STEROIDS AND THE STIFFNESS SYNDROME IN GUINEA PIGS

By J. J. OLESON, EVELYN C. VAN DONK, SEYMOUR BERNSTEIN, LOUIS DORFMAN,* AND Y. SUBBAROW

(From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York)

(Received for publication, June 9, 1947)

Wulzen and Bahrs (1–3) first demonstrated the "stiffness syndrome" in guinea pigs. Van Wagtendonk and coworkers (4–7) reported studies of the metabolic changes resulting from the deficiency of the fat-soluble factor involved. The isolation of the active factor from raw cream and cane juice has been described by van Wagtendonk and Wulzen (8, 9). However, several investigators have been unable to demonstrate the characteristic deficiency symptoms in guinea pigs (10, 11).

Using a diversity of diets, we were able to produce deficiency symptoms in guinea pigs which were apparently identical with those described by the Oregon State College group. Several synthetic diets, the skim milk diet of van Wagtendonk (4), and a commercial type of pelleted diet, were used for the study of this deficiency disease. The syndrome appeared the most rapidly and severely on the pellet diet. This is partly due to the poor growth and physical condition of the animals fed the synthetic or skim milk diets and partly to some property of the pellet diet which markedly induces a severe deficiency. This phenomenon may be caused by the presence in this diet of the "antagonistic factor" mentioned by van Wagtendonk and Wulzen (9).

EXPERIMENTAL

The pellet diet was used in all of the experiments reported here. Its composition is given in Table I. The guinea pigs were obtained from various commercial breeders and weighed from 200 to 400 gm. at the start of the experiment. The animals were housed in individual cages on raised screens and were given food and water *ad libitum*.

Signs of muscle stiffness usually developed in 1 to 3 weeks, depending on the age and source of the guinea pigs. In fact some animals were already deficient when we received them, indicating that the previous dietary history of the animals is an important factor in determining the time of onset of the disease in any given experiment.

* Present address, William R. Warner and Company, Inc., New York.

The procedure used for determining the severity of stiffness is the same as that described by van Wagtendonk and Wulzen (9). The fore leg of the animal is extended posteriorly along the body wall, supported from below by the operator's fingers, and held rigid by downward pressure on the olecranon process with the operator's thumb. By use of the other hand, the paw is flexed upward by gentle pressure. This bending at the wrist to a 90° angle is accomplished very easily in a normal animal. With deficient animals, bending becomes more difficult and the angle to which the paw can be bent decreases. In very severely deficient animals, the paw will not bend at all.

TABLE I

*Composition of Basal Diet**

	lbs.
Ground wheat	20
" whole yellow corn	15
Feeding oat meal	20
Wheat bran	5
Soy bean oil meal (41% expeller)	20
Linseed oil meal	5
Fish meal (60% vacuum)	2
Alfalfa leaf meal (dehydrated 20%)	10
Bone meal (steamed)	1
Limestone (feeding)	1
NaCl	0.25
Delsterol (900,000 I.U. vitamin D)	0.125
	gm.
Ascorbic acid†	12

* Manufactured by the Derwood Mills, Derwood, Maryland.

† Each animal received 20 mg. of ascorbic acid orally three times a week as additional ascorbic acid.

The 1+ to 4+ system, described in detail by van Wagtendonk and Wulzen (9), was used to indicate the severity of the stiffness. A 3+ animal was found most suited for assay purposes. In the 3+ deficiency, the paw bends with some difficulty to approximately a 60° angle. Curative tests were used entirely. Responses to therapy were designated ++, +, ±, or −, depending on whether complete, marked, very little, or no alleviation of the symptoms occurred in the 5 day test period. Only ++ or + responses were regarded as positive and a supplement was considered active when 50 per cent or more of the animals in a group gave this response.

The compounds to be tested were dissolved in refined cottonseed oil and daily doses were given orally for 5 days to test animals showing a 3+ deficiency. Most compounds were tested at a level of 5 γ per day. Final read-

TABLE II

New Esters of Ergostanol

Ester	M.p.	Empirical formula	Analytical data	
			Calculated	Found
	°C.		*per cent*	*per cent*
Formate	111 –112.5	$C_{29}H_{50}O_2$	C 80.87, H 11.70	C 80.50, H 11.98
Propionate	150 –151	$C_{31}H_{54}O_2$	" 81.16, " 11.87	" 81.02, " 11.88
Isovalerate	111.5–113	$C_{33}H_{58}O_2$	" 81.42, " 12.01	" 81.71, " 12.14
Isocaproate	97 – 98	$C_{34}H_{60}O_2$	" 81.33, " 12.08	" 81.38, " 12.42
Pelargonate	99 –100.5	$C_{37}H_{66}O_2$	" 81.85, " 12.25	" 81.87, " 12.52
Laurate	102 –103	$C_{39}H_{70}O_2$	" 82.04, " 12.36	" 81.76, " 12.74
Palmitate	105 –106*	$C_{44}H_{80}O_2$	" 82.27, " 12.58	" 82.14, " 12.91
Stearate	103 –105*	$C_{46}H_{84}O_2$	" 82.54, " 12.68	" 82.15, " 13.03
Ethyl carbonate	127 –128	$C_{31}H_{54}O_3$	" 78.42, " 11.47	" 78.49, " 11.41
Ethyl adipate	113.5–115	$C_{36}H_{62}O_4$	" 77.36, " 11.18	" 77.47, 77.56, H 11.76, 11.69
Cinnamoate	183.5–184.5	$C_{37}H_{56}O_2$	" 83.40, " 10.59	" 83.41, H 10.94
Cyclohexane carboxylate	143 –146.5	$C_{35}H_{60}O_2$	" 81.97, " 11.79	" 82.21, " 12.16
Phenylurethan	178.5–179.5	$C_{35}H_{55}O_2N$	" 80.56, " 10.62, N 2.68	" 81.06, 80.89, H 10.98, 11.18, N 2.73
α-Tetraacetylglucoside	195 –196.5	$C_{42}H_{58}O_{10}$	" 68.82, " 9.36	" 68.58, H 9.69

* Cloudy melt.

TABLE III

Action of Steroids on Stiffness Syndrome

Compounds tested	Level fed, per day	No. of times tested	5 day response (No. of guinea pigs)				Activity No. positive / Total
			++	+	±	−	
Ergostanol	5 γ	4	5	3	3	8	8/19
"	25 "	1	4	1		1	5/6
Ergostanyl acetate	1 "	3	5	5	3	8	10/21
" "	5 "	12	24	16	12	12	40/64
" "*	5 "	1	3	2			5/5
" "	50 "	1	2		1		2/3
" "	100 "	1	1	3			4/4
" formate	5 "	1			1	6	0/7
" propionate	5 "	1	3	1		1	4/5
" isovalerate	5 "	2	4	6	4	2	10/16
" isocaproate	5 "	1				8	0/8
" pelargonate	5 "	1	1	3	1	2	4/7
" laurate	5 "	1		3	1	4	3/8
" palmitate	5 "	1	1	3	2		4/6
" stearate	5 "	1			3	4	0/7
" ethyl carbonate	5 "	1			2	6	0/8
" " adipate	5 "	1		2	1	4	2/7
" cinnamoate	5 "	1			1	7	0/8
" benzoate	5 "	1	3		1	1	3/5
" cyclohexane carboxylate	5 "	1		1		6	1/7
" phenylurethan	5 "	1		5	2	1	5/8
" *p*-toluenesulfonate	5 "	1		1	3	4	1/8
" α-tetraacetylglucoside	5 "	1			2	5	0/7
α-Ergostenol	5 "	1	1		2	1	1/4
"	100 "	1	1	2		1	3/4
β-Ergostenol	5 "	1	1		1	1	1/3
"	100 "	1		1	1	1	1/3
γ-Ergostenol	5 "	1		2	1	1	2/4
γ-Ergostenyl acetate	5 "	1			3	1	0/4
Dehydroergostenyl acetate	5 "	1	1		1	2	1/4
" "	100 "	1	1	2		1	3/4
γ-Dihydroergosterol	5 "	1		2	2	4	2/8
γ-Dihydroergosteryl acetate	5 "	1				5	0/5
Ergosterol	5 mg.	1		2	1	1	2/4
"	10 "	1			1	3	0/4
"	10 "	1				2	0/2
Dehydroergosteryl acetate	5 γ	1	1		1	2	1/4
" "	100 "	1	2	2			4/4
β-Ergostenol oxide	5 "	1		2	2	4	2/8
Epiergostanol	5 "	1				4	0/4
Epiergostanyl acetate	5 "	1			2	2	0/4
Chlorergostane	5 "	1				8	0/8

TABLE III—*Concluded*

Compounds tested	Level fed, per day	No. of times tested	5 day response (No. of guinea pigs)				Activity No. positive / Total
			++	+	±	−	
Ergostane	5 γ	1			4	2	0/6
Cholesterol	5 mg.	1			1	3	0/4
Cholesteryl propionate	5 γ	1			2	5	0/7
Cholestanol	5 "	1			2	3	0/5
Cholestanyl acetate	5 "	1		1		3	1/4
7-Ketocholestanyl acetate	5 "	1		1	3	3	1/7
7-Ketocholesteryl "	5 "	1			2	6	0/8
Cholestanone	5 "	1				7	0/7
Δ^4-Cholestenone-3	5 "	1			2	6	0/8
Stigmastanol	5 "	1		2	1	2	2/5
Stigmastanyl acetate	5 "	1			1	4	0/5
Stigmasteryl "	5 "	1			1	7	0/8
Phytosterol	5 mg.	1	1		1	2	1/4
α_1-Sitostanol†	5 γ	1	1	1		6	2/8
α_1-Sitosterol†	5 "	1	1	1	2	3	2/7
β-Sitosterol	5 "	1	1		1	4	1/6
α_3-Sitosterol	5 "	1		1	2	4	1/7
Cholanic acid	5 "	1		1	2	2	1/5
Cholic acid	1 mg.	1			1	4	0/5
Methyl cholate	5 γ	1			3	5	0/8
Lithocholic acid	5 "	1	1	1	1	3	2/6
Desoxycholic acid	5 "	1		2		5	2/7
3-Ketocholanic acid	5 "	1				7	0/7
Methyl 3(α)-12-ketocholanate	5 "	1		2	2	2	2/6
Estrone	2 "	1	1	1	1	4	2/7
Crystalline vitamin D_2	5 "	1			1	5	0/6
Vitamin D_3 (delsterol)	5 "	1	1	1	1	3	2/6
Dihydrotachysterol	5 "	1			2	7	0/9
Crystalline "antistiffness" factor	1 "	5	7	5	2	6	12/20
" " "	2 "	3	4	6	1	7	10/18
" " "	5 "	1	1	1	1	1	2/4
Negative controls		23	1	14	19	79	15/113

* Injected intraperitoneally.

† Bernstein *et al.* (12) have indicated the possibility that α_1-sitosterol may be non-steroidal.

ings were taken late on the 5th day, and the responses were read by the operator, who did not know from which group the animals were taken. Cottonseed oil was found to be inactive.

In the course of testing various natural materials and extracts, "anti-stiffness" activity was found in crude sterol preparations made from *Penicillium notatum*. A number of pure steroids were then assayed and certain

ones were found to be very potent. This paper is concerned only with tests made on pure compounds.

Of the pure compounds assayed biologically, fourteen are new esters of ergostanol. The physical properties of these compounds which were prepared by the standard procedures are summarized in Table II.

The biological activities of the compounds tested are presented in Table III. The individual tests are given. The control groups are also listed to indicate variations in the assay over the test period. In addition to the known compounds tested, a sample of crystalline "antistiffness" factor, kindly supplied by Dr. A. L. Caldwell, was also assayed several times and served as a positive control.

Results

The most active compounds tested were found to be ergostanol and certain of its esters. Since ergostanyl acetate appeared to be more active than the free sterol, our attention was mainly directed to this derivative.

On the basis of this preliminary study of a large number of steroids, certain correlations between activity and structure were suggested. Obviously more experiments will be required to substantiate some of the following relationships. (1) Sterols having the ergostane carbon skeleton were most active, since, *e.g.*, cholestanol, stigmastanol, bile acids, and estrone were inactive. (2) Replacement of the hydroxyl group at the C-3 position of ergostanol with chlorine or hydrogen gave inactive compounds. (3) Inversion of the hydroxyl group of ergostanol decreased the activity, since epiergostanol and its acetate were inactive. (4) Complete saturation of the ergostane ring system was found to be necessary for maximum activity since ergosterol was inactive. It would appear in this connection that compounds of less degree of saturation in the ergostane series are less active than ergostanol and its acetate. (5) The crystalline "antistiffness" factor showed good activity in our tests. While a precise comparison of activity is difficult at this time, its activity was approximately the same as that of ergostanyl acetate.

DISCUSSION

It cannot be said that any one of the pure compounds which we have found active is identical with the crystalline "antistiffness" factor isolated by van Wagtendonk and Wulzen, since we have not isolated any material from a natural source and the chemical nature of their active substance has not been revealed. However, under our experimental conditions, ergostanyl acetate is approximately as active as their crystalline material.

The degree of structural specificity exhibited by the series of compounds tested is of considerable interest and has been discussed above.

SUMMARY

1. A severe deficiency of the "antistiffness" factor was produced in guinea pigs fed a natural diet.

2. Of the fifty-nine steroids tested, certain esters of ergostanol showed the greatest activity. The relationship between structure and activity has been discussed.

3. The preparation and physical constants of fourteen new esters of ergostanol have been presented.

4. A sample of the crystalline "antistiffness" factor isolated by van Wagtendonk and Wulzen was assayed and found to be highly active.

We are indebted to Dr. W. J. van Wagtendonk and Dr. R. Wulzen, Oregon State College, for very helpful advice. We are also indebted to Dr. A. L. Caldwell of Eli Lilly and Company for a sample of the crystalline "antistiffness" factor, and to Professor Everett S. Wallis, Princeton University, for specimens of α_1-, α_3-, and β-sitosterol and α_1-sitostanol.

We wish to thank Messrs. Louis Brancone, William Fulmor, and Samuel Modes for the microanalyses, and Mr. Karl J. Sax for assistance in the preparation of several of the compounds.

BIBLIOGRAPHY

1. Wulzen, R., and Bahrs, A. M., *Physiol. Zool.*, **9**, 508 (1936).
2. Bahrs, A. M., and Wulzen, R., *Proc. Soc. Exp. Biol. and Med.*, **33**, 528 (1936).
3. Wulzen, R., and Bahrs, A. M., *Am. J. Physiol.*, **53**, 312P (1941).
4. van Wagtendonk, W. J., *J. Biol. Chem.*, **155**, 337 (1944).
5. van Wagtendonk, W. J., and Lamfrom, H., *J. Biol. Chem.* **158**, 421 (1945).
6. van Wagtendonk, W. J., Freed, A. M., and Ballou, C. E., *Arch. Biochem.*, **5**, 329 (1944).
7. van Wagtendonk, W. J., and Zill, L. P., *J. Biol. Chem.*, **159**, 247 (1945).
8. van Wagtendonk, W. J., and Wulzen, R., *Arch. Biochem.*, **1**, 373 (1943).
9. van Wagtendonk, W. J., and Wulzen, R., *J. Biol. Chem.*, **164**, 597 (1946).
10. Homburger, E., and Reed, C. I., *Federation Proc.*, **4**, 34 (1945).
11. Kon, S. K., Bird, M. J., Coates, M. E., Mullen, J. E. C., and Shepheard, E. E., *Arch. Biochem.*, **11**, 371 (1946).
12. Bernstein, S., Wilson, E. J., Jr., and Wallis, E. S., *J. Org. Chem.*, **7**, 103 (1942).