Preparation and Properties of a Purified Ragweed Extract.* (20847)

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The isolation and purification of the active principle of ragweed pollen has been the subject of considerable study. The earlier work has been adequately reviewed by Newell(1). More recently, separations have been made by electrophoresis(2,3), precipitation at pH 3-4(4), phosphotungstic acid precipitation (5), and adsorption on alumina gel(6). In continuation of the work of Abramson *et al.* (3), we have found that the colorless fractions prepared electrophoretically still contain considerable impurity. It was also found that pigment could be removed in a variety of ways including electrophoresis and solvent extraction. A method of removing the pigment is described which involves the extraction of the pollen grains with 90% methanol before preparing the aqueous extract in the usual way.

Materials and equipment. Giant ragweed pollen was used after being defatted with ether. Reagents were all A.C.S. or C.P. grade. The electrophoretic separation cell was similar to that described previously(3). The method of Tiselius, with a Perkin Elmer instrument (9), was employed to study a methanol purified extract by analytical electrophoresis. Absorption spectra were obtained on a Beckman D. U. Spectrophotometer. The values given for the region 2050 Å to 2200 Å are not corrected for scattered radiation(7), but the values are qualitatively comparable.

Experimental. a) *Electrophoretic separation.* Trifidin, as well as the migrating pigment, was prepared as described by Abramson *et al.*(3), and the absorption spectra of these were compared with those of the crude extracts. These are given in Fig. 1 where two main absorption peaks are observed for the crude extract at about 2600 Å and 3700 Å (Curve A). On electrophoretic separation, the curve for the "colorless" Trifidin (Curve B) still shows the marked band at 2700 Å and a flat portion at 3600 Å. The spectrum of the colored fractions which migrated away from Trifidin gives a curve (Curve C) closely following the shape of the crude extract. b) *Solvent studies.* It follows from the rationale of the electrophoretic method that the crude extract should have as low a conductance as is possible(3). It was found by conductometric measurements that extraction of pollen in the cold with 90% methanol (V/V) removed a large fraction of conducting salts from pollen. At the same time, a large amount of color was also removed. Extraction with water of the methanol-leached pollen, however, still yielded a faint yellow solution. The difference between this pigment and that extracted with 90% methanol is shown by the reaction with alkali. The color of solutions of the latter (90% methanol extracted pigment) deepened in alkali (pH 9)

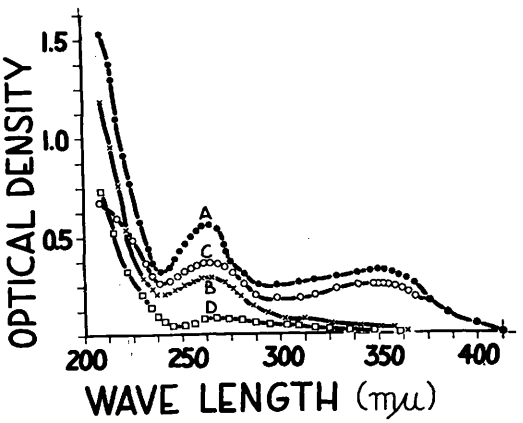

FIG. 1. Absorption spectra of electrophoretically separated pollen extracts. A: crude extract. B: Trifidin fraction. C: pigmented fraction. D: Trifidin precipitated with methanol.

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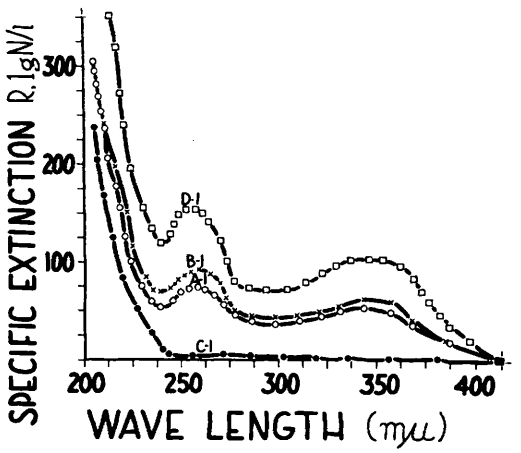

FIG. 2. Specific extinction absorption curves (k, N = 1 g/l). B-1: crude lyophilized fraction. A-1: MLL. C-1: precipitate from reprecipitation of MLL. D-1: methanol extract from reprecipitation of MLL.

and was lighter in acid (pH 2). The former pigment requires relatively stronger alkali before any change is noted and seems to be unaffected by acid. For biological testing, samples were prepared as follows: 5 g of defatted giant ragweed pollen was leached 6 times for 10 minutes in 50 ml 90% methanol. The sixth extract was essentially colorless. The air-dried pollen was then extracted with 100 cc water in the refrigerator for 48 hours. The mixture was filtered and the filtrate lyophilized. The lyophilized residue was a light yellow powder which, on dissolving in water, gave curve A-1, Fig. 2. The general shape of curve A-1 is the same as the crude lyophilized fraction (curve B-1), but the 2600 Å and 3600 Å bands were depressed. This material is called MLL (Methanol Leached Lyophilized). In general, about 100 mg of dry powder was obtained from 5 g of pollen. This powder was soluble in water and had approximately the same nitrogen content as the crude extract on a weight basis (Table I). A sample of MLL was dissolved in water and precipitated with 9 volumes of absolute methanol. The precipitate was separated by centrifugation. The supernatant gave curve D-1 and the precipitate, curve C-1, Fig. 2. The dilutions were made so that curve C-1 plus D-1 should give A-1 and this is correct within 5%. Again the alcohol solution had a predominance of the impurity with peaks at 2600 Å and 3600 Å whereas the precipitate showed neither band.

TABLE I. Analyses of Ragweed Extracts.

Preparation	Nitrogen (%) dry wt
Crude extract	4.85
MLL	5.1
MLL precipitated with methanol	6.3

Clinical study of biological activity of methanol washed, lyophilized giant ragweed pollen. This study was performed on 5 patients all of whom had a clinical history of ragweed hay fever. The patients ranged in age from 10 to 15 years. All patients were scratch-tested with alternating controls to giant ragweed pollen. Of the 5 subjects tested, 2 were found to give positive reactions. All 5 subjects were tested intradermally in triplicate with various dilutions of 1) MLL and 2) control ragweed. Carefully measured quantities of 0.03 cc were injected intradermally. The dilution range was from 0.001 (W/V), 0.001, 0.01, 0.1 and 1 mg/cc respectively.

TABLE II. Biological Tests of MLL in 5 Subjects.

mg/cc (W/V)	Methanol washed ragweed (cm)	Control ragweed (cm)
.0001	0	0
.001	.3, w†	.3, w
.01	.7, w—1 knob	.6, w—1 knob
.1*	1.4, w—2 p†	1.5, w—3 p
.0001	0	0
.001	.5, w	.4, w
.01	.8, w	.8, w
.1*	1.8, w—3 p	1.6, w
.0001	0	0
.001	0	0
.01	.6, w	.5, w
.1	.9, w	.9, w
1.0	1.2, w	1.1, w
.0001	0	0
.001	0	0
.01	0	0
.1	.3, w	.3, w
1.0	.6, w	.6, w
.0001	0	0
.001	0	0
.01	0	0
.1	0	0
1.0	.4, w	.5, w

* In these two subjects, because of the size of the 0.1 mg/cc reaction, no higher concentration was used.

† w = wheal; p = pseudopods.

Comparative dilutions of (1) and (2) were used in the testing. The results are summarized in Table II, which disclosed no difference between crude extract and MLL. A qualitative test of activity was made on the precipitate obtained by methanol precipitation and the sample was extremely active. In view of the spectroscopic results, it was concluded that the material which shows marked 2600 Å and 3600 Å bands is an inactive impurity.

Electrophoretic analysis. MLL was compared with extracts of pollen which had not been previously methanol leached. The electrophoretic analyses are illustrated in Fig. 3. The typical descending electrophoretic pattern of ragweed pollen extract, previously described(2). is shown. The figure on the right is the pattern for the aqueous extracts after treatment of the pollen with 90% methanol. The concentrations were approximately 16 mg/cc. The methanol extracted material is somewhat less concentrated. The buffer was 0.02 M with respect to phosphate and 0.15 M NaCl with a pH of 7.4. It may readily be observed that the unpigmented fraction previously described(2) is the main component and still present with an extremely low mobility. However, there is a new, essentially unpigmented fraction with a mobility of 0.3 μ per second per volt/cm which is a new component that appears after removal of the pigments with 90% methanol.

Discussion. The argument presented above that the "colorless" Trifidin forms the basis of biological activity is well borne out not only by the electrophoretic analysis, but also by the fact that the data in Table II demonstrate unequivocally that pollen extracts containing markedly reduced pigment show the same biological activity on ragweed sensitive individuals as pollen extracts containing the large variety of pigments previously reported. One of the pigments of giant ragweed pollen has been identified as isoquercitrin(8) and was found to possess no biological activity. We believe that removal of the pigments with 90% methanol brings us closer to the understanding of the basis of the biological activity. It would appear that the leaching of defatted pollen with 90% methanol may aid in obtaining sufficient material for further chemical studies of the nature of the "colorless" molecules.‡

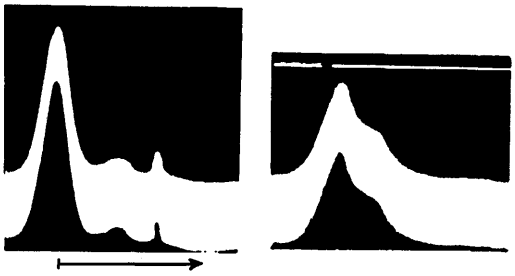

FIG. 3. The typical electrophoretic pattern of ragweed extract is shown on the left. On the right is the aqueous extract after treatment of the pollen with 90% methanol. There is a new, essentially unpigmented fraction with a mobility of 0.3 μ per sec. that forms after removal of the pigments.

Summary. Absorption spectra of ragweed pollen extracts prepared by a new electrophoretic preparative method and by extracting the pollen with methanol are reported. Two main absorption bands were observed for crude aqueous extracts at about 2600 Å and 3600 Å. The 2600 and 3600 Å bands may be correlated with an impurity. The main

‡ It is of interest to speculate on the nature of the binding forces between pigments and colorless fraction. This new essentially unpigmented fraction is active and conceivably would have the mobility of Trifidin if *all* the pigment could be removed. Since a very small amount of pigment is still present in this methanol extract an attempt was made to clear up the ambiguity related to pigment content since it was not certain what happens to the pigment. Direct observation of the electrophoresis cell did not result in any different decision because the intensity of the pigment is so low. However, it would appear that the formation of a new essentially unpigmented component following the removal of pigments supports our previous concepts(2) that the colorless material, the major practically uncharged component, represents the biologically active molecule with pigment groups lending the colorless molecules mobility but not biological activity. In other words, we believe that Trifidin is the basis of the biological activity of giant ragweed extract and that the various pigmented active molecules are in a steady state reacting with pigment groups that are readily disrupted by solvent extraction or as previously observed, electrophoresis itself. However, this must be supported by further studies with the ultracentrifuge.

components separated electrophoretically by the preparative method still showed a marked absorption band at 2600 Å, but none at 3600 Å. Ninety percent methanol leached, lyophilized ragweed pollen extracts (MLL) show a marked depression of the 2600 Å and the 3600 Å bands. Biological activity of the essentially pigment-free preparation is identical with the preparation containing all of the pigment thus confirming our previous point of view that the pigments do not contribute substantially to the biological activity. Electrophoretic analysis reveals that a new substantially colorless major component is formed, on removal of the pigment, with an electric mobility of approximately 0.3 μ per second at pH 7.4.

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Effect of 200 Roentgens Fractional Whole Body Irradiation in the Burro. (20848)

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In a previous investigation of the response of the burro to 400 r fractional whole body irradiation(1), it was observed that this species responded to such irradiation in the same manner as swine(2) and goats(3). In addition the burro demonstrated a pronounced hyperferremia one hour after the completion of the first 400 r exposure and this condition reached a peak on the sixth irradiation day. The present investigation was designed to compare the possible differences in response which might occur when the total daily dose was reduced and to compare the overall response with that seen after a single acute dose in the LD_{50} range.

Methods. Ten male burros were exposed to 200 r Co^{60} gamma ray whole body irradiation daily for 16 days using the multicurie irradiation site described by Wilding *et al.*(4). The radiation flux was approximately 48 r/hr. Control blood samples were obtained 3 times prior to irradiation and daily within one hour after the completion of each irradiation. Hemoglobin was determined colorimetrically by a modified Dupray method(5-7). Platelet counts were made with the method of Brecher and Cronkite(8) and the other hematological values were obtained by the methods of Wintrobe(9). Plasma iron values were determined by the method of Kitzes *et al.*(10) using 2 ml aliquots from plasma samples kept frozen until analyzed. Records were kept of daily body temperatures, food and water consumption and total body weight loss during the experimental period.

Results. General effects. During the first irradiation day the animals appeared normal, but were listless after 400 r and became more so with each subsequent irradiation. The

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