remaining groups. Here a clear distinction between Cernilton-treated and placebo-treated experimental persons has proved demonstrable both with relation to visits to the doctor and sick-certification.

The roborant effect of Cernilton has been evaluated on the basis of a question about condition. Here also groups C and D, and possibly F, give the clearest indication. It should be observed that the distinction is primarily expressed in a lower frequency of ,,tired'' amongst the Cernilton-treated persons. This occurs, naturally, in relation to the situation of the experimental persons, in which the burdens and the occurrence of common colds gives the least encouragement for individuals to report themselves as ,,more alert'' than usual. The results of the condition-question has also been considered separately for those individuals who declared themselves as suffering from some symptoms of the common cold. The object with this was to obtain a specially afflicted group for which any effect of Cernilton would have been particularly valuable. It is found that the effect in this analysis is most clearly expressed where the frequency of ,.more tired'' is lower throughout for all four units. The effect is most marked in group D, where none of the 26 sick persons in the Cernilton-treated group complain of having been ,,more tired''. The number of sick persons is admittedly relatively low, but the overall tendency gives nevertheless an unequiocally significant picture.

As we have already said, the results should not be generalized, at all events not to the extent that quantitative evaluations of the protective effect are given. It should also be remembered

in this connection that the experimental situation for military personel in training is an extremely specialized one.

It would be expected that in this situation, particularly when those concerned are aware that an experiment is being undertaken, that such persons would be extremely observant about their condition of health, and that tendencies to exaggeration may be found. This would not, however, be the reason for the observed effect of Cernilton, but it would make any quantitative evaluation very hazardous. All that should therefore be said for the present, therefore, is that the preparation under certain conditions combats the symptoms associated with infection of the upper air passage, and might for this reason be a useful prophylactic. The preparation has in addition shown during this investigation a roborant effect, in accordance with the observations already reported by Ask-Upmark [1], Glömme and Rasmussen [6] and Graudal [7].

Further elucidation of the conditions under which this effect arises, or the principle on which it is based, could not be provided by this field experiment, nor was this envisaged when it was undertaken.

REFERENCES

1. Ask-Upmark, E.: Sv. Läkartidn. 1959 56, 1840.

2. Ask-Upmark E.:Fol. Clin.Int. 1962, 12, 120.

3. Ask-Upmark. E.: Acta Med. Scand. 1967, 181, 355.

4. Cederlöf, R. Statistisk utvärdering av resultaten fran en klinisk prövning ac Cernilton. Rapport. Stockholm 1964.

5. Denis, L.J. Acta Urol. Belg. 1966, 34, 49.

6. Glömme, J., Rasmussen E.W.: Virkningen av Cernitin (pollen) i kosten ved dyreeksperimentelle forsök. Rapport. Oslo. 1965.

7. Grudal, H.: Sammenligning mellem semunil och placebo ved behandling af rheumatoid arthritis. Rapport. Kong Christian den Tiondes Gigtsanatorium i Graasten 1966.

8. Jönsson, G.: Sv. Läkartidn. 1961, 58, 2487.

9. Jönsson, G.: Nord. Med. 1962, 68, 1152.

10. Leander, G.: Sv. Läkartidn. 1962:59, 3296.

11. Leander, G., Palmstierna H: Nord. Med. 1963, 69, 436.

12. Noyes, C.E. The use of Cernitin, an extract of organic pollen, to increase body weight and to increase resistance toward infections. Report. Florida, 1955.

Physicochemical and immunochemical characterization of allergenic proteins from rye-grass (*Lolium perenne*) pollen prepared by a rapid and efficient purification method

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Three fractions of rye-grass (Lolium perenne) pollen extract have been isolated by preparative isoelectric focusing (i.e.f.) and characterized in terms of physicochemical and immunochemical properties. The purified components were designated 'R7' and 'R14' on the basis of their positions in relation to other rye-grass pollen extract components on SDS/polyacrylamide-gel electrophoresis and their apparent molecular masses were assessed as 31 and 11 kDa respectively. On i.e.f., R14 split into two components, one acidic (pI 5.0) and one basic (pI 9.0), termed 'R14a' and 'R14b' respectively, and R7 focused at pI 5.8. R7 and R14a were shown to be allergenic by skin-prisk test and all three components were recognized by rye-grass-pollen-specific human IgE. On SDS/polyacrylamide-gel electrophoresis and i.e.f., R7 behaved in a manner identical with that shown by an authentic sample of Rye I and gave an amino acid analysis similar to published data [Johnson & Marsh (1966) Immunochemistry 3, 91-100] for Rye group-I isoallergens; the amino acid sequence of the first 27 N-terminal amino acids was also determined. Physicochemical analysis revealed that R14a was equivalent to Rye II and 14b to Rye III. Preparative i.e.f. followed by gel-permeation chromatography proved to be a rapid and efficient method for purifying the allergenic components of Rye I (R7), Rye II (R14a) and Rye III (R14b) from rye-grass pollen extract.

Introduction

Extracts of pollen aeroallergens are usually composed of heterogeneous mixtures of protein and glycoprotein components. Such complexity has compromised both the investigation of the immunological events underlying the clinical manifestations of pollenosis and the standardization of pollen extracts for diagnostic and therapeutic use (Randolf, 1981; Yunginger, 1983). For progress to be made in these areas, therefore, it is evident that the availability of rapid, reproducible and efficient fractionation procedures allowing for the isolation and preparation of major allergen components is highly desirable.

Early fractionations of pollen extracts utilized conventional protein-chemistry separation procedures such as salt precipitation, and ion-exchange and gel-permeation chromatography (see, e.g. Johnson & Marsh, 1965). These multi-step methods, although often yielding relatively homogeneous materials, are commonly limited in usefulness by protracted preparation times and low yields. Preparative i.e.f. using either polyacrylamide gels or granulated gel beds now offers the potential of preparing components of

preparing components of high quality in good yield (Topping *et al.,* 1978; Ekramaddoullah *et al.,* 1981; Chakrabarty *et al.,* 1981).

The present paper describes the use of such methodology in the fractionation of rye-grass (*Lolium perenne)* pollen extract, the characterization of three major allergenic components, and their relationship to the earlier classification of Johnson & Marsh (1965).

MATERIALS AND METHODS

Materials

Rye grass (*Lolium perenne)* pollen was supplied by Bencard (Worthing, Sussex, U.K.). Authentic samples of rye groups I, II, and III were obtained from the Bureau of Biologics (Bethesda, MD, U.S.A.). Sephadex G-75 (superfine) and i.e.f. standards were obtained from Pharmacia AB; Ampholines, PAG-Plates for analytical i.e.f. and TSK 2000 SW h.p.l.c. columns were from LKB-Produkter AB; cellulose discs (size 0.6 cm; lot no. 541) were from Whatman. Diafiltration membranes were purchased from Amicon U.K. and membrane filters from Millipore Corp. $Na^{125}I$ was obtained from Amersham International. Other chemical reagents were from Sigma Chemical Co. or BDH.

Amino acid analysis

Protein samples that had been hydrolysed with 6 M-HCl for 24 h *in vacuo* were separated by cation-exchange chromatography, using a citrate/borate buffer (pH gradient of 2-11.5), on a Chromospek analyser. Amino acids were detected by post-column derivatization with ninhydrin.

Analytical gel-permeation h.p.l.c.

This was performed on a TSK 2000 SW column with either 0.3 M-sodium phosphate, pH 6.9, or 0.08 M-sodium phosphate/0.32 M-NaCl/20% (v/v) ethanol buffer, pH 7.0, as eluent.

Automatic *N*-terminal sequence analysis

This was performed by Mr. B. Dunbar and Professor J.E. Fothergill (Department of Biochemistry, University of Aberdeen, Aberdeen, Scotland, U.K.) using a Beckman 890c sequencer in conjunction with a Waters 5μ spherical C18 reverse-phase h.p.l.c. column. The methodology was described by Smith *et al.* (1982) and Carter *et al.* (1983).

Carbohydrate analysis

The phenol/H_2SO_4 method of Dubois *et al.* (1956) was used to determine the total carbohydrate content relative to a glucose standard.

Detection of contaminating Ampholines

T.l.c. was performed on protein samples with 10% (w/v) trichloroacetic acid as the solvent. Under these conditions the protein was precipitated at the origin, whereas any Ampholines present migrated with the solvent front and could be detected with ninhydrin.

Electrophoretic analysis

Polyacrylamide-gel electrophoresis in the presence of SDS was performed on a vertical slab-gel apparatus (Bio-Rad Laboratories) by the method of Laemmli (1970). The apparent molecular masses of the allergens were estimated by using the following marker proteins: bovine serum albumin (68 kDa), H-chain (50 kDa) and L-chain (23.5 kDa) of human IgG, ovalbumin (43 kDa), myoglobin (17.2 kDa) and cytochrome *c* (11.7 kDa) or prestained protein standards (3-43 kDa) from Bethesda Research Laboratories.

Analytical i.e.f. was performed on PAG-Plates, pH range 3.5-10, according to the manufacturer's instructions.

Preparation of rye-grass pollen extract

Rye-grass pollen (100 g) was defatted with sodium-metal-dried diethyl ether (2x1 liter) and extracted for 24 h with 10 mM-NH_4HCO_3 (litre, pH 7.0). Clarification of the solution was

achieved by centrifugation (10000 *g* for 30 min) and by sequential filtration through 1.2 µm-down to 0.22 µm-pore-size membrane filters. The resulting aq. 1% (w/v) extract was either stored at –20 °C before further purification, or dialyzed against 10 mM- NH_4HCO_3 and freeze-dried. This latter material is referred to as 'rye-grass pollen extract' in the text.

Purification of rye fractions

The aq. 1% (w/v) rye-grass pollen extract (100 ml) was diafiltered against distilled water over an Amicon PM10 membrane (10000-M_r nominal cut off). Pre-washed and dried Sephadex G-75 SF (5.5g) was suspended in the dialysis residue together with a 40% Ampholine buffer solution (5.5 ml), pH range 3.5-10. Preparative i.e.f. was performed on an LKB 2117 Multiphor apparatus as described by Winter *et al.* (1975). Subsequently, the gel bed was sectioned, fractions were eluted with distilled water (10 ml), and freeze-dried before characterization.

Fractions from the preparative i.e.f. run were further purified by gel-permeation chromatography on Sephadex G-75 SF in 50 mM-NH_4HCO_3, pH 8.0. Although a single passage of the three fractions yielded essentially homogeneous materials, as assessed by SDS/polyacrylamide-gel electrophoresis, a second chromatographic elution was routinely employed to ensure the removal of carrier ampholytes. The absence of contaminating ampholytes in the three purified fractions was confirmed by t.l.c., amino acid analysis and SDS/polyacrylamide-gel electrophoresis.

There appeared to be no further advantage in subjecting R7, R14a and R14b to ion-exchange chromatography after the gel-filtration step.

Determination of allergenic activity

Skin testing was performed, with informed consent, on the forearm of grass-pollen-sensitive human volunteers as described by Marsh *et al.* (1966), a range of concentrations of the starting rye-grass pollen extract and the purified components being used.

Radioallergosorbent test (RAST) inhibition assays were performed by incubating various concentrations of inhibitor (grass pollen extract and purified fractions) with a serum pool obtained from five grass-pollen-sensitive individuals (25 µl at 1:5 dilution) for 4 h at ambient temperature, before carrying out the RAST assays described by Ceska *et al.* (1972). Bound IgE was detected with ^{125}I-labelled rabbit anit-human IgE, raised against the YUIgE myeloma, purified and iodinated as described by Johansoon *et al.* (1971). Assay methodology used and calculations of the results have been described in detail by Gleich *et al.* (1974) and Chakrabarty *et al.* (1981).

Determination of antigenic activity

This was performed by a modification of the micro immunoassay procedure described by Moran *et al.* (1978) to measure allergen-specific IgG. A serum pool obtained from 23 grass-pollen-sensitive humans and known to contain rye-specific IgG antibodies was pre-incubated with various dilutions of rye or purified rye components before the addition of purified ^{125}I-R7. After incubation, the radiolabelled R7 bound to IgG antibody was immunoprecipitated with Protein A-Sepharose, and the radioactivity present on the solid phase counted in a counter. All reagents were prepared and used in the same proportions as described previously (Moron *et al.,* 1978).

RESULTS AND DISCUSSION

Physical and chemical characterization of purified components

Rye-grass pollen extract is composed of a mixture of protein-staining components with M_r (by SDS/polyacrylamide-gel electrophoresis) values between 11000 and 88000, which have been numbered sequentially R1-R14 in order of decreasing M_r (Moran *et al.,* 1982).

Preparative i.e.f. of rye-grass pollen extract was carried out over the pI range 3-10, since whole extract exhibited protein-staining components on analytical i.e.f. of pI values over the range 3.5-9.3. This yielded at least seven discrete fractions, as shown by SDS/polyacrylamide-gel electrophoresis. The major fraction, focusing around pH 5.8, was designated 'R7', by comparison of its SDS/polyacrylamide-gel electrophoresis profile with that of whole pollen extract (approx. M_r 31000). Two other main components, both with apparent M_r values of 11000, were recovered, one in the acidic region (approx. pI 5.0), and one in the basic region (approx. pI 9.0). These components, designated 'R14a' and 'R14b' respectively, are the main constituents of the R14 band of whole pollen extract. Electrophoretic analysis of other fractions obtained from preparative i.e.f. revealed the partial separation of other components of rye-grass pollen extract, including R4 (M_r 64000), R8 (M_r 27000), R9 (M_r 25000) and a basic red material of apparent M_r 10000. No attempt was made to purify or characterize these latter components further.

The preparative i.e.f. fractions containing R7 and the two R14 components were subjected to gel-permeation chromatography on Sephadex G-75 SF to remove contaminating proteins. The R7, R14a and R14b thus prepared had electrophoretic mobilities identical with those of authentic samples of Rye groups I, II, and III respectively (Fig. 1).

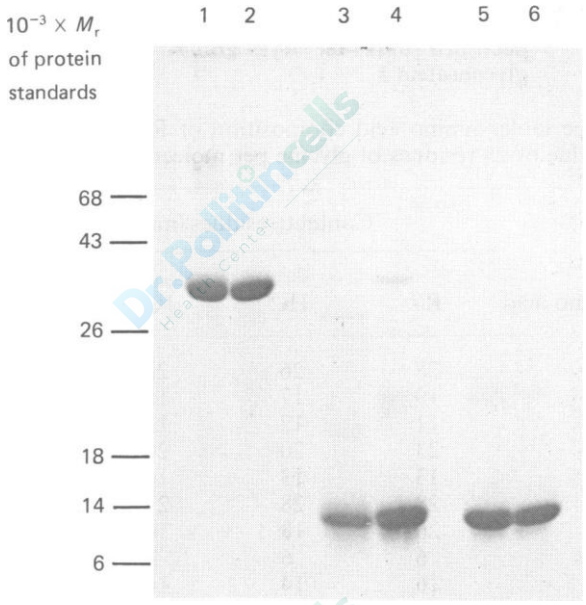

Fig. 1. Analysis of the purified components of rye-grass pollen extract by SDS/polyacrylamide-gel electrophoresis and protein staining
Rye I (1), R7 (2), Rye 11 (3), Rl14a (4), Rye III (5) and Rll4b (6) were electrophoresed on a 12.5% (w/v) polyacrylamide gel under reducing conditions. A 5 1ag sample of protein was loaded per track.

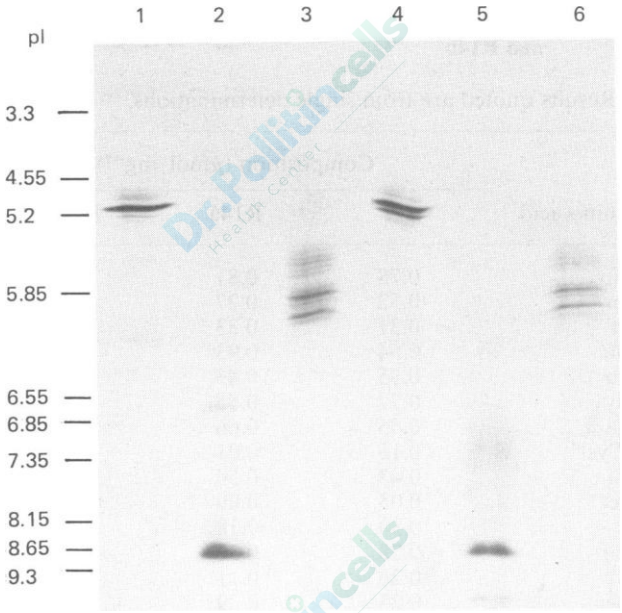

Fig. 2. Analytical i.e.f. of purified rye components
Rl4a (1), Rl4b (2), R7 (3), Rye 11 (4) Rye III (5) and Rye 1 (6) were subjected to i.e.f. on PAG-Plates (LKB-Produkter AR), pH range 3.5-10; 7 /sg of each protein was used. The pl values for the protein standards (Pharmacia AR) are indicated.

The contamination of these components by other rye proteins was estimated at less than 2% (w/w) by consideration of the limits of sensitivity of Coomassie Blue staining.

The average yield of purified R7 from freeze-dried dialyzed rye-grass pollen extract was found to be 8% (w/w); its M_r of 11000; the R14b component, recovered in 2% yield, had an M_r of 11000, but migrated very slightly in front of R14a (Fig. 1). The yields reported for fractions of rye-grass pollen extracts of 1.3% for rye group 1B (Johnson & Marsh, 1965) and 2.7% for glycoprotein 1 (Howlett & Clarke, 1981) compare favorably with these.

All three purified rye components chromatographed as single entities on a pre-calibrated TSK 2000 SW gel-permeation h.p.l.c. column. Both R14a and R14b were eluted with 0.3м-phosphate buffer, pH 6.9, and found to have a M_r of 12000. R7 aggregated in this buffer, but was successfully eluted in 0.08м-phosphate/0.32м-NaCl/20% ethanol, pH 7.0, with an M_r of 32500.

Analytical i.e.f. revealed microheterogeneity of R7 and R14a, arising from minor charge variations (Fig. 2). Fraction R7 had a pI over the range 5.5—6.0, R14a focused over the pI range 4.6—5.2 and R14b appeared as a diffuse band at a pI of 9.0. In agreement with the SDS/polyacrylamide-gel electrophoresis data, R7, R14a and R14b were found to correspond to Rye groups I, II and III respectively (Fig. 2). The charge inhomogeneity for R7 and Rye group I shown on i.e.f. is consistent with previously reported date for both Rye group I (Johnson & Marsh, 1966) and glycoprotein I (Howlett & Clarke, 1981). This latter material is reported to show poor affinity for the lectin concanavalin A; R7, also glycosylated, behaved similarly (R. Standring, unpublished work).

Typical amino acid contents of R7, R14a and R14b are shown in Table 1. When the amino acid composition of R7 was compared with published data (Table 2) for rye groups 1B and 1C (Johnson & Marsh, 1966) and for glycoprotein I (Howlett & Clarke, 1981), a good correlation was found that was consistent with these proteins being equivalent.

The carbohydrate contents of the three fractions R7, R14a and R14b, determined against a glucose standard, were 4, 2, and 2% respectively. The low carbohydrate content of R14b is especially surprising in the light of the low recovery in the amino acid analysis and the apparent absence of any salt contamination as determined by chromatographic analysis. A similar low – M_r highly basic fraction isolated Kentucky-blue-grass pollen extract (allergen C) (Chakrabarty *et al.,* 1981) had a carbohydrate content of up to 500 µg/mg of protein, as assayed by a similar phenol/ H_2SO_4 method.

Partial primary sequence data for fraction R7 were also obtained (Fig. 3). Despite the microheterogeneity in R7, indicated by charge variations evident in the i.e.f. pattern, 27 of the first 30 *N*-terminal amino acids were readily identified. The unique *N*-terminus was identified as isoleucine and was in accord with that reported for rye group I (Johnson & Marsh, 1966). Of the three unidentified residues, positions 5 and 8 were tentatively assigned as cysteine (R7 was not submitted for sequence analysis as the carboxymethylated form) and position 9 was possibly glycosylated. Sequence data were not obtained for fractions R14a and R14b. The ability to assign the partial sequence of R7 positively, despite the obvious inhomogeneity as seen on i.e.f., supports the view that the charge variations seen in the rye group-I isoallergens arise from minor differences in glycosylation or amidation rather than major differences in the primary structure.

Table 1. Typical amino acid composition of fractions R7, R14a and R14b

Results quoted are from single determinations.

Amino acid	Composition (μmol• mg⁻¹) R7	R14a	R14b
Asp	0.78	0.81	038
Thr	0.52	0.27	0.41
Ser	0.31	0.33	0.16
Glu	0.64	0.93	0.44
Pro	0.35	0.43	0.18
Gly	0.77	0.55	0.34
Ala	0.55	0.66	0.15
½-Cys	0.16	0.03	
Val	0.43	0.50	0.30
Met	0.05	0.09	0.10
Ile	0.30	0.18	0.07
Leu	0.28	0.36	0.31
Tyr	0.26	0.11	0.11
Phe	0.23	0.29	0.16
His	0.14	0.13	0.06
Lys	0.72	0.78	0.43
Arg	0.21	0.22	0.13
Total recovery (μ• mg⁻¹)…	840	842	480

Table 2. Comparison of the amino acid content of R7 with published date on Rye groups 1B and 1C and glycoprotein 1

The molar amino acid composition of R7 is based on a value of 28 residues of glycine per molecule of R7.

Amino acid	Content (residues/ molecule) R7	1B*	1C*	Glyco-protein 1 †
Asp	28	26	26	26
Thr	19	17	16	19
Ser	11	12	11	14
Glu	23	20	20	22
Pro	13	13	14	14
Gly	28	28	27	28
Ala	20	18	18	24
½-Cys	6	6	6	4
Val	16	14	14	15
Met	2	2	2	2
Ile	11	10	10	11
Leu	10	9	11	11
Tyr	9	9	9	8
Phe	8	8	8	9
His	5	3	3	3
Lys	26	26	26	25
Arg	8	6	6	6

* Data from Johnson & Marsh (1966).
† Data from Howlett & Clarke (1981).

Immunochemical/biological characterization

Since R7, R14a and R14b were found to resemble major rye-grass allergens Rye I, II and III with regard to their physicochemical properties, their interaction with human IgE (allergenicity) was investigated. Two methods were employed: skin testing (components R7 and R14a only) and radioallergosorbent test (RAST) inhibition. Table 3 shows the minimum concentration of allergen required to elicit a weal of area greater than 20 mm² in the skin-prick test performed on six grass-pollen-sensitive individuals. Overall the R7 component elicited a response at lower concentrations than that required for the whole rye-grass pollen extract; in three of six subjects studied 10-fold lower concentrations of R7 than whole extract were required. The R14a component, however, although matching these responses in four volunteers, did not elicit any significant reaction in two individuals (C and F, Table 3) up to a concentration of 10 μg•ml⁻¹. It is noteworthy that, on ummunoprecipitation of ^{125}I-rye-protein-IgG complexes from the sera of these two individuals followed by SDS/polyacrylamide-gel electrophoresis and autoradiography [by the methodology described previously (Moran *et al.,* 1982)], there was no apparent serological response to the R14a/R14b band at 11000 M_r.

Typical RAST inhibition data is shown in Fig. 4. In general, although there were slight batch-to-batch variations in the actual inhibition curves, the three fractions R7, R14a and R14b showed a loss in inhibitory activity when compared with whole rye-grass pollen extract. Even at high concentrations (1 mg • ml⁻¹), the purified components did not completely inhibit the uptake of the grass-pollen-specific IgE on to the rye-protein coated discs. The human serum pool

employed in the assay was shown to contain IgE antibodies against the R7, R14a and R14b components by using the Western-blotting method, as described by Sutton *et al.* (1982). This confirmed that the loss of ability to inhibit 100% of the IgE-reactive property of the whole extract coupled to the disc was due to removal of allergenic specificities on purification.

From the electrophoretic analysis (Fig. 1) and analytical h.p.l.c. results, any cross-contamination between R7 and R14a appeared to be at low levels. Further confirmation of this and any possible antigenic cross-reactivity between these two components was obtained by a ^{125}I-R7 inhibition assay (Fig. 5). The uptake of ^{125}I-R7 on to human IgE from grass-pollen-sensitive individuals was inhibited by R7 than whole rye-grass pollen extract, whereas R14a was a much less effective inhibitor. From these results, it could be calculated that rye-grass pollen extract contained approx. 18% (w/w) of R7 antigenic activity and R14a contained no more than 1% (w/w) of R7 activity.

```
Ile – Ala – Lys – Val – * – Pro – Gly – * – † – Ile –
 1                       5                      10

Thr – Ala – Glu – Tyr – Gly – Asp – Lys – Trp – Leu – Asp
11                      15                      20

Ala – Lys – Ser – Thr – Trp – Tyr – Gly – Lys – Pro – Thr
21                      25                      30
```

Fig. 3. Automatic *N*-terminal sequence analysis of R7

*****, Residures 5 and 8 tentatively assigned as Cysteine; †, residue 9 is possibly glycosylated.

Table 3. Comparison of the allergenicity of Rye, R7 and R14a by prick test in human volunteers

Minimum concentration for weal 20 mm² (μg • ml⁻¹)

	(a)		(b)	
Volunteer	R7	Rye	R14a	Rye
A	2.5	3.1	1.25	3.1
B	1.25	12.5	0.63	6.3
C	1.25	50	No response at 10	12.5
D	10	25	0.63	6.3
E	2.5	3.1	0.63	1.6
F	1.25	25	10	6.25

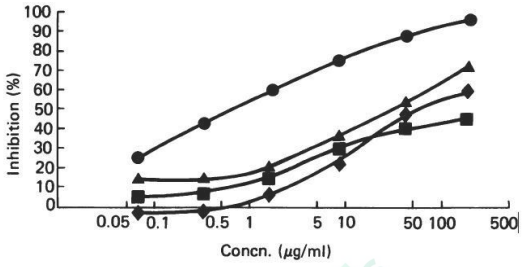

Fig. 4. Allergenicity of purified rye components relative to whole rye-grass pollen extract assessed by the RAST inhibition assay

The uptake of rye-pollen-extract-specific IgE from a human serum pool on to whole-extract-coated cellulose discs is inhibited by whole rye-grass pollen extract (●), R7 (▲), R14a (■) and R14b (♦).

In agreement with results obtained for rye groups I and II (Marsh *et al.*, 1966), both R7 and R14a were shown to have potent allergenic activities as assessed by skin test, RAST inhibition and direct IgE binding. The R14b component was tested only by 'in vitro' techniques, but was shown to be recognized by human IgE on Western blotting and RAST inhibition. The apparent lower activity of all three components relative to whole rye-grass pollen extract by RAST

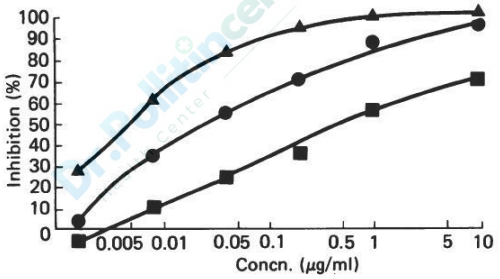

Fig. 5. Inhibition of the uptake of ^{125}I-R7 binding to human IgG from a pool of serum containing antibodies directed against antigenic components of whole rye-grass pollen extract. Inhibitors were whole-rye-grass pollen extract (●), R7 (▲), and R14a (■).

inhibition suggested that a significant part of the response to rye-grass pollen is directed against components other than R7, R14a or R14b. However, no attempt was made to confirm this point with a mixture of all three components. Partial denaturation of the purified fractions during the extractive procedures could not be excluded as an explanation for this effect. Nevertheless, similarly reduced RAST inhibitory activity was apparent with authentic Rye groups I, II and III, obtained from the Bureau of Biologics. The poor skin-test response of two individuals to R14a (Table 3) could be explained by variations in the response of humans to individual allergenic components in rye-grass pollen and accords with the findings of Marsh *et al.* (1970), who showed that only 70% of grass-pollen-sensitive individuals responded to rye group II (R14a).

Conclusions

Three fractions of rye-grass pollen extract were readily purified from whole extract by a combination of preparative i.e.f. and gel-permeation chromatography. The purities of the fractions were formally assessed as being not less than 98%, with no contaminating ampholytes being detected. The three fractions R7, R14a and R14b were identified with the previously described rye groups I, II and III respectively, on the basis of physicochemical and immunological properties. This rapid and reproducible procedure for obtaining relatively large quantities of purified major allergens has obvious applications in the development of improved diagnostic and therapeutic reagents.

We thank Mr. B. Dunbar and Professor J.E. Fothergill, Department of Biochemistry, University of Aberdeen, for carrying out the sequence analysis and Miss S. J. Porter and Mr. Y.K. Davé for their excellent technical assistance.

References

Carter, P.E., Dunbar, B. & Fothergill, J.E. (1983) Biochem. J. 215, 565-571

Ceska, M., Eriksson, R. & Varga, J.M. (1972) J. Alergy Clin. Immunol. 49, 1-9

Chakrabarty, S., Ekramaddoullah, A.K.M., Kisil, F.T. & Sehon, A.H. (1981) Int. Arch. Allergy Appl. Immunol. 65, 377-389

Dubois, M., Gilles, K.A. Hamilton, J.K., Rebers, P.A. & Smith, F. (1956) Anal. Chem. 28, 350-356

Ekramaddoullah, A.K.M., Kisil, F.T. & Sehon, A.H. (1981) Int. Arch. Allergy Appl. Immunol. 65, 367-376

Gleich, G.J., Larson, J.B., Jones, R.T. & Baer, H. (1974) J. Allergy Clin. Immunol. 53, 158-169

Howlett, B.J. & Clarke, A.E. (1981) Biochem. J. 197, 695-706

Johansson, S.G.O., Bennich, H. & Berg, T. (1971) Int. Arch. Allergy Appl. Immunol. 41, 443-451

Jognson, P & Marsh, D.G. (1965) Eur. Polymer J. 1, 63-77

Johnson, P. & Marsh, D.G. (1966) Immunochemistry 3, 91-100

Laemmli, U.K. (1970) Nature (London) 227, 680-685

Marsh, D.G., Milner, F.H. & Johnson, P. (1966) Int. Arch. Allergy Appl. Immunol. 29, 521-535

Marsh, D.G. Haddad, Z.H. & Campbell, D.H. (1970) J. Allergy 46, 107-121

Moran, D.M., Dupe, B.E. & Guantlett, S. (1978) J. Immunol. Methods 24, 183-191

Moran, D.M., Strandring, R. & Henderson, D.C. (1982) Int. Arch. Allergy Appl. Immunol. 69, 120-126

Randolf, W.F. (1981) Fed. Regist. 46, 39135-39136

Smith, M.A., Gerrie, L.M., Dunbar, B. & Fothergill, J.E. (1982) Biochem. J. 207, 253-260

Sutton, R., Wrigley, C.W. & Baldo, B.A. (1982) J. Immunol. Methods 52, 183-194

Topping, M.D., Brighton, W.D., Stokell, M. & Patterson, J.M. (1978) J. Immunol. Methods 19, 61-67

Winter, A., Perlmutter, H. & Davies, H. (1975) LKB Application Note 198, LKB-Produkter A.b>, Stockholm

Yunginger, J.W. (1983) Symp. Pediat. Allergy: Pediat. Clin. North Am. 30, 225-23

EATING DISORDER SUPPORT:

GRAMINEX Flower Pollen Extract

A New Appetite Stimulant Drug based on Pollen Extracts, with no Hormonal or Antihistamine action, in a Pediatric Practice

Report on 100 observations

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91 – Limeil-Brévannes

The trial covered 101 children.

The overall indication for admission to the trial was low weight due either to chronic malnutrition or to recent weight loss.

The children's ages ranged from 4 months to 12 years and 3 months, with a mean of 3 years 7 months.

The sex distribution was approximately even: 53 boys and 48 girls.

Results were assessed on the basis of weight gain, with correction of any concomitant anorexia or asthenia.

In the absence of precisely known aetiologies such as malabsorption due to intestinal villous atrophy or pancreatic insufficiency, we are not able to give a precise metabolic cause for most of our cases of chronic malnutrition, nor for any weight gain registered (save that no cases of oedema were noted at any time).

Sthénorex** (** -- Ozothine Laboratories) was given in one of two forms:

- Gelules containing only 6 mg of lipid-soluble pollen extract and 120 mg of water-soluble extract;
- Sachets (not yet marketed) containing a one-third greater dose of the active principles, with a flavored vehicle.

The dose was one gelule morning and night for all ages, or of one sachet (above the age of 5) or half a sachet (below this age), morning and night; the duration of treatment in all cases was one month.

No clinical or laboratory evidence of intolerance was seen. 10 of the 101 children had a soft stool at some stage of treatment. This digestive disturbance was always slight, and recovered without any need for stopping treatment with Sthénorex or for giving symptomatic treatment.

Overall Results

Expressing results as follows: +++ = very good; ++ = good; + = average; 0 = negative, we can draw up the following table:

- Weight gain:

+++	28
++	42
+	14
0	17

The addendum to this paper gives a more detailed appreciation of the weight study.

- Anorexia: 87 anorexic children:

+++	23
++	41
+	5
0	10

- Asthenia: 77 asthenic children:

+++	13
++	41
+	5
0	18

Results in relation to clinical indications

When weight gain is related to clinical indication, a number of points can be noted:

- Chronic patients: 24 cases:

+++	6
++	7
+	5
0	6

But a limited number or conditions account for almost all the very good and good results: malformations of the urinary tract, compensated chronic neurological disease, chronic blood disorders, nonspecific intestinal disease. These were present in 15 children, who showed the following results:

+++	6
++	6
+	1
0	2

In contrast, children with cariac disease, well-defined metabolic disorders (Lesch-Nyhan syndrome, mucoviscidosis, Schwochwan syndrome), numbering 9 in all, showed the following results:

+++	1
++	4
0	4

Children convalescing from acute illness: 40 cases:
The overall results appear very good:

+++	15
++	20
+	1
0	4

The nature of the infectious illness does not appear to play a part: there were 5 cases of measles, 3 of varicella, 11 of acute respiratory infections and 6 of viral hepatitis.

Results in viral hepatitis were as follows:

+++	4
++	1
0	1

- Malnutrition of unknown aetiology

This group comprised 37 children, characterized by the absence of any precisely known cause for their low weight.

Results in this group were:

+++	7
++	15
+	8
0	7

These results appear relatively less good than in the preceding group, but the way in which cases were collected divides the children (37 in number) into two groups:

- Cases of isolated low weight, noted in hospital: 18 cases
- Cases of low weight noted at consultations in a child psychiatry clinic (i.e.) with coexistent psychiatric or personality disorder): 19 cases.
-

The first group showed:

+++	6
++	7

The second group showed:

+++	1
++	8

General Conclusions

The general conclusions relating to weight gain show that this highly acceptable and well-tolerated product has a very high degree of overall effectiveness, with over 74% of positive results.

The absence of any side-effects is particularly worthy of note, in comparison to other appetite stimulant drugs.

It is regrettable that no weight gain was seen in infants with cardiac disease (in whom weight gain is of vital importance), in our limited series of four children.

The efficacy of the product appears to be slightly less in low weight of unknown cause associated with personality disorder.

Observations

Serum proteins were measured before and after treatment in 40 children. Total serum protein rose by 3 g/l in 27 children (no account was taken of small increases).

We cannot offer any precise explanation for this observation.

In this context we must point out that a double-blind trial on 6 children receiving Sthénorex and 6 receiving placebo showed a rise in serum protein of 4.30 g/l (mean) in the treatment group, as compared to 0 in the controls.

The other results of the double-blind trial were as follows:

Weight gain over one month:	treatment group……..0.561 kg controls…….0.080 kg
Appetite gain (+++) by end of treatment:	treatment group……..4 out of 6 controls……0

Conclusion

Although the mode of action of the product cannot yet be rigorously explained in pharmacological terms, it appears to us to be widely effective, and – let us repeat – entirely free from side-effects, a point worth stressing in the context of appetite stimulant drugs.

The absence of side-effects and allergic reactions appears to us to eliminate any contra-indication to its use.

Addendum

Wherever possible, body weight was recorded during the month proceeding of following the treatment month, or in all three. This depended on the duration of admission.

- 8 children showed a weight gain during the preceding month of 85 g (mean); during the treatment month their mean gain was 510 g.
- 8 children weighed during and after treatment showed:

mean weight gain during treatment: 450 g
mean weight gain after treatment: 220 g

- 15 children observed over 3 months showed:

mean weight gain during pre-treatment month: 190 g
mean weight gain during treatment month: 450 g
mean weight gain during post-treatment month: 190 g.

Naturally, in the case of infectious illnesses, the weighings of the pre-treatment month did not include the period of the acute illness itself.

All these figures, particularly the last set, suggest that the drug has a direct action, rather than that weight gain was due to convalescence or a change in diet alone.

The Effect of Cernitin on Upper Respiratory Tract Infections

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A number of reports have given as the definite opinion of a number of well-known urologists that the carefully digested pollen extract called Cernitin, constituting the active principle in "Cernitin", has a good effect on chronic prostatitis when the definitions (1,2,3) and the indications are made clear (4,5,2,6).

There are also a number of reports stating that Cernilton has beneficial effect upon infections in the upper respiratory tract (7,8,9,10).

Experimentally a great number of investigations have shown that Cernilton is practically non-toxic (11,12,13). It has also been proved that there is a streptolysing inhibitory factor in Cernilton T 60 which is the main constituent of Cernilton (14, 15).

In animal experiments a statistically probable significant effect of Cernilton on the frequency of spontaneous lung infects in rats (17) may be present.

The above mentioned results, especially when having a certain support in well-controlled animal experiments, made it natural to go more detailed into the problems as to the possible anti-infectious effect of Cernilton regarding the upper respiratory tract. This problem was studied in some detail by Malstrom and Cederlof (7) when administering in a double-blind experiment Cernilton and placebo respectively to a fairly great number of military personnel. This type of clinical trials have many definite advantages especially for double-blind studies: the examined persons are of the same sex and age and are generally speaking under the same influence by the surroundings and the climate and they may be looked upon as randomized selection as to motivation. This should therefore a priori be acceptable as a group well suited for comparison. In the report of Malstrom and Cederlof (1966-1967) (7) there is some striking features. In total there were 615 observed military persons. The Cernilton and placebos respectively which were identical as to taste and appearance, there was by the dechiffration proved to be 294 who had got the placebos and 321 who had got Cernilton. The distribution was made in a way which gave very good randomizing of the distribution within every small group and section which made it highly improbable that there should be any significant difference because of the distribution of the preparations used.

The most striking features is the results as to the number of persons during the 14 days of observation where all the preparations used when regarding the number of sick absences and the number of visiting doctors for upper respiratory tract troubles.

Among the 294 men in the placebo-treated group 17 were visiting the doctor for upper respiratory tract diseases. This makes 5.8 percent ± 1.39. In the Cernilton-treated group there were only 8 visits to the doctor because of upper respiratory tract diseases that makes 2.5 percent ± 0.87. The difference as to visiting the doctors because of upper respiratory tract diseases is 3.3 percent ± 1.64. This gives a t-value (according to Students-t-test) = 2.01 and a

probability of statistical significance on the 5 percent level.

As to the frequency of sick-leave this occurred in the placebo-treated group in 26 cases (among the 294 persons) which makes 8.8 percent ± 1.67. In the Cernilton-treated group there were altogether 11 cases of sick-leave among the 321 men, which makes 3.4 percent ± 1.01. This gives a difference between placebo-treated group and the Cernilton-treated group on 5.4 ± 1.95. According to Students-t-test this gives a t-value of 2.77 and a statistical significant difference on the 1 per cent level (0.01>p>0.001).

As to the single symptoms treated within each of the 4 different groups separately, there is a difference as to the frequency of sore throat on the 2 percent level in 2 out of the 4 groups in favor of Cernilton. It is also a difference as to the frequency of coughing between Cernilton and the placebo-treated groups in 2 of the separated divisions on respectively the 10 percent level and the 5 percent level. As to the rhinitis symptoms there is no certain difference of trend in any of the groups. The trend in the results of the enquete gives partly 10 percent partly 20 percent significance in favor of the Cernilton group generally speaking when regarding most of the symptoms from the upper respiratory tract but no definite or probable statistical significance except for the symptoms in the few groups mentioned above.

Even more interesting when regarding the generally roborating effect, which has been presumed for Cernilton, is the results given by the 615 men as to their general condition and feeling of well-being. It is as to these subjective symptoms a difference on the 10 percent level in favor of Cernilton as the total groups are regarded and when regarding only about 40 percent which have had any symptoms or signs indicating an upper respiratory tract disease, it is significant difference on the 2.5 per cent level in favor of the Cernilton-treated group compared with the placebo-treated group.

As this report has never been published it has been found of interest to give a fairly extensive extract of the results.

Dr. med. H. Klapsch (8) has in his report as to the effect of the “Grippen-Tabletten Fluaxin” stated that this tablet which contains a small amount of acetylsalicylic acid together with the pollen extract, was given as prophylacticum or as therapy to people occupied in a heavy industry where he was the industrial physician when the actual candidates had a feeling of getting sick in influenza or upper respiratory tract infections.

Altogether the tablets were given to 510 persons. In addition to the about 52 percent of the total number of employees who got the Cernilton-containing tablet there was 5.5 percent getting another so called “Grippen-Tableitte A” and 6.3 percent another type of so called “Grippen-Tablette B”.

It may be of some interest to give a few of the observations reported by Dr. H. Klapsch. Of the 510 (52 percent) who got the Cernitin-containing tablets, 6 (2.4 percent) who got the tablets only once (6 tablets specially prepared) got an upper respiratory tract infection which caused sick-leave. Among those who asked for the tablets only 1.1 percent had a sick-leave because of an upper respiratory disease during the period of observation.

The total frequency of sick-leave because of upper respiratory tract infections thus was about the same in the group which got the Cernitin-contained tablets as in the group getting one of the two other tablets which should serve the same purpose about: a total of about 2 percent sick-leave because of “Grippe” or upper respiratory tract diseases.

The fairly small difference between sick-absence: 2.4 percent and 1.1 percent respectively gives a t-value according to Students-t-test on 1.20 which is not statistically significant (0.3>p>0.2).

Only among a few of the patients getting the Cernitin-containing tablets Dr. Klapsch ask about the patients' reactions and in those cases about 8 percent gave a good or very good effect as about 14 persons gave a bad effect.

Altogether 7 patients (1.4 per cent) gave side effects as cephalalgia, extreme tiredness, feeling of being unwell and sweating. As there are given as the only spontaneous side-effects and all belong to most of the upper respiratory tract infections there does not seem to be any real side effect of the tablets as reported by Dr. Klapsch.

Although Dr. Klapsch himself is drawing the conclusion that there is a very good effect of the Cernitin-containing Fluaxin tablets as prophylacticum and therapeutics against upper respiratory tract disease and/or "influenza" there is no definite evidence in his report which supports his opinion.

As to the investigation of Lindberg and Sorensen (1968) (8) "A tentative treatment of the common cold with Cernitin", this is carried out as "prophylactic" treatment when the first symptoms of a common cold was observed by the test persons themselves. All the patients were treated according to double-blind controlled clinical trial technique. All of them got 30 tablets of which they should take 10 immediately as soon as they observed any symptoms or signs of a beginning common cold, then 10 tablets again after 8-12 hours and finally 10 tablets again after another 8-12 hours. Further supply of tablets could then be given by the physician in charge of trial. Neither the physicians not the patients knew anything about the type of tablets besides that they were quite harmless and contained vitamins, minerals and amino acids and other quite harmless substances. After dchiffation of the test it turned out that in the Cernitin-treated group there were 83 percent who have given that they were either completely free from symptoms within the first day or that the symptoms of the common cold or upper respiratory tract disease lasted for a shorter period and the symptoms were less severe than usual. Only 17 percent hold the opinion they could not observe any effect at all. In the control group which had got placebos, there were 63 percent who gave that they're signs and symptoms disappeared completely within the first day or that the signs and symptoms were easier or had a shorter duration than usual. The Cernilton=treated group proved to consist of 39 persons while the control group consisted of 24 persons. (If treating these results statistically the author got the following table:

Table 1.

Subjective estimate of symptoms of common cold or from the upper respiratory tract in Cernilton-treated and placebo-treated groups.

Subjective estimate of symptoms	Cernilton-treated group		Control-group (placebo-group)	
	No.	Per cent	No.	Per cent
Symptoms disappeared completely within 1 day	13	45	8	33
Symptoms disappeared more rapid or were easier than usual	11	38	7	30
No effect at all	5	17	9	37
Total	29	100	24	100

It is obvious that we have a fairly high frequency of "placebo-effect" in both groups. If trying to find out whether there is a real difference between the groups it may be reasonable to treat together the groups which found that their symptoms and signs disappeared completely within one day and the group which found their symptoms and signs were easier or of shorter duration.

Table 2

Subjective estimate of symptoms	Cernilton-treated group		Control-group (placebo-group)	
	No.	Per cent	No.	Per cent
Definite improvement of signs and symptoms	24	83	15	63
No effect	5	17	9	37
Total	29	100	24	100

This makes a difference in favour of the Cernilton-treated groups fo 83 per cent $\pm$ 7.1 against 63 per cent $\pm$ 10.1.

The difference is 20 per cent $\pm$ 12.3 which according to Student-t-test gives a t-value on 1.63 and a probability of $0.2 > P > 0.1$. This does not give a significant difference.

In 1970 and 1972 Glømme has carried through a systematic clinical trial of Cernilton in cases which are bordered by infections as continuous or very often occurring recidives of sore throat or what they may call common cold. Most of these patients are known by the author from before and many of them have been interested in trying vaccination with standard-vaccine or auto vaccine to try to get rid of their troubles from the upper respiratory tract. Altogether these tests have been carried out as a prophylactic treatment in 180 cases and lasted 4 months. The patients in these controlled trials were all given medication according to the double-blind technique and the dechiffration was carried out unknown to the author who was treating the patients. Seventy nine percent did return and satisfied the requirements of the total examination. Sixty six (47%) belonged to the placebo-group. The difference in favor of Cernilton of 52 percent compared with 45 percent gives a t-value of 1.71 and (according to the students-t-test) and is significant on the 10 percent level that this difference may not only be occurring by chance.

In these cases there are carried out very careful clinical- and laboratory investigations which also include measuring of heights and weight (some of the patients are claiming that the appetite increases when using the preparations) sedimentation rate, hemoglobin amount, iron in serum, transferrin, cholesterol, electrocardiogram as to state as far as possible the normal condition of the heart, bacteriological examination from the nose and throat and examination as to the antistreptolysintiter. As to all these parameters these is no difference between the groups.

The four investigations referred to cannot be dealt with combined.

They are all aiming at somewhat different approaches. Malstrom and Cederlof have carried through a prophylactic treatment in a not definitely stated period of time and Lindberg has treated his patients at the onset of a common cold with a very high dosage but during a very short period of time. Glomme in contrast to the three others has, in his report, carried through a long-term prophylactic treatment on clinically very carefully examined patients who have been troubled in the past by upper respiratory tract diseases, more or less chronically or with frequent recidives. Therefore it is impossible to get a common statistical view on the possible effect of Cernilton on upper respiratory tract diseases by combining results from these four papers. It seems, a difference in favor of the Cernilton-treated groups in all of these papers but the difference is not great enough to make it statistically reliable or statistically significant.

On the other hand, as to frequency in sick-leave and visits to the doctor for upper respiratory tract diseases and even to general well-being and less pronounced symptoms with shorter duration in the paper reported by Malstrom and Cederlof statistical significant difference has been established.

However, these results seem so interesting that it must be worth-while to carry through careful and sufficiently extensive controlled clinical trials to try to find out whether there exists proved positive effect of Cernilton in cases of upper respiratory tract diseases. The results higher to

reported seem to tally fairly well with the opinion given by the author in connection with the experimental investigation on the effect of Cernilton on spontaneous lung infections in rats: that the most reasonable explanation may be that Cernilton gives a general roborating effect which may support the organism in its own resistance against infections. There is no indication that there are any definite and specific effects.

References

1. Romanus, R: Nordisk Medicine 7, 111. (1952). Chronic prostatovesiculitis.
2. Jonsson, G.: Svenska Läkartindningen (Swedish Medical Journal) 53 (1961) 2497. Prosatitis and Pollen.
3. Schnierstien, J.: Der Urologe 3:4 (1964) 202-208. Zur Termiologie der sogenannten chronischen Prostatitis.
4. Ask-Upmark, E.: Acta Medica Scand. 181 (1967) 355-357. Prostatitis and its treatment.
5. Alken, C.E., Röhl, L. And Jönsson, G: Unpublished report 1966. Report on a clinical trial of Cernilton in chronic prostatitis.
6. Heise, G.W.: Urologischen Klinik der Medizinischen Akademie, Magdeburg. 24.11.1970. Die Chronische Unspezifische Prostatitis.
7. Malstrom,S. And Cederlof, R.: Report 1965-1966. Om pollen som forkyl-ningsprrofylaktikum. (Pollen as a prophylactic against the common cold).
8. Klapsch, H.: From the Department of Industrial Medicine of a heavy Industry Concern, 1967. Experiences of Fluaxin, an anti-influenza medicine in tablet form.
9. Linderberg, E. and Sorensen, S.: A tentative treatment of the common cold with Cernilton. 1968.
10. Glomme, J: University Health Service, University of Oslo, Norway. Unpublished report. 1971. The effect of Cernilton on upper respiratory tract infections.
11. Magnusson, T. and Carlsson A.: Unpublished report, 1960. Report of experiments concerning the toxicity of the pollen preparations Cernitin D 30 UK and GBX-comp.
12. Nilsson, A and Jarplid, B.: Unpublished report. A summarized report of the results of pathological-anatomical studies of mice during long-term trials with Cernilton
13. Huntington Research Centre: Acute oral toxicity to mice. 1970.
14. 13b. Huntington Research Centre: Acute oral toxicity to rats. 1970.
15. Kienholz, M.: Municipal Hospital Offenbach a.M. Central Laboratory and Department for Medical Examinations, 1967. Streptolysin inactivating effect in Cernitin T 60.
16. Kvanta, E.: Institute of Chemistry, Teknikum, Vaxjo, Sweden. 1970. Stretolysin Inhibitory Factor in Pollen, (in press).
17. Karkkula, H.: Cernelle Symposium, 1963, pp. 28.
18. Glomme, J.: University Health Service, University of Oslo, Norway. Unpublished report 1971, 1973. A study on the effect of digested pollen extract on the frequency of spontaneous lung infections in rats.