again formulations containing castor oil in the vehicle proved to be less irritating than similar preparations containing sesame oil. Physically and chemically both oil solutions were stable. Based on acceptable preliminary data, formulations such as those listed in Table VI were prepared and tested. Acceptability in humans was confirmed by clinicians and described in the literature (22, 23) and in case reports.[4]

SUMMARY

1. The development and testing of parenteral steroid hormone formulations has been described, using castor oil as a vehicle.

2. After ascertaining stability and animal muscle irritation, selected formulations were evaluated in humans. They exhibited a prolonged action, were effective and well tolerated.

3. Examples of commercially available products are the estrogen, estradiol valerate[5] at 20 mg./ml. and 40 mg./ml., and the progestogen, 17-hydroxyprogesterone caproate[6] at 250 mg./ml.

[4] Case reports: estradiol valerate, 20 mg./ml., in castor oil 78%, benzyl benzoate 20%, benzyl alcohol 2%—90 injections in 46 patients. Two mild local reactions. Estradiol valerate 40 mg./ml. in castor oil 58%, benzyl benzoate 40%, benzyl alcohol 2%—51 patients. Number of injections not completely tabulated. One report is in press.

[5] Marketed as Delestrogen by E. R. Squibb & Sons, New York, N. Y.

[6] Marketed as Delalutin by E. R. Squibb & Sons, New York, N. Y.

REFERENCES

(1) Deanesly, R., and Parkes, A. S., *J. Physiol.*, **78**, 155 (1933).
(2) Brown, W. E., Wilder, V. M., Schwartz, P., *J. Lab. Clin. Med.*, **29**, 259(1944).
(3) "Hormone Therapy in Practice," 2nd ed., Schering, A. G., Erich Blaschker, Berlin, 1954, p. 109.
(4) Master, W. H., Grody, M. H., and Magallon, D. T., *J. Clin. Endocrinol.*, **12**, 1445(1952).
(5) "Modern Trends in Endocrinology," H. Gardiner-Hill, ed., P. B. Hoeber, 1958, p. 233.
(6) Junkmann, K., *Arch. Exptl. Pathol. Pharmakol.*, **215**, 85(1952).
(7) Davis, M. E., and Wied, G. L., *J. Clin. Endocrinol. Metab.*, **15**, 923(1955).
(8) Boschann, H. W., *Arst. Wochenschr.*, **9** (25) 591 (1954).
(9) Richter, U. S. pat. 2,822,316.
(10) Brit. pat. 817,241.
(11) Ercoli, A., U. S. pat. 2,983,649.
(12) Eckey, E. W., "Vegetable Fats and Oils," A.C.S. Monograph, Reinhold Publising Co., New York, N. Y., Series No. 123, pp. 587–597.
(13) Roberts, H. R., and Florey, K., THIS JOURNAL **51**, 794 (1962).
(14) Short, C. L., *Am. Practitioner Dig. Treatment*, **11**, 149(1960).
(15) Greenblatt, R. B., and Dutta, S. N., *Ariz. Med.*, **18** (4), 107(1961).
(16) Kelley R. M., and Baker, W. H., *New Engl. J. Med.*, **264**, 216(1961,).
(17) Kennedy, B. J., *J. Am. Med. Assoc.*, **184**, 758(1963).
(18) Danforth, D. N., and Buckingham, J. C., *Postgrad. Med.*, **32**, 345(Oct. 1962).
(19) Kistner, R. W., *Clin. Obstet. Gynecol.*, **5**, 1166(1962).
(20) Siegel, I., *Obstet. Gynecol.*, **21**, 666(1963).
(21) Pellegrino, L., *Current Therap. Res.*, **4**, 301(1962).
(22) MacDonald, I., and Yettra, M., *Med. Clin. N. Am.*, **43**, 971(1959).
(23) Eichner, E., Brown, M., and Sable, M., *J. Intern. Coll. Surgeons*, **32**, 394(1959).

Isolation of Marrubiin, a Sterol, and a Sesquiterpene from *Marrubium vulgare*

By HAROLD J. NICHOLAS*

A simple column chromatographic method for isolating the bicyclic diterpene marrubiin from acetone and ethanol extracts of *Marrubium vulgare* L. is described. An unsaturated sterol of the stigmastanol series, present in esterified form, and a sesquiterpene ($C_{15}H_{22}O_2$) have been isolated from the extracts.

IN PREPARATION for radioactive tracer work on the biosynthesis of marrubiin it was necessary to examine extracts of the plant for associated terpenoid substances. A convenient column chromatographic method was therefore devised for separating relatively pure marrubiin from crude acetone extracts. Two new terpenoid substances were detected in the extracts.

EXPERIMENTAL

Materials and Methods.—Ground *M. vulgare* L. was obtained from the Wunderlich-Diez Corp., Hasbrouck Heights, N. J.[1] This material was exhaustively extracted with hot acetone or hot ethanol. Either solution on removal of solvent by distillation (the last stages *in vacuo*) yielded black, viscous material which was used for further examination. Melting points were determined on a Fisher-Johns melting point apparatus. Optical rotations (in $CHCl_3$) and C—H analyses were determined by Drs. G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford, England. An infrared spectrum of the unidentified diterpene was determined on a Perkin-Elmer spectrophotometer by the KBr disk method.[2] An infrared spectrum of the sterol was determined in chloroform solution in a 0.1-mm. sealed cell, compensated with $CHCl_3$, on a Beckman IR-4 recording infrared spectrophotometer,[3] and by the KBr disk method. The

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[1] This firm has given assurance that the material investigated was *M. vulgare* or white horehound, not *Ballota hirsuta* (black horehound).

[2] We are indebted to the Department of Pathology, University of Kansas, for this determination.

[3] Determined by Sadtler Research Laboratories, Philadelphia, Pa.

TABLE I.—CHROMATOGRAPHY OF CRUDE *M. vulgare* ACETONE EXTRACTS

Fraction[a]	Eluting Solvent[b]	Material Eluted, Gm.
1	...	11.8, reddish, crystalline wax-like substance
2	Petroleum ether	0.2, orange wax-like substance
3	5% ethyl ether	0.5, orange wax-like substance
4	5% ethyl ether	0.6, orange wax-like substance
5	10% ethyl ether	0.4, orange wax-like substance
6	10% ethyl ether	0.3, orange wax-like substance
7	20% ethyl ether	0.2, orange wax-like substance
8	20% ethyl ether	1.8, brown wax-like substance
9	40% ethyl ether	3.2, blackish, crystalline wax-like substance
10	40% ethyl ether	1.3, blackish, crystalline wax-like substance
11	40% ethyl ether	0.7, black glass-like substance
12	80% ethyl ether	1.3, black glass-like substance
13	100% ethyl ether	3.6, blackish-green, crystalline solid
14	100% ethyl ether	5.0, blackish-green, crystalline solid
15	100% ethyl ether	1.4, dark green glass-like substance
16	100% ethyl ether	0.8, black wax-like substance
17	100% ethyl ether	1.4, black wax-like substance
18	100% ethyl ether	1.5, black wax-like substance
19	100% ethyl ether	1.1, black wax-like substance
20	100% ethyl ether	0.5, black wax-like substance
21	100% ethyl ether	1.2, black wax-like substance
22	100% ethyl ether	0.8, dark green, semicrystalline solid
23	100% ethyl ether	0.2, dark green, semicrystalline solid
24	100% ethyl ether	0.1, dark green, semicrystalline solid
25	5% ethanol in benzene	0.1, greenish solid
26	5% ethanol in benzene	2.0, black glass-like substance
27	5% ethanol in benzene	2.2, black glass-like substance
28	5% ethanol in benzene	1.9, black glass-like substance
29	1:1 ethanol-benzene	1.6, black wax-like substance

[a] Each fraction 800 ml. in volume. [b] The percentages of ethyl ether refer to mixtures with petroleum ether (v/v). Ethanol-benzene mixtures are also expressed as v/v.

alumina used was Merck, acid-washed. The silicic acid was Mallinckrodt A.R. grade, 100 mesh, heated to 100–110° for 1 hour just prior to use. Except for material used to perform the initial extraction of plant material, all solvents were distilled before use. The petroleum ether had a boiling point of 30–60°.

Chromatography of Crude Extract.—Acetone or ethanol proved satisfactory for extracting marrubiin, sterol, and sesquiterpene from the plant, but a comparison of yields from each solvent was not made. The black viscous concentrate from either solvent contained some benzene-insoluble tar[4] which interfered with chromatography. Since hot benzene effectively separated the materials under investigation from the tar, a preliminary separation was made, based on this differential solubility in hot benzene. A description of isolation from acetone concentrates follows.

Benzene soluble material (78 Gm.) in 200 ml. of benzene was diluted with an equal volume of petroleum ether and poured onto a 5.5-cm. diameter column containing 1000 Gm. of alumina. After the solvent was all on the column, an additional 600 ml. of petroleum ether was added and the eluate was collected as Fraction *1*. Additional 800-ml. fractions containing varying quantities of ethyl ether or ethanol-benzene were subsequently collected. Table I gives the composition of the eluting fractions and a description and yield of material eluted after removal of solvent. Only about 55% of the original benzene-soluble material was recovered from the column.

Isolation of Marrubiin.—Fractions *13* to *15* were combined and crystallized to constant melting point from absolute ethanol, giving rods, m.p. 160–161°; $[\alpha]_D^{21°} = +42.9°$ (1). Yield: 8.0 Gm. Marrubiin also is slowly removed from alumina with 80% ethyl ether in petroleum ether. An additional small amount was recovered from Fraction *12*. In a control experiment 50 mg. of pure marrubiin in 30 ml. of 1:1 benzene-petroleum ether[5] was placed on a 1.2-cm. diameter column containing 15 Gm. of alumina. The eluate was collected as Fraction *1*. An additional 30 ml. of petroleum ether was poured through as Fraction *2*. Three 30-ml. fractions each of 5, 10, 20, 40, and 80% ethyl ether in petroleum ether (v/v), respectively, were poured through, followed by three ethyl ether fractions, each also 30 ml. in volume. A small amount of solid was removed by the second and third 80% ethyl ether fractions. The bulk of the material was recovered from the 100% ethyl ether fractions. Total recovery from the column: 37.4 mg. (74.8%).

ISOLATION OF STEROL FRACTION AND PREPARATION OF DERIVATIVES

The material in Fractions *9* and *10* (40% ethyl ether fraction) had the characteristics of a steroid ester. It readily became semifluid at steam bath temperature and gave no precipitate with digitonin. On saponification with 10% KOH in ethanol, a nonsaponifiable fraction was obtained, representing a large portion of the original wax. After several crystallizations of the nonsaponifiable fraction from acetone-methanol, crystals melting in the range 100–300° were obtained; these slowly gave characteristic colors with the Liebermann-Burchard reagent and gave a precipitate with digitonin. Since a nonsaponifiable fraction of the original acetone or ethanol concentrates proved a more ready source of the sterol mixture, purified sterol was obtained

[4] Examination of this hot benzene-insoluble fraction yielded no products of interest.

[5] At benzene-petroleum ether ratios, less than 1:1 the marrubiin precipitated.

in the following manner. Black gummy extractive from ethanol concentrates of *M. vulgare* was refluxed for 2 hours in 2 L. of 10% alcoholic KOH. After cooling, the mixture was diluted with H_2O and extracted thoroughly with ethyl ether. The latter was washed with H_2O and distilled, leaving 104 Gm. of orange, semisolid nonsaponifiable fraction. This was shaken thoroughly with 1.5 L. of petroleum ether and decanted from 22.5 Gm. of insoluble red oily substance which was not further examined. The petroleum ether solution was poured onto a 5.5-cm. diameter column containing 1000 Gm. of alumina. The column was washed with petroleum ether until only traces of yellow gummy substance were eluted. It was then washed with 3200 ml. of 2% ethanol in petroleum ether which yielded on distillation 32.8 Gm. of a red wax-like substance. This proved to be the source of the sterol and unidentified diterpene. On trituration with acetone-methanol and standing overnight, the solid precipitated. This was crystallized from acetone, yielding 2.3 Gm. of yellow solid as crude sterol. The filtrate on removal of solid gave viscous, red oily substance with a faintly fragrant odor, the crude diterpene fraction.

Purification of Sterol.—Crude sterol (9.9 Gm.) was converted to the acetate with acetic anhydride and anhydrous pyridine under reflux. The acetylated crystalline product so obtained could not be purified to a constant melting point by chromatography on silicic acid and crystallization from several solvents. It was therefore saponified with 5% alcoholic KOH. The free sterol was dissolved in 20 ml. of warm benzene, diluted with 100 ml. of petroleum ether, and poured onto a 2.2-cm. diameter column containing 100 Gm. of alumina. The eluate was collected as Fraction *1*. Seven 50-ml. fractions of petroleum ether were taken (yielding a small amount of colorless oily substance: discarded), followed by sixty 50-ml. fractions of benzene. Of the latter, Fractions *15* to *60* yielded white crystalline solid, combined weight 4.0 Gm.

Sterol Acetate.—The above product was acetylated with pyridine and acetic anhydride under 0.5-hour reflux, then processed in the usual manner. After three crystallizations from acetone-methanol, 2.6 Gm. of scales were obtained, m.p. 133–134°. Four additional crystallizations from the same solvent mixture or acetone-ethanol gave scales, m.p. 134–136°; $[\alpha]_D^{21°} = -45.61°$.

Anal.—Calcd. for $C_{31}H_{50}O_2$: C, 81.88; H, 11.08. Found: C, 81.81; H, 11.11.

Free Sterol.—Saponification of the above acetate with 5% alcoholic KOH (steam bath, 2 hours) gave rods after five crystallizations from either acetone, ethyl acetate–methanol, or acetone-methanol, m.p. 158–160°. An additional crystallization from acetone-methanol gave needles, m.p. 159–160°; $[\alpha]_D^{21°} = -46.52°$. The compound exhibited only nonspecific end absorption in the region 220–300 mμ. In the Liebermann-Burchard test ($CHCl_3$–HAc underlayed with concentrated H_2SO_4) the compound gave no immediate color in either liquid phase, although a pink interface developed within a few minutes. After 30 minutes, a light green developed in the $CHCl_3$ layer, remaining several hours. The substance, therefore, falls in the category of a "slow-reacting" sterol (2). It gave a negative Tortelli-Jaffe reaction (4).

Anal.—Calcd. for $C_{29}H_{48}O$: C, 84.40; H, 11.73. Found: C, 84.45; H, 11.51.

The infrared spectrum obtained in chloroform solution showed a close similarity to that of β-sitosterol, but was characterized by general poor resolution of the bands. The spectrum obtained by the KBr disk method was identical with that of β-sitosterol, except for the appearance of an additional band at 14.3 μ.

Sterol Benzoate.—Free sterol (290 mg.), benzoyl chloride (1 ml.), and 3 ml. of anhydrous pyridine were refluxed 1 hour. The cooled mixture was diluted with H_2O, filtered, and washed thoroughly with 5% Na_2CO_3 and H_2O, respectively. The product was then crystallized to a constant m.p. of 151–153° from benzene-ethanol, acetone-methanol, or chloroform-acetone; $[\alpha]_D^{21°} = -21.67°$.

Anal.—Calcd. for $C_{36}H_{52}O_2$: C, 83.68; H, 10.15. Found: C, 83.42; H, 9.90.

Reduction of Sterol Acetate in Neutral Medium.—The sterol acetate (70 mg., m.p. 134–136°) was shaken for 8 hours under 60 p.s.i. hydrogen pressure in the presence of 50 mg. of PtO_2 and 30 ml. of ethyl acetate. After three crystallizations from acetone-methanol, the product so obtained gave scales, m.p. 122–124°, unchanged by an additional crystallization from chloroform-methanol; $[\alpha]_D^{21°} = \pm 0°$. Yield: 45 mg.

Reduction of Sterol Benzoate in Neutral Medium.—The sterol benzoate (93 mg., m.p. 151–153°) was hydrogenated as described for the above acetate. The product crystallized readily from acetone-methanol giving microscopic needles, m.p. 122–124°, after four crystallizations; $[\alpha]_D^{21°} = 26.74°$. Yield: 80 mg.

Free Sterol After Reduction in Neutral Medium.—The benzoate (m.p. 122–124°, 60 mg.) was saponified with 5% KOH in ethanol. The product was recovered by dilution with H_2O, extraction with ethyl ether, etc. After two crystallizations from acetone-methanol, needles (m.p. 136–137°) were obtained, unchanged after an additional crystallization from acetone-ethanol; $[\alpha]_D^{20°} = +23.5°$. The compound gave a melting point depression of 3–5° on admixture with stigmastane-3β-ol.

Reduction of Sterol Acetate in Acidic Medium.—Sterol acetate (107 mg.) in 15 ml. of glacial acetic acid was shaken under 60 p.s.i. hydrogen pressure in the presence of 50 mg. of PtO_2 for 8 hours. After filtration, acetic acid was removed *in vacuo* and the product crystallized four times from either aqueous acetone or acetone-methanol giving needles, m.p. 134–135°; $[\alpha]_D^{21°} = +15.36°$. The melting point was undepressed on admixture with stigmastanol acetate.

Free Sterol After Reduction in Acidic Medium.—Saponification of the above acetate with 10% alcoholic KOH (steam bath, 1 hour) and work-up of the product gave needles, m.p. 137–139°, after four crystallizations from acetone-methanol; $[\alpha]_D^{21°} = +19.06°$. The melting point was undepressed on admixture with stigmastane-3β-ol.

Examination of Mother Liquors.—Extensive examination of the combined filtrates from crystallization of the original sterol mixture revealed free sterol, m.p. higher than 142°, acetate 133°+. It appeared likely that little or no β-sitosterol was present in the nonsaponifiable fraction of the plant.

TABLE II.—CHROMATOGRAPHY OF CRUDE ACETYLATED SESQUITERPENE PREPARATION

Fraction[a]	Eluting Solvent[b]	Material Eluted, Gm.
1 to *5*	Petroleum ether	None
6 to *20*	Petroleum ether	1.1, colorless oily substance
21 to *25*	1% ethyl ether	0.8, light yellow oily substance
26 to *39*	1% ethyl ether	0.5, partially crystalline yellow oily substance
40 to *44*	2% ethyl ether	5.0, semicrystalline oily substance
45 to *50*	2% ethyl ether	11.6, yellow crystalline solid
51 to *64*	2% ethyl ether	5.2, yellow gummy substance
65 to *70*	5% ethyl ether	0.2, yellow gummy substance
71 to *75*	10% ethyl ether	1.2, yellow gummy substance
76 to *80*	20% ethyl ether	3.0, yellow gummy substance
81 to *90*	50% ethyl ether	17.0, red oily substance
91	100% ethyl ether	1.5, yellow gummy substance

[a] Each fraction 100 ml. in volume. [b] The percentages of ethyl ether refer to admixture in petroleum ether.

TABLE III.[a]—MOLECULAR ROTATION DIFFERENCES BETWEEN DERIVATIVES OF STEROL FROM *M. vulgare* AND Δ^5- AND Δ^7-STENOLS

	Molecular Rotation (M_D) of—			—M_D (deriv.)	$-M_D$ (sterol)—
	Free Sterol	Acetate	Benzoate	Acetate	Benzoate
From *M. vulgare*	−192	−207	−113	−15	+79
Δ^5-Stenols[b]	−153 ± 8	−190 ± 12	−145	−35 ± 16	+81 ± 16
Δ^7-Stenols[c]	+35 ± 8	+31 ± 8	+68 ± 8	−15 ± 15	+20 ± 14

[a] Data, other than for *M. vulgare* sterol, largely from References *5* and *6*. [b] Δ^5, 11(?)-Stigmastadiene-dβ-ol (6). [c] Δ^7,11(?)-Stigmastadiene 3β-ol (6).

ISOLATION OF TERPENES

A quantity of the viscous red oily substance obtained from the filtrates in the preliminary crystallization of the crude sterol mixture was refluxed with excess acetic anhydride and anhydrous pyridine for 1 hour. The cooled mixture was diluted with H_2O and extracted thoroughly with ethyl ether. The latter was washed alternately with dilute HCl, H_2O, 5% NaOH and H_2O, then distilled, yielding 59 Gm. of viscous red oily substance. In 100 ml. of petroleum ether this oily substance was poured onto a 4.5-cm. diameter column containing 300 Gm. of silicic acid. The column was subsequently washed with 100-ml. volumes of petroleum ether and ethyl ether in petroleum ether. Yields and a description of the products are given in Table II.

Most of the crystalline solids (Fractions *40* to *50*) proved to be sterol acetate retained in the original filtrates. The unidentified diterpene was found in Fractions *81* to *90*. This material was heated on the steam bath in 5% KOH in ethanol and a neutral fraction obtained with ethyl ether in the usual manner as 15.9 Gm. of deep red oily substance. On distillation at 0.6 mm. Hg, a fraction boiling at 200–205° was obtained as a pale yellow, viscous gummy substance.[6] The color was not removed by further distillation. Yield: 2.8 Gm.; $n_D^{25°} = 1.5231$.

Anal.—Calcd. for $C_{15}H_{22}O_2$ (bicyclic, 2 double bonds): C, 76.88; H, 9.42. Found: C, 76.92; H, 9.63. The product exhibited only nonspecific end absorption in the region 230–300 mμ. Molecular weight (Rast): 244,257; no optical rotation in chloroform. The presence of a hydroxyl group(s) was substantiated by an infrared curve of the compound (absorption band, 3.0 μ).

In the Liebermann-Burchard test the substance gave colors in the H_2SO_4 layer ranging from orange to pink. A faint green $CHCl_3$ developed after about 1 hour. These reactions were interpreted as additional evidence for unsaturation. Attempts to form a crystalline phenyl urethane, acetate, or benzoate were unsuccessful.

Reduction of Unidentified Sesquiterpene.—Purified sesquiterpene (1.25 Gm., b.p. 200–205°/0.6 mm. Hg) in 50 ml. of ethyl acetate was shaken for 7 hours under 60 p.s.i. hydrogen pressure with 100 mg. PtO_2. The product was recovered and distilled under 0.6 mm. Hg pressure. The bulk of the material (0.80 Gm.) was received as a pale yellow oily substance at 268–270°; $n_D^{24°} = 1.5134$.

Anal.—Calcd. for $C_{15}H_{26}O_2$: C, 75.58; H, 10.91. Found: C, 75.66; H, 11.00.

No optical rotation was found in $CHCl_3$. Oxidation of 520 mg. of this product with CrO_3 in glacial acetic acid gave 211 mg. of neutral material and 234 mg. of acidic product, both as yellowish gummy substances. Neither product yielded an identifiable substance.

DISCUSSION

In addition to the bicyclic diterpene marrubiin (1) and ursolic acid (3), two additional substances may now be included as major lipid constituents of *M. vulgare* L. One of these, a sterol, is present in the plant in esterified form. Although the presence of other sterols was not excluded, the unidentified sterol appeared to be the major one present in *Marrubium.* β-Sitosterol appeared to be the major sterol of several other members of the *Labiatae* family (4). Reduction of the sterol in acidic medium gave stigmastane-3β-ol, proving the relationship of the substance to members of the sitosterol series.

[6] An additional viscous oily substance boiling unsharply at this pressure and at a considerably higher temperature (250°+) was obtained from the residue remaining after distillation of the sesquiterpene. It was not further examined.

It appears that two double bonds are present in the sterol, although unequivocal evidence must await further data. One double bond is at position 5(6)-; rotational differences between the new sterol and its acetate and benzoate coincide with established differences for Δ^5-stenols (Table III). This is substantiated by the similarity of the infrared spectrum to β-sitosterol and the rate of the Liebermann-Burchard reaction. The exact position of the second double bond cannot be ascertained with the available data, which were limited by the supply of sterol on hand. However, nuclear positions 7(8)-, 8(9)-, 8(14)-, and 9(11)- can be ruled out because of the rotational values in Table III and because of the negative Tortelli-Jaffe reaction (Reference 5, p. 101). Positions 7(8)- and 3(4)- can be eliminated since no conjugation was indicated in the ultraviolet spectrum, and position 11 can be eliminated because of the lack of identity with the $\Delta^{5,(11^7)}$-stigmastadienol of Idler *et al.* (6). Hydrogenation in neutral medium converted the sterol to a compound (unidentified) that was not Δ^5-stigmastenol (β-sitosterol). Therefore, the second (presumed) double bond must have been more resistant to hydrogenation than Δ^5. This eliminates the side chain positions 22(23)-, 24(25)-, or 24(28)- (6). It also eliminates position 14(15)- (7). With these eliminations ring A, positions 1(2)- or 2(3)- become possibilities, since some "vicinal" effect (Reference 5, p. 209) is indicated by the M_D for both the free sterol and acetate (Table III). The significance of the peak at 14.3 μ in the infrared shows promise for later identification; at the moment its significance is unknown.

The other substance new to *M. vulgare*, a strongly polar sesquiterpene which probably contains two nonconjugated double bonds, was not isolated from the crude extracts but was readily isolated from nonsaponifiable fractions of extracts of the plant. Neither the sterol nor the sesquiterpene has yet been equated with known substances; we are describing them as "unidentified," pending further experimentation.

REFERENCES

(1) Cocker, W., Cross, B. E., Duff, S. R., Edward, J. T., and Holley, T. F., *J. Chem. Soc.*, 1953, 2540.
(2) Moore, P. R., and Baumann, C. A., *J. Biol. Chem.*, 195, 623(1952).
(3) Pourrat, H., and LeMen, J., *Ann. Pharm. Franc.*, 11, 190(1953).
(4) Nicholas, H. J., THIS JOURNAL, 47, 731(1958).
(5) Fieser, L., and Fieser, M., in "Natural Products Related to Phenanthrene," Reinhold Publishing Corp., New York, N. Y., 1949, p. 201.
(6) Idler, D. R., Nicksic, S. W., Johnson, D. R., Meloche, V. W., Schuette, H. A., and Baumann, C. A., *J. Am. Chem. Soc.*, 75, 1712(1953).
(7) Cornforth, J. W., Gore, I. Y., and Popjak, G., *Biochem. J.*, 65, 94(1957).

Preservatives for Poliomyelitis (Salk) Vaccine III

2-Phenoxyethanol

By HILLIARD PIVNICK, J. M. TRACY, A. L. TOSONI, and D. G. GLASS

Poliomyelitis vaccine contains antibiotics, but the antibiotics are inadequate for preventing the growth of heavy contamination with bacteria or light contamination with fungi. The addition of 0.375 per cent v/v of 2-phenoxyethanol to poliomyelitis vaccine furnished a stable mixture of preservatives (streptomycin, neomycin, and 2-phenoxyethanol) which was inhibitory to both bacteria and fungi. This mixture was also effective when poliomyelitis vaccine was mixed with diphtheria and tetanus toxoids and pertussis vaccine.

VACCINE packaged in multiple-dose vials must contain a preservative to prevent the growth of microbial contaminants which may be introduced when samples are withdrawn. In studies of preservation of poliomyelitis vaccine and DPT polio vaccine (1, 2) we showed that antibiotics, benzethonium chloride, formaldehyde, and esters of *p*-hydroxybenzoic acid (parabens) alone, or in various combinations, had deficiencies as preservatives.

The present report concerns 2-phenoxyethanol[1] (ethylene glycol monophenyl ether) (2-POE) as a preservative for poliomyelitis vaccine. This compound was chosen for study because of its activity against *Pseudomonas aeruginosa* (3–6) a potential pathogen which may grow in vaccines and other medicinals (7–9).

MATERIALS AND METHODS

2-Phenoxyethanol was sterilized by Seitz filtration, added to vaccines or media and the mixture was stirred slowly for a few hours to effect solution.

A method of assay for 2-POE in poliomyelitis

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[1] Marketed as Phenoxetol by Nipa Laboratories Ltd., Treforest Industrial Estate, near Cardiff, Wales, U.K.