was obtained by summation of radioactivity in each spot from a sample applied to the thin-layer plate.

As may be observed in Table V, from five to seven labeled compounds were separated from urine samples collected at various intervals after dosing. The compound at R_f 0.50 corresponded to intact furosemide while the compound at R_f 0.41 was 4-chloro-5-sulfamyl anthranilic acid. The remaining labeled compounds were not identified. The relative amount of furosemide decreased with the passage of time, while metabolites of furosemide increased. The 24-hr. urine sample contained 40 to 47% of total radioactivity as furosemide and approximately 30% as 4-chloro-5-sulfamyl anthranilic acid. The labeled compound at R_f 0.24 contained up to 20% of the total radioactivity in the 24-hr. urine sample. The methanol solution of ^{35}S-labeled furosemide applied as a reference to the chromatographic plate contained 2.5% 4-chloro-5-sulfamyl anthranilic acid as a radiochemical impurity.

SUMMARY

The data observed during the investigation of the distribution of labeled furosemide would seem to suggest that a portion of the diuretic agent and/or metabolites was distributed in various tissues, amassed in the body, and released gradually over a period of time. The daily administration of labeled furosemide resulted in increased concentrations of the drug and/or metabolites in the liver, kidney, and urine of experimental animals in relation to rats given a single dose of the compound, thus suggesting accumulation in the rat from chronic administration.

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* Sankyo Co., Tokyo, Japan.

Isolation of the Anti-inflammatory Principles from *Achillea millefolium* (Compositae)

ARTHUR S. GOLDBERG*, EVANGELYNNE C. MUELLER, EDWARD EIGEN, and SALVATORE J. DESALVA

Abstract □ An aqueous extract of the dry flower heads of *Achillea millefolium* (yarrow) has been found to possess anti-inflammatory activity as measured by the mouse paw edema test. Fractionation has resulted in the isolation of a material which reduces inflammation by 35%. This concentrate is water-soluble, nonsteroidal, and has a very low order of toxicity. Physical and chemical studies show this active fraction to be a mixture of protein-carbohydrate complexes.

Keyphrases □ Anti-inflammatory constituents—*Achillea millefolium* flower heads □ Column chromatography—separation, identity □ Electrophoresis, disc—identification □ Colorimetry—analysis □ Pharmacological screening—*A. millefolium* extracts

The isolation, partial characterization, and pharmacology of the water-soluble, nonsteroidal, anti-inflammatory constituents of *Achillea millefolium* are reported.

Achillea millefolium L. (colloquially referred to as yarrow) is a wild-growing flowering plant belonging to the family Compositae and is found abundantly throughout America and Europe (1). The many uses of yarrow in folk medicine date back to Achilles who used the plant to heal the wounds of his warriors (2). The chemical investigation of *A. millefolium* for the past 200 years has led to the isolation of a large number of compounds, but the principles responsible for the anti-inflammatory properties of the plant are still unknown. The volatile oil of *A. millefolium* has been tested for anti-inflammatory activity and found to be inactive (3).

Preliminary screening in these laboratories of an aqueous extract of the flower heads of *A. millefolium* showed that it possessed both topical and systemic anti-inflammatory activity.

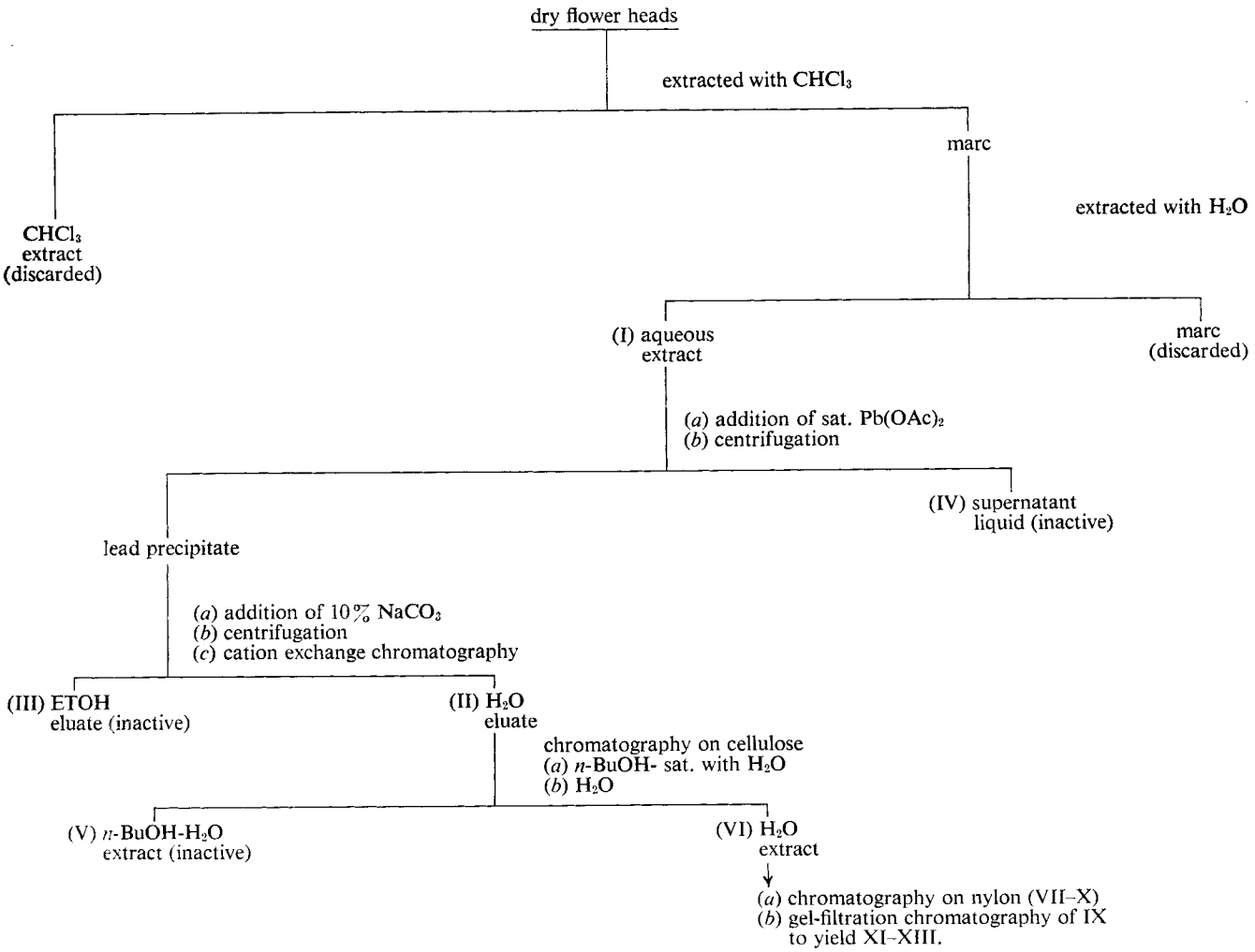
EXPERIMENTAL

Materials—The flower heads of *A. millefolium*, collected in New Jersey during the summer of 1966, were used in this investigation.

Preparation of the Initial Extract for Biological Testing—The initial evaluation of *A. millefolium* for anti-inflammatory activity was carried out on a cold aqueous extract of the dry flower heads. The extract was dried by lyophilization and tested.

Isolation and Characterization of the Active Principles—The dry flower heads of *A. millefolium* (1.3 kg.) were extracted with $CHCl_3$ for 24 hr. in a continuous extraction[1] apparatus. The marc was air-

[1] Soxhlet.



Schematic Presentation of Fractionation of A. millefolium

dried and extracted with 12 l. of water at 65–75°. The aqueous extract was evaporated under vacuum to a small volume and lyophilized (Freeze-Mobile Virtis model 10), yielding 42 g. of brown material (Fraction I). Fraction I gave positive tests with ferric chloride, lead acetate, and Prussian blue reagents, suggesting the presence of phenolic-type compounds. Negative tests with Mayer's and Hager's reagents indicated the absence of alkaloids.

Twenty grams of I was added to 1 l. of water and the insoluble residue removed by centrifugation and discarded. A saturated solution of $Pb(OAc)_2$ was slowly added to the above solution until no further precipitation occurred. The precipitate was collected by centrifugation and the supernatant was placed on a cation exchange column[2] to remove any lead present as $Pb(OAc)_2$. The combined aqueous and ethanolic eluate (IV) from this column was tested biologically and found to be inactive. The lead precipitate was washed with distilled water, and decomposed by addition of a 10% solution of Na_2CO_3 which was added until a pH of 9 was attained. The $PbCO_3$ which resulted was removed by centrifugation.

The supernatant was placed on a cation exchange column[2] which was eluted with water resulting in a 29% yield of active material (II). Elution of the column with 95% ethanol resulted in another fraction (III) which was inactive.

When II was tested with ferric chloride, lead acetate, and Prussian blue reagents, positive tests were again obtained. This fraction was tested, therefore, for the presence of flavonoid compounds using the color reactions described by Venkataraman (4). The test reagents used were aqueous sodium hydroxide, concentrated sulfuric acid, magnesium-hydrochloric acid, and sodium amalgam followed by hydrochloric acid. All of these tests were negative, thereby excluding the presence of flavonoid-type compounds.

It was observed that, when an aqueous solution of II was shaken, a stable honeycomb froth formed which is characteristic of saponins (5). Fraction II was then tested for steroidal and triterpenoid saponins using the blood hemolysis test of Wall (6). No hemolysis of red blood cells occurred, thus eliminating the above classes of compounds.

Chromatography of II on a cellulose column[3] resulted in two fractions (V, VI). Fraction V was obtained by elution with *n*-butanol saturated with water and was found inactive. This was followed by elution with water which resulted in a 61% yield (18% overall yield) of active material (VI).

Fraction VI was chromatographed on a nylon column[4] yielding four fractions (VII–X). Elution of the column with 100% MeOH resulted in VII which was inactive. Fractions VIII–X were obtained by elution of the column with MeOH–NH_3 (10:1), MeOH–H_2O–NH_3 (5:5:1), and H_2O–NH_3 (10:1), respectively. They represent a combined yield of 34% (6% overall yield), and all were active. These fractions were tested with Molisch and Lowry reagents to detect the presence of carbohydrates and proteins, respectively. Fraction VII gave negative tests with both reagents while VIII–X gave positive tests with these reagents. It should be mentioned that all of the previous fractions isolated were then tested with these reagents. Negative tests resulted with all of the inactive fractions while the active fractions reacted positively.

Purification was continued on the most active of the above frac-

[2] Amberlite CG-50, hydrogen form, Rohm & Haas Co., Philadelphia, Pa.

[3] MN-2100 cellulose powder, Brinkmann Instruments, Dist., Westbury, N. Y.

[4] Ultramide powder, BASF in Ludwigshafen, Alupharm Chemicals Dist., New Orleans, La.

Table I—Amino Acid Analysis of Fraction XII

Compounds Present	moles/10^3 moles
Cysteic acid	16.4
Hydroxyproline	59.1
Methionine sulfoxide	9.4
Aspartic acid	130.2
Threonine	49.3
Serine	68.6
Glutamic acid	125.4
Proline	46.8
Glycine	168.6
Alanine	64.6
Valine	50.0
Cystine (1/2)	49.8
Methionine	4.3
Isoleucine	20.9
Leucine	35.4
Tyrosine	10.1
Phenylalanine	13.4
Hydroxylysine[a]	—
Ornithine	2.3
Lysine	24.1
Histidine	7.1
Unknown amino acid	25.0
Arginine	19.2

[a] Only a trace peak was observed.

tions (IX), which was isolated in a 13% yield (2.3% overall yield). Gel filtration chromatography on synthetic polysaccharide[5] using distilled water yielded three fractions (XI–XIII), all of which were active. The second fraction (XII) eluted from the column possessed more than twice the activity of the other two fractions, and characterization of this material was undertaken.

Physical and Chemical Characteristics of Fraction XII—Fraction XII gave positive tests with Lowry and Molisch reagents indicating the presence of proteins and carbohydrates, respectively. It also gave positive tests with ferric chloride, lead acetate, and Prussian blue reagents which may now be attributed to the presence of proteins. A negative Benedict's test showed the absence of free reducing sugars. Ninhydrin reagent also gave a negative test suggesting that the presence of free amino acids in Fraction XII is unlikely. Elemental analysis showed the following results: N, 4.07; P, 0.28; S, 1.56%. A pure protein contains an average of 16% nitrogen. If the percent nitrogen obtained is attributed only to the presence of proteins, then XII contains a maximum of 25% protein based on this nitrogen value.

Disc Electrophoresis—Fraction XII was subjected to disc electrophoresis (Canalco model 12). Electrophoresis was carried out in 7.5% acrylamide gel using tris-glycine buffer at pH 8.3 with ionic strength of 0.01 and a current of 5 ma. per column. The time required for the separations was approximately 35 min. The gels were stained with Coomassie Brilliant Blue[6] which detects proteins and with periodic acid (Schiff reagent) which is used to detect glycoproteins, polysaccharides, and high molecular weight carbohydrates. Two bands were observed and each gave positive tests with both of these reagents.

A molecular weight for the material comprising XII was estimated between 30,000–70,000 using gel filtration chromatography on synthetic polysaccharide G-25, G-50, and G-75[7] gels. A small variation of these range limits is possible, however, since the above values were obtained using the fractionation ranges of these gels which are based on globular proteins (7). The molecular weights of some glycoproteins calculated from calibration curves using globular proteins have been found to deviate from their expected values (8).

Amino Acid Analysis—A sample of XII was hydrolyzed by treating it with 6 *N* hydrochloric acid in a sealed tube at 110° for 24 hr. Amino acid analysis was carried out using an automatic analyzer (Technicon) and the results are shown in Table I. Seven unknown peaks with very short elution times were observed which absorbed more strongly at 440 mμ than at 570 mμ. Zacharius (9, 10) has shown that such peaks are characteristic of non-nitrogenous compounds related to carbohydrates.

Sugar Analysis—A sample of XII was hydrolyzed with 3 *N* hydrochloric acid in a sealed tube for 3 hr. at 110°. The hydrolysate was neutralized by passing it through an anion exchange column[8] in the carbonate form. Sugar analysis was performed using the automatic analyzer according to the method of Catravas (11). The major sugars characterized from the chromatogram obtained were glucose, xylose, and arabinose. Rhamnose, mannose, and galactose were also detected along with five unknown peaks.

PHARMACOLOGICAL TESTS

The major portion of the pharmacological tests was carried out on intermediate fractions which eventually led to the isolation of the most active Fraction XII, and not on this latter fraction. This was necessitated because of the large amounts of material needed for the various chemical tests.

Mouse Paw Edema Test—A modification of the method of DeSalva and Evans (12) was employed to evaluate anti-inflammatory activity and to follow the course of fractionation. Female mice (Swiss-Webster) (18–25 g.) were injected with 0.125 mg. yeast in the left foot pads and immediately thereafter the test solution was injected subcutaneously into the scapular area. The assay was terminated after 3 hrs. and all animals were sacrificed by cervical fracture and their feet were severed at the ankle joints and weighed. The greater the difference between the weights of the right and left feet, the larger the edema. Eight mice were used for each fraction tested. All yarrow fractions were tested at a dose level of 40 mg./kg., since initial tests at this dosage of the crude extract produced a significant response.

Mouse Paw Edema Test Results—The activities of the major fractions isolated are reported in Table II along with the activities of some known anti-inflammatory agents.

Topical Anti-inflammatory Activity—This was evaluated by a modification of the rabbit skin irritation test of Draize (13). Irritation was induced with sodium lauryl sulfate using a ratio of test material to sodium lauryl sulfate of 5:20 (mg./ml.). Fraction VI produced a 50% reduction of inflammation when subjected to this test.

Effect of Adrenalectomized Mice—Fractions VI and IX were tested for activity in adrenalectomized and nonadrenalectomized mice following the same procedure used in the mouse paw edema test. Identical activity resulted in both types of mice when each of these fractions was tested.

Acute Toxicity Studies—Toxicity studies on VI showed that both the oral LD_{50} and subcutaneous LD_{50} in mice are equal to or greater than 1,000 mg./kg. A diuretic effect was also observed at a dose level of 100 mg./kg. and the urine at this dosage was dark brown.

DISCUSSION

An investigation of *A. millefolium* has revealed that the constituents responsible for the anti-inflammatory properties of the plant are present in the aqueous extract of the flower heads. Fractionation of this extract has led to a number of active and inactive fractions. All of the fractions possessing activity are water-soluble and give positive tests for proteins and carbohydrates. Tests for alkaloids, saponins, flavonoids, and reducing sugars throughout the isolation procedure showed that these classes of compounds are absent. No proteins or carbohydrates were detected in any of the inactive fractions.

The most active fraction isolated to date (XII) reduced inflammation by 35% at a dose level of 40 mg./kg. It is difficult to account for the fact that the extensive purification achieved in going from I to XII did not result in any substantial increase in activity (Table II). Two plausible explanations are offered. Denaturation of the protein might have occurred during the course of fractionation. This assumes that the protein in the active fractions is associated with the anti-inflammatory properties of these fractions. It is also possible that a contaminant may have been concentrated along with

[5] Sephadex G-75, Pharmacia Fine Chemicals Inc., Piscataway, N. J.
[6] Mann Research Lab., Inc., New York, N. Y.
[7] Sephadex.
[8] Dowex 1-X4, J. T. Baker Chemical Co., Phillipsburg, N. J.

Table II—Anti-inflammatory Activities in the Mouse Paw Edema Test

Compound	Dose	Decrease in Inflammation, %
Corticotrophin	10 USP units/kg.	−53
Acetylsalicylic acid	40 mg./kg.	−19
Phenylbutazone	40 mg./kg.	−26
Dexamethasone-21-phosphate	10 mg./kg.	−42
Hydrocortisone acetate	40 mg./kg.	−62
Indomethacin	40 mg./kg.	−44
Fractions[a]		
II[5]	40 mg./kg.	−34
VI[18]	40 mg./kg.	−29
IX[14]	40 mg./kg.	−25
XI[3]	40 mg./kg.	−10
XII[3]	40 mg./kg.	−35
XIII[3]	40 mg./kg.	−15

[a] Superscripts designate the number of times a fraction was tested.

the active material and that this material exerted an inhibitory effect on the active components. In view of the chemical tests performed and the isolation procedure used, the latter explanation is plausible only if the contaminant was bound to the active components or was very similar in nature to the active material.

In order to ascertain whether the active principles act as true anti-inflammatory compounds or counter irritants, tests were performed with both adrenalectomized and nonadrenalectomized mice. The results of these studies show that the presence of the adrenal glands is not needed for activity. These fractions most likely exert a true anti-inflammatory response. If they function as counter irritants, the adrenal gland is a critical intermediate site of action which may be activated either indirectly by the release of ACTH from the hypophysis or directly through an unknown mechanism in the adrenal cortex.

A preliminary characterization of the most active fraction (XII) isolated has shown that it contained approximately 25% protein based on an elemental analysis for nitrogen (4.07%). Since no compounds other than proteins and carbohydrates were detected, XII should contain a very high percentage of carbohydrates. Disc electrophoresis of XII gave two bands demonstrating that it was not homogeneous. Each of these bands gave positive tests for protein and carbohydrates indicating that the protein was linked to the carbohydrate.

Additional evidence to support the linkage of the protein to the carbohydrate was the use of nylon chromatography in the fractionation procedure which eventually led to the isolation of XII. Separation of compounds on nylon is based on the different hydrogen-bonding affinities of these compounds toward nylon. The presence of a mixture of protein and carbohydrate components in the same fraction would require that they possess the same hydrogen-bonding characteristics toward nylon. Similar hydrogen-bonding properties of these compounds are highly unlikely since proteins will hydrogen-bond mainly through their amide groups while carbohydrates will hydrogen-bond mainly through their hydroxyl groups. This strongly suggests that the active principles are a number of closely related protein-carbohydrate complexes or glycoproteins. The use of the term protein-carbohydrate complexes is preferred by the authors, rather than glycoproteins to describe the active principles because glycoproteins usually contain a higher percentage of protein than was found in XII.

Glycoproteins are known to be involved in the inflammation process and have been found to accumulate at the site of inflammation (14, 15) but their role is still unclear. Varga (14) has postulated that they are retained at the site of inflammation for tissue repair. Proteolytic enzymes have also been used as anti-inflammatory agents (16). This, however, is the first report of protein-carbohydrate complexes acting as anti-inflammatory agents. It is hoped that future investigations will determine the exact nature of the protein-carbohydrate complexes and will lead to a better understanding of the role of glycoproteins and related compounds in the inflammatory process.

SUMMARY

Chemical and pharmacological investigation of the flower heads of *A. millefolium* has resulted in the isolation of a number of fractions which possess anti-inflammatory activity. These fractions are water-soluble, nonsteroidal, and appear to be of very low toxicity. The most active fraction isolated reduced inflammation by 35%. Physical and chemical evidence showed that this fraction is not homogeneous but consists of a mixture of protein-carbohydrate complexes.

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* Present address: Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029

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Requests for reprints should be sent to Edward Eigen, Colgate-Palmolive Research Center, Piscataway, NJ 08854