

solution gave a deep red color indicating the presence of a sulfhydryl group.

Hopkins reagent was of no value in isolating this product. When added to an acidified aqueous solution of the residue after the liquid ammonia was removed, it sometimes caused

a yellow color but gave no precipitate. The final crystalline hydrochloride also failed to give a precipitate with this reagent.

MADISON, WISCONSIN

[CONTRIBUTION FROM THE LABORATORY OF CHEMISTRY OF NATURAL PRODUCTS, NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, U. S. PUBLIC HEALTH SERVICE, DEPARTMENT OF HEALTH, EDUCATION AND WELFARE]

## Studies on the Occurrence and Structure of Acetylandromedol (Andromedotoxin)

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A procedure for the detection of acetylandromedol in plant extracts was developed through use of paper electrophoresis with borate buffer solutions. Positive results were found for some but not all species of *Kalmia*, *Leucothoe*, *Lyonia*, *Pernettya*, *Pieris* and *Rhododendron*. A particularly good source was found in *K. angustifolia* var. *caroliniana*. New evidence indicated that the empirical formula for andromedol is  $C_{20}H_{34}O_6$ , and that acetylandromedol, grayanotoxin I and rhodotoxin are identical and have the formula  $C_{22}H_{36}O_7$ .

The reported toxicity to stock of *Andromeda*, *Kalmia*, *Leucothoe* and *Lyonia* species indicates that a number of plants of the *Ericaceae* in addition to those of the genus *Rhododendron* contain physiologically active compounds. Acetylandromedol,<sup>1</sup> isolated<sup>2</sup> from leaves of *Rhododendron maximum*, was found to be a potent hypotensive agent,<sup>3</sup> and in order to determine if commonly occurring species of these genera contained acetylandromedol or related substances a survey was made of a number of plants of the *Ericaceae* with reported or potential physiological activity. These included *Andromeda japonica* from which Eykman<sup>4</sup> isolated in 1882 a crude toxic substance to which the name asebotoxin was given, and *Rhododendron luteum*, reported by Xenophon to be the source of a poisonous honey given to Grecian soldiers in an early campaign in Asia Minor. It was necessary to develop a method for the identification of acetylandromedol in plant samples, and this work is described in Part I together with the results of a limited survey made for ten genera in the *Ericaceae*.

Part II describes further chemical studies on acetylandromedol.

### I. Plant Studies

Acetylandromedol is known to contain a 1,2-glycol structure.<sup>2</sup> This suggested the possibility of utilizing paper electrophoresis with a borate electrolyte for the separation and identification of plant components in the acetylandromedol fraction. The rate of migration of a borate diol complex during paper electrophoresis depends on the borate-diol equilibrium and upon the absorption characteristics of the complex with regard to cellulose. In dilute borate solutions a 2:1 diol-borate complex may be formed in addition to a 1:1 complex, and this effect usually can be recognized by study-

(1) This is proposed in Part II as a change in name from andromedotoxin.

(2) H. B. Wood, V. L. Stromberg, J. C. Keresztesy and E. C. Horning, *THIS JOURNAL*, **76**, 5689 (1954).

(3) N. C. Moran, P. E. Dresel, M. E. Perkins and A. P. Richardson, *J. Pharmacol. Exp. Therap.*, **110**, 415 (1954); N. C. Moran, M. E. Perkins and A. P. Richardson, *ibid.*, **111**, 454 (1954). In normal dogs, intravenous administration of 5-10 mcg./kg. led to 20-40% lowering of blood pressure. Levels required for hypotensive action in humans were slightly higher.

(4) J. F. Eykman, *Rec. trav. chim.*, **1**, 225 (1882).

ing the effect of borate concentration changes. The 2:1 diol-borate complex is the stronger acid of the two.<sup>5</sup>

It was found that acetylandromedol migrated toward the anode on paper electrophoresis with sodium tetraborate solutions. The vanillin-perchloric acid spray of Godin<sup>6</sup> was an excellent visualizing reagent. With 0.05 *M* sodium tetraborate concentration, a single migrating species was observed (curve B, Fig. 1), and with 0.01 *M* electrolyte concentration two ionic species were observed (1a and 1b in curve D, Fig. 1). The principal species under the latter conditions was assumed to be the expected 1:1 borate-diol complex. The secondary species with higher mobility occurring in very dilute borate solution was assumed to be the 2:1 complex. An alternate but less likely explanation for the two ionic species is that they represent borate complexes with two different pairs of hydroxyl groups of acetylandromedol.<sup>5a,5e</sup>

When this procedure was used with crude acetylandromedol fractions from *Rhododendron maximum* leaves, the characteristic behavior of acetylandromedol with different borate concentrations was exhibited (curves A and C in Fig. 1), and compounds responsible for two additional colored areas (marked 2 and 3 in curves A and C) were indicated by the spray reagent. The recognition of these and other compounds in successive runs was aided materially by the strong and distinctive colors produced by the Godin reagent.

This procedure was applied to a number of plant specimens from the U. S., Cuba and Costa Rica. Figure 2 shows examples of the results obtained after electrophoresis. Data taken from such electrophoretograms are summarized in Table I. The presence of acetylandromedol was checked, where it occurred, in two concentrations of sodium tetraborate electrolyte (0.01 and 0.05 *M*) in order to confirm the characteristic equilibrium behavior.

(5) Details on the effect of borate concentration and other factors on borate-diol complex formation are given in (a) H. S. Isbell, J. F. Brewster, N. B. Holt and H. L. Frust, *J. Research Nat. Bur. Standards*, **40**, 129 (1948); (b) J. Boeseken, *Advances in Carbohydrate Chem.*, **4**, 189 (1949); (c) C. A. Zittle, *Advances in Enzymology*, **12**, 493 (1951); R. Conden and W. M. Stanier, *Nature*, **169**, 783 (1952), and (e) D. J. Bell and D. H. Northcote, *Chemistry & Industry*, 1328 (1954).

(6) P. Godin, *Nature*, **174**, 134 (1954).

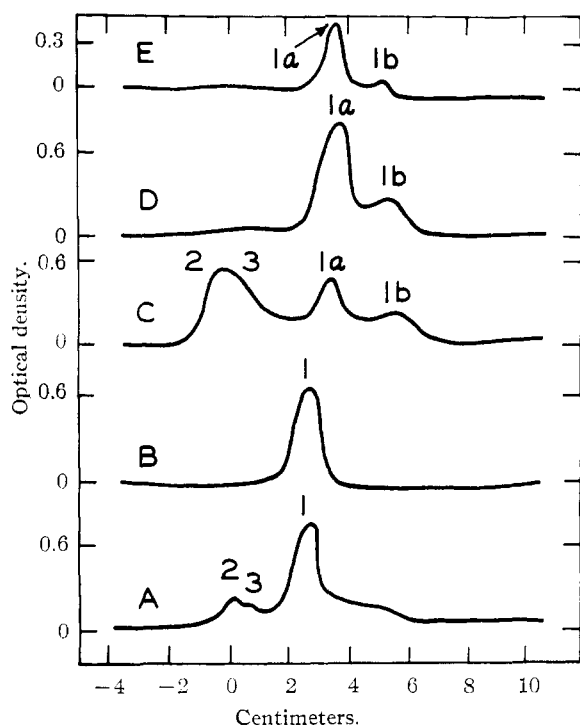


Fig. 1.—Densitometer tracings of paper electrophoretograms obtained with 0.05 *M* sodium tetraborate solution for crude *Rhododendron maximum* extract (curve A) and acetylandromedol (curve B) and of paper electrophoretograms obtained with 0.01 *M* sodium tetraborate solution for crude *Rhododendron maximum* extract (curve C) and acetylandromedol (curve D). Grayanotoxin I from *Leucothoe grayana*<sup>11</sup> was used for curve E with 0.01 *M* sodium tetraborate solution. The times were 4 hours for A and B, and 6 hours for C, D and E. A vanillin-perchloric acid spray was used as a visualizing reagent. Colors are noted in Table II.

It was not possible to secure quantitative data relating to acetylandromedol, but by working with selected quantities of the extracts and by estimating the intensity of color it was found that some degree of differentiation could be made between plants containing much acetylandromedol and those containing little. This is indicated by the ratings of + to +++ in Table I. The highest concentration observed for acetylandromedol was in *K. angustifolia* var. *caroliniana*, and in separate isolation experiments (on a large scale) it was found that the yield of acetylandromedol from fresh leaves of this plant was about 0.06–0.09%.<sup>7</sup>

*Rhododendron* species showed a considerable variation in their acetylandromedol content; *R. calendulaceum*, *R. canadense* and *R. canescens* gave completely negative results. *R. luteum*, believed to be the source of the poisonous *Rhododendron* honey mentioned by Xenophon and Dioscorides, contained acetylandromedol. It was not possible to examine the flowers of this species, but flowers of *R. catawbiense* contained more acetylandromedol than the leaves. Two common species of the genus *Kalmia* were examined. *K. latifolia* ("mountain laurel"), although reported to be

(7) Yields from *R. maximum* collected in North Carolina averaged about 0.008%. This was estimated as a ++ concentration.

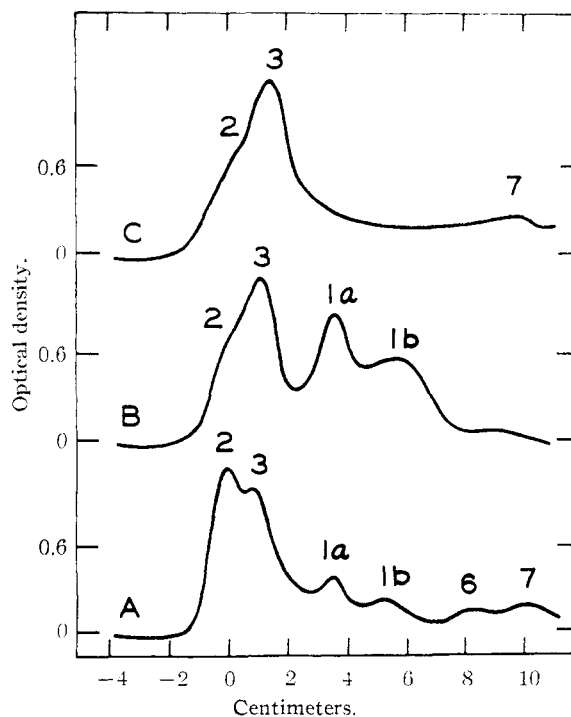


Fig. 2.—Densitometric tracings of paper electrophoretograms obtained with 0.01 *M* sodium tetraborate solution (6 hours) for extracts of *R. japonicum* (curve A), *K. angustifolia* (curve B) and *Leucothoe axillaris* (curve C). A vanillin-perchloric acid spray was used as a visualizing reagent.<sup>8</sup> Colors are in Table II. Bioassays for hypotensive activity were negative for the sample used for C, while a low order of activity was found for A. The plant used for B contained the highest amount of acetylandromedol of all of those investigated.

toxic to stock, contained far less acetylandromedol than *K. angustifolia* ("sheep-kill"). *Lyonia* and *Leucothoe* species are also regarded generally as being poisonous to stock, and *Leucothoe editorum* was found to contain acetylandromedol in a quantity comparable to *R. maximum* and *R. luteum*. *Lyonia* species contained little (*L. mariana*) or no acetylandromedol.

There has been uncertainty about the identity or non-identity of the substances described by Eykman<sup>4</sup> (asebotoxin, from *A. japonica*), de Zaayer<sup>8</sup> (andromedotoxin, m.p. 228–229°, from *Andromeda* species), Hardikar<sup>9</sup> (andromedotoxin, m.p. 258°, from an unidentified *Rhododendron* species), Makino<sup>10</sup> (rhodotoxin, m.p. 245°, from *R. hymenanthes*), Takemoto<sup>11</sup> (grayanotoxin I, m.p. 259–260°, from *L. grayana*) and acetylandromedol from *R. maximum*<sup>2</sup> and *K. angustifolia*. Through the courtesy of Dr. Takemoto, samples were exchanged and compared. The infrared spectra of all samples were identical<sup>11</sup> (rhodotoxin from *R. metternichii*, grayanotoxin I, and acetylandromedol). The borate electrophoresis behavior

(8) H. G. de Zaayer and P. C. Plugge, *Arch. ges. Physiol.*, **40**, 480 (1886–1887).

(9) S. W. Hardikar, *J. Pharmacol. Exptl. Therap.*, **20**, 17 (1922).

(10) M. Makino, *Okayama-Igakki-Zasshi*, **39**, 2099 (1927); **40**, 138 (1928); [*C. A.*], **23**, 1691, 3027 (1929)].

(11) T. Takemoto, Y. Nishimoto, H. Meguri and K. Katayama, *J. Pharm. Soc. Japan*, **75**, 1441 (1955), and ref. 15.

TABLE I  
 EXAMINATION OF ERICACEAE PLANT EXTRACTS

Plant <sup>a</sup>	Origin	Acetyl- andromedol	Un- identified <sup>b</sup> compd.
<i>Befaria racemosa</i> Vent.	Florida	—	2
<i>Gaultheria donnellii</i> Sleumer	Costa Rica	—	2
<i>Gaultheria donnellii</i> Sleumer <sup>c</sup>	Costa Rica	—	2
<i>Kalmia angustifolia</i> L. (var. <i>caroliniana</i> )	North Carolina	+++	2,3
<i>Kalmia hirsuta</i> Walt.	Florida	—	2,3
<i>Kalmia latifolia</i> L.	North Carolina	+	2,3,6
<i>Kalmia latifolia</i> L. <sup>c</sup>	North Carolina	—	2,3,4,5,7,8,9
<i>Leiophyllum buxifolium</i> (Berg.) Ell.	New Jersey	—	2,3,7
<i>Leiophyllum buxifolium</i> (Berg.) Ell. <sup>c</sup>	New Jersey	—	2,3,4
<i>Leucothoe axillaris</i> (Lam.) D. Don	North Carolina	—	2,3,7,8
<i>Leucothoe editorum</i> Fern. et Schub.	Georgia	++	2,3,8
<i>Lyonia ferruginea</i> (Walt.) Nutt.	Florida	—	2,5,10
<i>Lyonia mariana</i> (L.) D. Don	North Carolina	+	2,3,4,6
<i>Lyonia myrtilloides</i> Griseb.	Cuba	—	2,3,6,8
<i>Lyonia myrtilloides</i> Griseb. <sup>d</sup>	Cuba	—	2,3
<i>Pernettya coriacea</i> Klotzsch	Costa Rica	++	2,3,7
<i>Pieris japonica</i> (Thunb.) D. Don <sup>e</sup>	Maryland	+++	2,3,7
<i>Rhododendron calendulaceum</i> (Michx.) Torr.	South Carolina	—	2
<i>Rhododendron canadense</i> (L.) Torr.	Pennsylvania	—	2
<i>Rhododendron canescens</i> (Michx.) Sweet	North Carolina	—	2,4
<i>Rhododendron catawbiense</i> Michx.	North Carolina	++	2,3,5,6
<i>Rhododendron catawbiense</i> Michx. <sup>e</sup>	Maryland	+++	2,3
<i>Rhododendron japonicum</i> (Gray) Suringar	Maryland	+	2,3,6,7
<i>Rhododendron luteum</i> Sweet	Maryland	++	2,3,7,8,9
<i>Rhododendron ponticum</i> L.	Maryland	++	2,3
<i>Rhododendron maximum</i> L.	North Carolina	++	2,3
<i>Vaccinium consanguineum</i> Klotzsch	Costa Rica	—	2,3,10
<i>Vaccinium myrsinites</i> Lam.	Florida	—	2

<sup>a</sup> Leaves or leaves and stems were used unless otherwise specified. <sup>b</sup> See Table II for additional data. <sup>c</sup> Flowers. <sup>d</sup> Bark. <sup>e</sup> *Andromeda japonica*.

for grayanotoxin I was identical with that observed for acetylandromedol (curve E, Fig. 1). The paper electrophoresis patterns for extracts of *A. japonica* also showed areas 1 and 1a and 1b, and it therefore seems likely that all of these descriptions relate to the same compound.

In carrying out this work, it was evident that certain reservations were required. Experiments with different species showed that a number of compounds giving positive color reactions were always present in the crude fractions (Tables I and II). One or more of these compounds, or possibly other substances which did not give a Godin color, might well exhibit physiological activity similar to that of acetylandromedol. In order to examine this possibility, several fractions from plants reported to be toxic and found to contain several Godin-positive compounds but little or no acetylandromedol were compared with fractions from *K. angustifolia* with regard to their physiological activity. These fractions included extracts of *L. axillaris* (leaves), *L. myrtilloides* (leaves) and *K. latifolia* (flowers), and in these cases it was found that the physiological activity was negligible. Extracts from *R. japonicum* (leaves), *K. latifolia*

(leaves) and *L. mariana* (leaves) were found to possess physiological activity qualitatively similar to that of acetylandromedol and quantitatively about as predicted by approximation of acetylandromedol content from paper electrophoretograms. Of the several Godin-positive compounds synthesized in these plants, those responsible for areas 2 and 3 (Table II) were by far the most widely distributed. Because it was a rich source of these compounds, *Leiophyllum buxifolium* was also examined for physiological activity and found to have none.

 TABLE II  
 COMPOUNDS PRESENT AFTER 0.01 M SODIUM TETRABORATE ELECTROPHORESIS OF ERICACEAE PLANT EXTRACTS

Compound <sup>a</sup>	Distance moved in 6 hr. (cm.) <sup>b</sup>	Color <sup>c</sup>
1a	3.5	Blue
1b	5.5	Blue
2	0.0	Gray
3	1.0	Blue-purple
4	3.5	Red
5	6.5	Red
6	8.5	Yellow
7	10.0	Purple
8	12.0	Green
9	13.5	Red
10	14.0	Blue

<sup>a</sup> Diol complexes 1a and 1b are derived from acetylandromedol (see Fig. 1). <sup>b</sup> Conditions: 7.5 volts per cm., 1–2 ma., 6 hours, corrected for electroendosmosis. <sup>c</sup> After spraying with vanillin-perchloric acid reagent.<sup>8</sup>

These results do not exclude the possibility that a second compound with the same characteristics as acetylandromedol may exist, but the chemical and bioassay data obtained up to the present time point to the identification of a common substance as the principal physiologically active agent of the Ericaceae.<sup>12</sup> The compounds responsible for colored areas described here as 2 and 3 are widely distributed through the family, but their presence or absence has no apparent relationship to the acetylandromedol content of the plant.

## II. Chemical Studies

The presence of physiologically active materials in *Rhododendron*, *Kalmia*, *Pieris* (*Andromeda*) and *Leucothoe* species has been confirmed in every laboratory that has studied the problem, but there has been no general agreement on the physical properties and empirical formulas of the agent or agents responsible for the activity. (The two most recent formulas are C<sub>19</sub>H<sub>30</sub>O<sub>6</sub> and C<sub>22</sub>H<sub>36</sub>O<sub>7</sub>.) The work in the previous section points to the occurrence of a common substance throughout these plants, and it is proposed that this be named acetylandromedol.<sup>13</sup>

Acetylandromedol (from *R. maximum*) was sub-

(12) Investigations of grayanotoxins I, II and III (see Part II) point to three substances with physiological activity. The Japanese workers used a fresh-water fish for their work; the activity described in this paper relates only to hypotensive action studied in anesthetized dogs. There is a possibility that andromedol (grayanotoxin III) accompanied acetylandromedol in crude fractions, but we have not observed this compound in our isolation work up to the present time.

(13) It is desirable to use a name which reflects the functional groups of the molecule and which permits a systematic naming of derivatives. The parent polyhydroxy compound has been named andromedol, following the plant first used by Lykman<sup>4</sup> and de Zaayer and Plugge.<sup>8</sup>

mitted to another laboratory for X-ray studies.<sup>14</sup> The molecular weight was found to be 410.2, calculated from unit cell and density measurements, with the assumption of four molecules per unit cell. This value corresponded to the previously suggested formula<sup>2</sup> ( $C_{19}H_{30}O_6$ ) and the elements of acetone ( $C_{22}H_{36}O_7$  has m.w. 412.5). The presence of acetone of crystallization was considered to be a possible explanation for this result; the crystals used in the X-ray work were obtained from an acetone-containing solvent mixture. However, it was known that the infrared spectra of acetylandromedol samples crystallized from solvent mixtures with and without acetone were identical, and new evidence relating to the empirical formula was therefore sought.

Three substances were brought under study. These were a hydrolysis product (andromedol), a benzoyl derivative (tribenzoylandromedol), and a dehydration-hydrolysis product (anhydroandromedol). The first of these substances was described previously<sup>2</sup> as a non-crystalline material which formed crystalline adducts of indefinite composition with methyl and ethyl acetate. The compound was obtained in this work as a crystalline, non-solvated but hygroscopic material, whose analysis corresponded to  $C_{20}H_{34}O_6$ . The structural relationship between andromedol and acetylandromedol is the same as that between grayanotoxin III and grayanotoxin I<sup>15</sup> (to which the formula  $C_{22}H_{36}O_7$  was assigned), and andromedol and grayanotoxin III should therefore be identical. A more direct comparison is not possible since grayanotoxin III has been described only as a solvated material with one-half molecule of water or ethyl acetate.<sup>15</sup> We have not observed this compound as a component of *Rhododendron* or *Kalmia* species, but it may be present in low concentration in these plants.

Miyajima and Takei<sup>15</sup> also described anhydrograyanotoxin III as a compound obtainable from both grayanotoxin III and grayanotoxin I. In our work, acetylandromedol was subjected to acid treatment in aqueous solution, and from the mixture of products there was isolated by counter-current distribution a crystalline substance of formula  $C_{20}H_{32}O_5$ . The melting point (and empirical formula) is in agreement with that given by Miyajima and Takei<sup>15</sup> for anhydrograyanotoxin III. Additional points of comparison are lacking. It is likely that these compounds are identical, but melting points in this series are not a reliable index of identity.

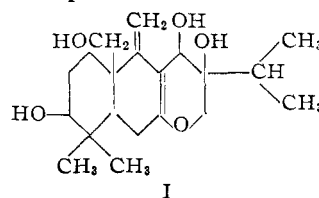
The calculated and found C, H analytical values for acetylandromedol, rhodotoxin and grayanotoxin I reported in this and other papers<sup>2,9-11,15</sup> do not distinguish between the formulas  $C_{19}H_{30}O_6$  and  $C_{22}H_{36}O_7$  (different acetyl analyses have been

reported; these may have been due to analytical difficulties). However, the reported derivatives of the isolated materials should be subject to correlation with the formula  $C_{20}H_{34}O_6$  for andromedol, if this formula is correct. We therefore reviewed the question of benzoyl and acetyl derivatives. A benzoyl derivative was prepared from acetylandromedol, and this compound was found by analytical and ultraviolet spectra data to correspond to tribenzoylandromedol (Table III). The tribenzoylacetylandromedol (tribenzoylgrayanotoxin I) of Miyajima and Takei and of Yamashita<sup>15</sup> represents another benzoyl derivative, and the analytical data indicate that our earlier benzoyl derivative (described as dibenzoylandromedotoxin<sup>2</sup>), confirmed by Takemoto<sup>11</sup> was a methanol solvate of tribenzoylandromedol. Further studies of the benzoylation reaction are required to determine the point at which ester exchange occurs. Acetyl derivatives have been reported in several papers; these include diacetylandromedol (Table III) and two apparently different tetraacetyl derivatives.<sup>15</sup> The carbon-hydrogen values for diacetylandromedol correspond so closely that either formula may be used; the acetyl values, however, point to the  $C_{20}H_{34}O_6$  formula for andromedol. The acetylation of andromedol to the naturally occurring acetylandromedol has not been accomplished, and a triacetylandromedol is not known.

A remaining problem concerns the structure of isopropylideneanhydroandromedotoxin,<sup>2</sup> which should now correspond, on the basis of its infrared spectrum, to an isopropylideneanhydroacetylandromedol with one double bond. The reaction leading to this compound is under investigation.

Table III contains newly determined analytical data for acetylandromedol and a number of derived compounds. These data, with the comparisons made as a consequence of Takemoto's recent reisolation of grayanotoxin I and rhodotoxin mentioned in Part I and the molecular weight determination for acetylandromedol,<sup>14</sup> indicate that the correct formula for andromedol is  $C_{20}H_{34}O_6$  and that the acetyl derivatives, grayanotoxin I, rhodotoxin and acetylandromedol, are identical.

Recent work on grayanotoxin II has resulted in a structural proposal (I).<sup>16</sup> It seems reasonable to accept a close structural relationship between acetylandromedol and a second polycyclic polyhydroxy compound isolated from *L. grayana*, but our observations do not particularly support structure I as a basic system subject to elaboration for acetylandromedol. Anhydroandromedol and andromedol were examined for evidence of unsaturation by ultraviolet absorption studies to 195 m $\mu$ . Both



(14) We are indebted to Dr. Ray Pepinsky, Pennsylvania State University, for cell measurements and the molecular weight determination.

(15) S. Miyajima and S. Takei, *J. Agr. Chem. Soc. Japan*, **10**, 1098 (1934); **12**, 497 (1936) [*C. A.*, **29**, 1133 (1935); **30**, 6747 (1936)]. Grayanotoxin I was first described by O. Kubo, *Arch. Exptl. Pathol. Pharmacol.*, **67**, 111 (1912) (as  $C_9H_{14}O_8$ ) and was later studied by M. Yamashita, *Sci. Repts. Tohoku Imp. Univ.*, **31**, 537 (1932) [*Chem. Zentr.*, **104**, [I] 1793 (1933); *C. A.*, **27**, 989 (1933)], *J. Chem. Soc. Japan*, **53**, 664 (1932) [*C. A.*, **27**, 303 (1933)].

(16) M. Nakajima and I. Iwasa, *Botyu Kagaku*, **13**, 11 (1949) [*C. A.*, **44**, 1090 (1950)]; **16**, 28, 32 (1931) [*C. A.*, **46**, 3986, 3987 (1952)]; M. Nakajima and S. Miyajima, *ibid.*, **15**, 28, 30 (1950) [*C. A.*, **44**, 7811 (1950)].

TABLE III  
 ANALYTICAL DATA FOR ACETYLANDROMEDOL AND RELATED COMPOUNDS

Compound	Empirical formula	Calcd.			Acetyl	C	Found <sup>a,b</sup>		
		C	H	O			H	O	Acetyl
Andromedol	C <sub>17</sub> H <sub>28</sub> O <sub>5</sub>	65.36	9.03	..	...	64.81, 64.93	9.11, 9.23	.....	...
	C <sub>20</sub> H <sub>34</sub> O <sub>6</sub>	64.84	9.25	..	...				
Acetylandromedol	C <sub>19</sub> H <sub>30</sub> O <sub>6</sub>	64.38	8.53	27.1	12.14	63.96, 64.04	8.82, 8.86	26.8, 26.9	10.66
	C <sub>22</sub> H <sub>36</sub> O <sub>7</sub>	64.05	8.80	27.2	10.43				
Diacetylandromedol	C <sub>21</sub> H <sub>32</sub> O <sub>7</sub>	63.61	8.14	28.3	21.71	63.55, 63.45	8.61, 8.53	28.1, 27.8	18.38
	C <sub>24</sub> H <sub>38</sub> O <sub>8</sub>	63.41	8.43	28.2	18.94				
Tribenzoylandromedol	C <sub>35</sub> H <sub>40</sub> O <sub>8</sub>	73.06	6.45	..	...	72.45, 72.57	6.64, 6.85	.....	None
	C <sub>41</sub> H <sub>46</sub> O <sub>9</sub>	72.12	6.79	..	...				
Anhydroandromedol	C <sub>17</sub> H <sub>26</sub> O <sub>4</sub>	69.36	8.90	21.7	...	68.33, 68.19	9.17, 9.15	22.8, 22.8	...
	C <sub>20</sub> H <sub>32</sub> O <sub>5</sub>	68.15	9.15	22.7	...				

<sup>a</sup> Carbon, hydrogen and acetyl determinations by Mr. J. F. Alicino, Metuchen, N. J. <sup>b</sup> Direct oxygen determinations by Dr. W. Zimmermann, Melbourne, Australia.

substances showed no ultraviolet absorption. Anhydroandromedol was unaffected by bromine and by hydrogenation conditions, and this may be taken to indicate an ether bridge possibly similar to that found for some steroids. If the empirical formula C<sub>20</sub>H<sub>34</sub>O<sub>6</sub> for andromedol is correct, a tetracyclic system must be allocated to the parent compound. One of the hydroxyl groups of the 1,2-diol structure evidently is involved in ether formation in anhydroandromedol, since the anhydro-compound does not form a borate-diol complex.

Terminal methyl determinations were made for andromedol, acetylandromedol and anhydroandromedol. These indicate that two or three methyl groups may be tentatively assigned to andromedol. We suspect that andromedol may be a hexahydroxy compound; positive evidence bearing on this point is an active hydrogen determination on anhydroandromedol (four active hydrogens were indicated). In the absence of additional supporting evidence this should not be taken as a definitive structural assignment. There is no strong evidence for the nature of the ring system of andromedol, but the cell measurements for crystalline acetylandromedol suggest that a steroid-like skeleton is possible. The most attractive part of this hypothesis lies in the possibility that suitably substituted steroids may have a direct effect on blood pressure control centers.

Oxidation and dehydrogenation experiments present additional possibilities for further study, and this approach is under investigation.

**Acknowledgments.**—We are indebted to the Section of Plant Introduction, Agricultural Research Service, U. S. Department of Agriculture, for the collection and identification of plant materials, and to Dr. N. C. Moran for the pharmacological data. Mr. D. L. Rogerson carried out the plant extraction work; Miss Catherine Monaghan and Miss Patricia Wagner performed the spectroscopic work, and the analytical data were supplied by Mr. J. F. Alicino, Dr. W. Zimmermann and the Clark Microanalytical Laboratories.

### Experimental<sup>17</sup>

**Plant Extractions.**—A 500-g. sample of undried plant material was ground in a Wiley mill and suspended in 5 l. of

water. The pH was adjusted to 7.0–7.5 with barium hydroxide, and the suspension was heated to 90–100° for 1 hour with efficient stirring. It was filtered (Supercel pad) and the filter cake was washed well with hot water. The combined filtrates were concentrated (reduced pressure) to 500 ml.; the pH was adjusted to 7.0–7.5 and 4.5 l. of ethanol was added. A precipitate was removed by filtration and washed with alcohol. The filtrate and washes were combined and concentrated to 300 ml. (reduced pressure). The solution was adjusted to pH 6.5 and filtered. The filter cake was washed with water, and the solution was concentrated to 300 ml. and continuously extracted with chloroform for 24 hours. The solvent was removed from the organic extract *in vacuo*, and the residue was examined for the presence of acetylandromedol and related compounds by paper electrophoresis.

After the observation was made that *K. angustifolia* was a particularly rich source of acetylandromedol, several large-scale isolation experiments were carried out according to the published procedure.<sup>2</sup> One run, on a 100 lb. scale, gave 28.6 g. of acetylandromedol. The yields were in the range 0.06–0.09% (about ten times that observed for *R. maximum*).

**Paper Electrophoresis.**—Strips of Whatman #1 paper 42 cm. long and 26.5 cm. wide were used. For solid samples, solutions containing 10 mg. per ml. in ethanol or chloroform were employed. Approximately 0.02 ml. of the solution was applied to the paper. For crude plant extracts, 0.06 ml. of solutions containing 25 mg. per ml. in chloroform or ethanol was used. The mixtures or compounds were spotted at 4 cm. intervals along a center line parallel to the short edge of the paper. The strip was supported at the center line by a glass rod, and the ends dipped about 1 cm. into electrode compartments containing sodium tetraborate solution. The entire system was enclosed in a Lucite box. The strip was allowed to wet by a capillary action, and the separation was carried out at 300 v. (a gradient of 7.5 v. per cm.). This resulted in a current of 1–2 ma. when 0.01 *M* sodium tetraborate was used and 5–6 ma. when the buffer concentration was 0.05 *M*. The time for electrophoresis was 6 hours for a 0.01 *M* buffer and 4 hours for a 0.05 *M* buffer. Acetylandromedol and related compounds were detected by spraying the air-dried strip with a solution prepared just before use by mixing equal volumes of solutions of 3% perchloric acid in ethyl alcohol and 1% vanillin in ethyl alcohol.<sup>6</sup> Electroendosmosis was determined by spotting urea (0.02 ml. of a water solution containing 10 mg./ml.) on the strip and determining its position after electrophoresis by cutting the appropriate part of the strip and spraying it with a 0.5% solution of *p*-dimethylamino-benzaldehyde in 1.0 *N* hydrochloric acid. A yellow spot resulted, the center of which represented "0 cm. moved" for all curves and data reported in this paper.

Optical density measurements were made with a Photo-a Perkin-Elmer Model 21 instrument. Infrared spectra of all new compounds described here have been deposited as Document No. 5130 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document number and by remitting \$1.25 for photoprints, or \$1.25 for 35 mm. microfilm, by check or money order payable in advance to Chief, Photoduplication Service, Library of Congress. Other spectra are available through a previous paper.<sup>2</sup>

(17) All melting points were taken on a Kofler stage. Optical rotations were determined with a Rudolph photoelectric-matching polarimeter. Ultraviolet spectra were taken with Cary Recording Spectrophotometers (Models 11 and 14). Infrared spectra were obtained with

volt Electronic Densitometer, Model 525. A tungsten light source (no filter) was used, and the sensitivity dial was at 2.

**Comparison of Samples.**—A sample of grayanotoxin I<sup>18</sup> (identical with rhodotoxin), was compared with acetylandromedol samples from *R. maximum* and *K. angustifolia*. The infrared spectra of all of these materials were identical. The behavior on electrophoresis, including the borate concentration effect, was identical for all samples. Curve E, Fig. 1, shows the characteristic result found with 0.01 *M* buffer solution for the grayanotoxin I sample.

**Physiological Activity.**—The hypotensive action of crude plant extracts and related materials was determined as described elsewhere.<sup>3</sup>

**Acetylandromedol.**—The molecular weight of acetylandromedol was determined by measurements of the crystal density and unit cell dimensions.<sup>14</sup> Assuming four molecules per unit cell, the value was 410.2 (calcd. for C<sub>22</sub>H<sub>36</sub>O<sub>7</sub>, 412.5; for C<sub>19</sub>H<sub>30</sub>O<sub>6</sub>, 354.4).

A C-methyl determination showed the presence of at least three such groups (one must be allocated to the acetyl group).

*Anal.* Calcd. for C<sub>22</sub>H<sub>36</sub>O<sub>7</sub>: C-CH<sub>3</sub>, 10.93 (for three). Found: C-CH<sub>3</sub>, 9.16, 9.19.

Additional analytical data are in Table III. The sample used for these analyses was prepared for clinical studies; the isolation followed our earlier work.<sup>2</sup>

**Diacetylandromedol.**—Newly determined analytical data are in Table III. The sample was from a previous preparation.<sup>2</sup>

**Tribenzoylandromedol.**—Benzoyl chloride (15 ml.) was added to a previously chilled (0°) solution of 500 mg. of acetylandromedol in 60 ml. of pyridine. After 12 hours at 0°, the mixture was added to stirred iced 10% sodium carbonate solution. The suspension was stirred for 1 hour, and the product was extracted with chloroform. The chloroform solution was washed (sodium bicarbonate solution and water), and the solvent was removed under reduced pressure. After trituration of the residue with benzene-hexane, 433 mg. remained; this was crystallized from benzene-methanol to give 295 mg. of colorless crystalline solid, m.p. 213–216°. Recrystallization from chloroform-benzene gave a sample melting at 217–219°, and this was not changed by further recrystallization from methanol-chloroform, chloroform-benzene, methanol-benzene, and ethyl acetate-benzene.

Optical rotation values were found to be  $[\alpha]^{24}_{589} + 85.5$ ,  $[\alpha]^{24}_{436} + 200.0$  (*c* 0.93, chloroform).

Analytical data are in Table III.

The ultraviolet spectrum of this substance in ethanol was qualitatively similar to that of ethyl benzoate. The maxima for the two spectra were at 230 and 228 m $\mu$ , respectively. The  $\epsilon_{\text{max}}$  values were  $3.71 \times 10^4$  and  $1.22 \times 10^4$ . The  $\epsilon_{\text{max}}$  value for the andromedol derivative is therefore in close agreement with that expected for three benzoyl groups (calcd. value  $3.66 \times 10^4$ ), and since the acetyl analysis showed no acetyl groups, the structure assigned to this compound is that of tribenzoylandromedol.

The infrared spectrum was similar to that of our previous benzoyl derivative (reported m.p. 226.5–227.5°) except for differences in bands at 9.70 and 9.80  $\mu$ . Three carbonyl bands were found at 5.80, 5.85 and 5.90  $\mu$ , and a sharp hydroxyl band at 2.80  $\mu$ . Since the earlier sample was used for analytical purposes, it was not possible to obtain mixed m.p. or additional analytical data on the original sample. It should be pointed out that the material described previously as "dibenzoylandromedotoxin" was prepared by benzoylation in pyridine at steam-bath temperature, and it was recrystallized for analysis from methanol-benzene. The analytical data and the great similarity in infrared spectra with that from the compound prepared at 0° suggest that the earlier product was a methanol solvate of tribenzoylandromedol (*Anal.* Calcd. for C<sub>42</sub>H<sub>60</sub>O<sub>10</sub>: C, 70.57; H, 7.05. Found: C, 70.27; H, 6.78). The occurrence of solvates in this series is not uncommon. The analytical sample for the new benzoyl derivative was recrystallized from chloroform-benzene immediately before analysis and solvation is not indicated. It is not possible to define the relationship of this tribenzoyl derivative to the "tribenzoylgrayanotoxin I" (tribenzoylacetylandromedol) of Yamashita,<sup>15</sup> except that the analytical data point to solvation as a possible ex-

planation. The experimental conditions described here were chosen to allow the use of a large excess of benzoyl chloride, but to avoid ester exchange or dehydration effects which might result from heating the reaction mixture. Since ester exchange occurred in this case at 0°, it is likely that it also occurred in cases where reaction mixtures were heated.

**Andromedol.**—A solution of 3.0 g. of acetylandromedol in water (135 ml.) and ethanol (30 ml.) containing 15 g. of sodium hydroxide was heated under reflux for 7 hours. The alkali was removed with Amberlite IRC-50 (H+) resin, and the product was extracted with chloroform (continuous extraction). The chloroform solution was reduced to a small volume, and a crystalline material separated slowly. This product, m.p. 224–227° dec., was hygroscopic and could not be recrystallized in satisfactory form.

The infrared spectrum showed no carbonyl band. A strong, broad band at 2.90  $\mu$  indicated a hydroxyl group or groups.

Optical rotations were found to be  $[\alpha]^{26}_{589} - 16.7$ ,  $[\alpha]^{26}_{436} - 29.6$  (*c* 0.96, ethanol).

Analytical data are in Table III. A C-methyl determination showed at least two such groups.

*Anal.* Calcd. for C<sub>20</sub>H<sub>34</sub>O<sub>5</sub>: C-CH<sub>3</sub>, 8.12 (for two). Found: C-CH<sub>3</sub>, 8.10, 8.18.

This compound was water-soluble, and its ultraviolet absorption properties were measured in water solution to 195 m $\mu$ . No absorption of any kind was found; this suggests that unsaturation was not present.

When examined by paper electrophoresis with a borate electrolyte, andromedol was found to migrate very much like acetylandromedol. The behavior was similar with different borate concentrations, indicating that the stereochemical relationships of the hydroxyl groups involved in the borate complexes were essentially the same for the two compounds. The Godin color was blue, and sufficiently similar to that from acetylandromedol to make differentiation difficult.

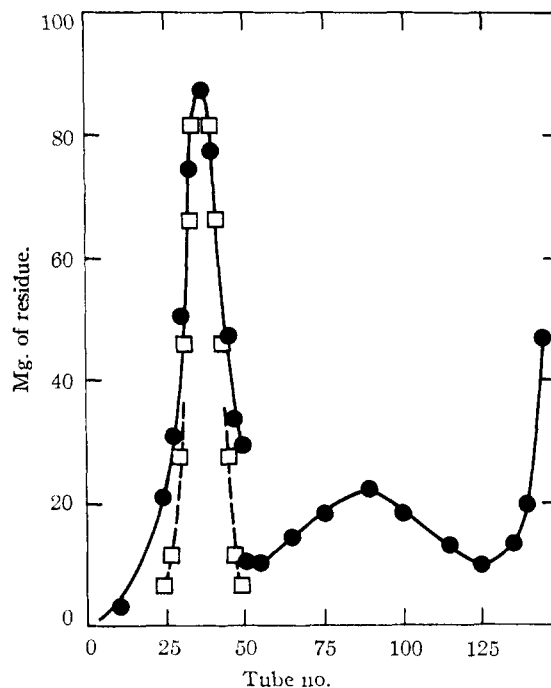


Fig. 3.—Countercurrent distribution of reaction products from treatment of acetylandromedol with acid. The solvent system used was prepared by mixing equal volumes of cyclohexane, ethyl acetate, ethanol and water. The operation was carried out with a 50 tube, 100 ml. per phase apparatus. Tubes 50 to 149 in the figure represent withdrawal fractions of top phase. The closed circles represent experimental points, and the calculated values for the theoretical curve are shown as open squares.

(18) We are indebted to Dr. Takemoto for this sample.

The physiological activity is under study. Preliminary work indicates that the compound has some degree of hypotensive action, and comparison studies are in progress with andromedol, acetylandromedol and the crystalline adduct of andromedol and ethyl acetate.

Andromedol has not been observed in isolation studies with *R. maximum* and *K. angustifolia* var. *caroliniana*, but it might have been present in low concentration in these plants. It has been reported to be present, along with grayanotoxin I (acetylandromedol) in extracts from *L. grayana*.<sup>15</sup>

**Anhydroandromedol.**—A solution of acetylandromedol (12.5 g.) in 2.5 l. of water containing 7.5 ml. of concd. sulfuric acid was heated under reflux for 4 hr. The dark mixture was neutralized with sodium hydroxide and filtered to remove polymerized material. The filtrate was extracted with chloroform; the chloroform was removed under reduced pressure to yield 3.8 g. of dark, amorphous material. The usual chromatographic methods (alumina, silica) failed to yield a pure substance. Preliminary small-scale studies indicated that purification by countercurrent distribution might be successful. With a solvent system containing equal parts of water, ethanol, ethyl acetate and cyclohexane (this mixture gives two phases of about equal volume) a separation of components was effected. The separation was carried out with 3.6 g., in a 50-tube apparatus with 100 ml. per phase capacity, and with an arrangement for collecting withdrawal fractions of top phase.

The shape of the major band with a peak concentration in tube 37 was compared with a theoretical curve; Fig. 3 shows the experimental and theoretical values. The close correspondence provides evidence for the purity and homogeneity of this product. The contents of tubes 30 to 45 were combined to yield 1.1 g. of crystalline material, m.p.

223–226° after recrystallization from benzene–ethyl acetate.

The infrared spectrum (Nujol) of this compound showed a broad hydroxy band (2.80  $\mu$ ) but no evidence of a carbonyl group or unsaturation. The ultraviolet absorption was examined to 195 m $\mu$  in water solution, but no evidence of unsaturation was found. The compound did not decolorize bromine in chloroform solution, and an ethanol solution did not absorb hydrogen in the presence of a 10% Pd–C catalyst (the compound was recovered unchanged).

The optical rotation was found to be  $[\alpha]^{25}_{393} -9.3$ ,  $[\alpha]^{25}_{436} -15.2$  ( $c$  0.76, ethanol).

When subjected to electrophoresis on paper with a borate solution, the substance gave a purple Godin color and had a mobility of zero. It displayed no physiological activity.

In addition to the analytical data in Table III, the compound was examined for C–CH<sub>3</sub> and active hydrogen.

*Anal.* Calcd. for C<sub>20</sub>H<sub>32</sub>O<sub>5</sub>: C–CH<sub>3</sub>, 8.53 (for two), 12.80 (for three); active H, 0.86 (for three), 1.14 (for four). Found: C–CH<sub>3</sub>, 9.84; active H, 1.04.

The broad band with maximum at tube 90 (Fig. 3) was not characteristic of a pure substance; when material from this tube was examined through paper electrophoresis with a borate solution, two compounds were indicated. Both gave purple Godin colors; one was uncharged and the other moved slightly toward the anode. The mixture showed ultraviolet absorption in ethanol solution ( $\lambda_{max}$  248 m $\mu$ ) and therefore consisted in part of an unsaturated compound. Tubes 60 to 110 were combined and attempts to separate the components were not successful.

Tubes 125 to 149 contained dark material which was not investigated.

BETHESDA 14, MD.

## COMMUNICATIONS TO THE EDITOR

### TRANSFER OF THE METHYL GROUP OF METHIONINE TO CARBON-24 OF ERGOSTEROL<sup>1</sup>

Sir:

In a previous communication it was demonstrated that the methyl group of methionine serves as a source of the 24-methyl group of ergosterol in yeast.<sup>2</sup> The mechanism of this reaction remained, however, in doubt. Since formate also has been established as a possible source of the 24-methyl group,<sup>3</sup> the oxidation of methionine-methyl to "active formate" with subsequent reduction provided one possible explanation, using only well established biochemical transformations.<sup>4</sup> On the other hand, while transfers of intact methyl groups from sulfur to nitrogen<sup>4</sup> and oxygen<sup>5</sup> were known, no transmethylation to carbon has been reported.

In our hands, formate, formaldehyde, NaHCO<sub>3</sub> and serine-3-C<sup>14</sup> gave lower C<sup>14</sup> incorporation than methionine<sup>2</sup> (also see Table I). Almost 6% of formate-C<sup>14</sup> was incorporated into the non-saponifiable fraction of yeast; however, addition of non-radioactive methionine cut the formate-C<sup>14</sup> incorporation to 0.3%. Under similar conditions, 24.8% of

methionine-methyl-C<sup>14</sup> was incorporated and addition of non-radioactive formate did not affect this incorporation significantly. Addition of homocysteine, which reacts with formate to give methionine,<sup>6</sup> doubled the yields from formate-C<sup>14</sup>. Folic acid, which is essential in *de novo* methyl synthesis, further increased the yield. On the other hand, aminopterin, a folic acid antagonist, decreased the formate-C<sup>14</sup> incorporation to 2.3%. While formate itself had only a slight effect on the incorporation of methionine-methyl-C<sup>14</sup>, formate and homocysteine cut this incorporation sharply. Aminopterin, which prevents the condensation of formate and homocysteine, counteracted the formate homocysteine effect. In the absence of exogenous formic acid, aminopterin increased the yields from methionine-methyl-C<sup>14</sup>, probably by preventing partial oxidation of the S-methyl groups.

Serine-3-C<sup>14</sup> was 50% as efficient as methionine but twice as efficient as formate as the source of C-28 of ergosterol. Non-radioactive serine raised the yield of C-28 from methionine, possibly by acting as an acceptor of the homocysteine moiety, left after the methyl transfer from methionine to ergosterol. The cystathionine thus formed has been shown to be biologically inactive.<sup>6,7</sup>

When doubly labeled methionine, made by mixing methionine-methyl-C<sup>14</sup> with methionine-

(1) This work was supported by the U. S. Public Health Service, Grant C321, The Jane Coffin Childs Memorial Fund, and an institutional grant from the American Cancer Society.

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