[CONTRIBUTION FROM THE DIVISION OF CHEMISTRY AND DIVISION OF PATHOLOGY AND MICROBIOLOGY, MEDICAL UNITS, UNIVERSITY OF TENNESSEE]

Seroflocculating Steroids. II.[1] General[2]

BY FREDERIC C. CHANG, ROBERT T. BLICKENSTAFF, AARON FELDSTEIN,[3a] JANE RANSOM GRAY, GEORGE S. MCCALEB[3b] AND DOUGLAS H. SPRUNT

RECEIVED NOVEMBER 20, 1956

The chemical basis for the possible use of a new type of seroflocculation reaction, either for the detection of a disease like cancer or as a general health clearance test, is discussed. In this paper a program of study of the seroflocculating activity of certain steroids is outlined with these alternatives as the goal, and a first report of screening results is given. Accompanying papers III and IV of this series deal with the chemistry and synthesis of compounds reported here.

Although seroflocculation reactions are widely used in medicine for detection of disease, little fundamental knowledge concerning these reactions is available.[4] An understanding of the mechanism of such a reaction entails studying initially: (1) the nature of the substances that function as seroflocculating agents (seroflocculants), (2) the nature of the serum components that are flocculated, (3) the composition of the flocculated material (flocculates) and (4) the conditions under which the reaction takes place. We have been considering some of these factors in a new type of seroflocculation reaction[1,5] which is given by a high percentage of the sera of patients with moderately advanced cancer and of moderately advanced cases of other diseases such as tuberculosis.

Tests on three seroflocculants, the non-saponifiable fraction of cancerous livers,[5] ethyl choladienate[6] and ethyl 3β-chloro-11-cholenate[1] show that they can all be used to detect moderately advanced cancer and other diseases with good precision, but their "sensitivity"[7] was low in many diseases at an early stage, particularly in cancer, and their "selectivity"[7] seemed to decrease with increase in sensitivity. Whether this inverse relationship between sensitivity and selectivity is an inherent property of this series of seroflocculants will determine what applications can be made of the reaction for detection of disease.

Development of the reaction could give rise to two different uses in the detection of disease, depending on which of two alternative chemical situations exists in the serum. Ideally, the reaction would be used to detect specific diseases, or secondarily it might be able to distinguish normal sera from those of a group of diseases and thus be useful as a general health screening test.

From a chemical point of view, the problem can be stated as follows: if the flocculable material in the serum is a component uniquely associated with a specific disease, it would be potentially possible to detect that disease by finding a seroflocculant which will react with the unique component. On the other hand, if the serum component participating in the flocculation reaction is one produced by any of a group of diseases or by a process not necessarily limited to a single disease, then the reaction will be non-specific. In this case, any differences in activity found in flocculants of varying chemical structure will merely be measures of how effectively they combine with the common serum component. Thus, sensitivity appears to be a problem basic to the chemical structure of the flocculant, whereas selectivity is one that concerns the serum component primarily.

There is *a priori* no way of knowing which of these alternative situations prevails for any particular disease. In syphilis, at least, a unique component (or situation) apparently exists since a serological reaction for detection of syphilis is known and widely used. For cancer, there is no previous evidence of a unique cancer component detectable serologically. But because of the obvious importance of a good test for the detection of cancer, the existence of evidence that can be interpreted favorably,[8] and because we have a feasible method of study through the finding of a seroflocculant of known structure, an investigation of this problem appears to be desirable.

Our program involves a search for a sensitive flocculant based on the assumption that the seroflocculation reaction is a useful method of detecting early disease. The completed research will furnish information as to whether it will be possible to detect a specific disease like cancer or whether it will be useful as a health clearance test.

The two substances[6] first studied in this reaction were both oils. The chemical composition of the liver fraction is complex and largely unknown,[9] and

(1) Paper I of this series, THIS JOURNAL, **76**, 3213 (1954).

(2) This investigation was supported in part by grants (CS-9053 and C-2249) from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

(3) (a) Worcester Foundation for Experimental Biology, Shrewsbury, Mass.; (b) Department of Chemistry, University of Arkansas, Fayetteville, Ark.

(4) L. J. Zeldis and S. C. Madden, *Ann. Review Biochem.*, **17**, 340 (1948); A. Saifer, *Am. J. Med.*, **13**, 730 (1952).

(5) H. S. Penn, *J. Natl. Cancer Inst.*, **12**, 1389 (1952).

(6) A. H. Dowdy, H. S. Penn, G. Hall and A. Bellamy, *Proc. Am. Assoc. for Cancer Research*, **1**, 12 (1954).

(7) The term "sensitivity" is used to indicate efficiency in confirming cancer (diagnosed by biopsy); "selectivity" to denote the degree with which a test is free from giving "false positives." Quotation marks will be omitted when these terms are used subsequently in these papers.

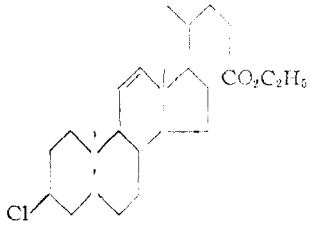
(8) C-Reactive protein antiserum parallels ethyl 3β-chloro-11-cholenate in activity [D. H. Sprunt, W. M. Hale, F. C. Chang, S. G. Richmond, C. C. Erickson, *Science*, **122**, 273 (1955)], but there are differences suggestive that C-Reactive protein and a cancer component are similar but not identical. Orosomucoid, a glycoprotein known to increase in the sera of patients with carcinoma [H. Goodman, S. Brennan and W. J. Simpson, *Proc. Am. Assoc. for Cancer Research*, **2** 111 (1956)], is immunologically distinct from C-Reactive protein. Both orosomucoid and C-Reactive protein seem to belong to a group of abnormal protein substances, another one of which might be unique for cancer. Furthermore, the inverse sensitivity and selectivity relationship mentioned earlier with seroflocculants tested does not hold strictly, suggesting that the serum components flocculated have chemical differences.

(9) R. F. Riley, M. Hokama, P. Kratz and H. S. Penn [*Federation Proc.*, **15**, 636 (1956)] recently reported preliminary results on the fractionation of this material.

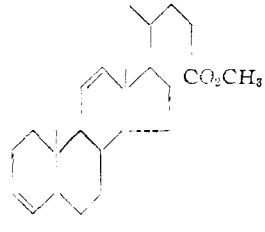
our experience with the desoxycholic acid-derived "antigen"[6,10]—an uncrystallizable oil of variable activity and stability—convinced us of its heterogeneous nature, although it is without doubt chiefly a mixture of ethyl choladienates. Thus it was desirable to find a stable active compound of known structure and our attention was first directed toward the preparation of a pure ethyl choladienate.

The pyrolytic methods used by Wieland[11] when applied to ethyl desoxycholate indeed gave active, crystalline material but appeared unpromising as a means of obtaining a pure isomer. Since elimination reactions involving ionic mechanisms have produced mixtures differing from products of pyrolysis, we decided to try dehydrotosylation, a method often used for introducing unsaturation in steroids.[12] When ethyl Δ^{11}-lithocholenate,[1] refluxed with *p*-toluenesulfonyl chloride in pyridine, yielded a crystalline product of constant melting point and good activity, we naturally assumed that we had in hand an ethyl choladienate. However, the material contained chlorine and was demonstrated to be a constant melting mixture of ethyl 3β-chloro-11-cholenate (28)[13] and unsaturated matter. A separate synthesis of 28 confirmed this. This compound 28, which showed the best seroflocculating activity of all the substances we had tested, was the first active compound of defined structure. Moreover, it had the requisite characteristics of stability, availability and reproducibility to use as a comparison standard for evaluation of other substances.

The preparation of a pure ethyl choladienate was pursued further and ethyl 3,11-choladienate (45) has been obtained by a satisfactory method.[14] Compound 45 when pure turned out to be somewhat too insoluble to be tested satisfactorily by the original test conditions, but the corresponding methyl ester (46) was found to have good activity. With 28 and 46, two compounds of known structure to serve as basic models, it was possible to plan a structure–activity study on a less empirical basis than a random screening would be.



28



46

The program followed is to prepare (or collect) accessible structural variations of these two substances and to test these related compounds for seroflocculating activity in a short screening test. The test, described briefly below, is designed to find compounds with the highest general sensitivity without regard in the first instance to their selectivity. This information, which has given some indications of the structural features associated with good activity, will be a guide in further work. When a sufficient number of representative compounds of high sensitivity but of different chemical structure is found, a parallel clinical test on a larger scale using these compounds will show whether they show selectivity and incidentally answer the question of the existence of a unique cancer component. The results, furthermore, should point to the significant molecular structural features which would be incorporated in the designing of more efficient flocculants. These in turn should facilitate the isolation of the hypothetical cancer component, the study of which would be of fundamental biochemical importance.

This paper is an interim report giving results of the screening to date. The two accompanying papers are concerned with the syntheses of compounds reported here.

Screening Method.—(Only a summary of the method is given; the testing particulars will be described in detail elsewhere.) The tests were performed by a modification of the original method[5]; only one-half the amount of flocculant and different serum dilutions are used. The test is a simple one to perform: a suspension, prepared from an alcoholic solution of the flocculant, a trace of cholesterol and buffered saline solution, is combined with the test serum, diluted serially and centrifuged. The tubes are shaken and read. A good positive test is one in which floccules are formed and the solution remains sparkling clear; a negative test is one in which there are no floccules and the solution is turbid.

Test sera used in the screening varied from three to ten depending on the supply of flocculant available. Cancer sera and normal sera were included in the same test run. Test cancer sera are from cancers (confirmed by biopsy) that give good tests with the standard flocculant, ethyl 3β-chloro-11-cholenate[14a] (28); test normal sera are from apparently healthy individuals and have given consistently negative tests with 28.

Screening Results.—Compounds are classified below in three groups. Group A consists of compounds which clearly can distinguish between test positives and normals and therefore have the same order of activity as the standard 28. Group B are compounds that show differences between the positive and normal standards but not as distinctly as those in group A. In general, there are floccules in the positive tubes but the solution is not clear. These are substances classified as lower activity compounds. Group C compounds do not distinguish between positive and normal sera; almost all give tests that remain turbid and have no floccules. A few flocculants which give positive tests on all sera indiscriminately are also included among this group.

(10) We are indebted to Drs. Dowdy and Penn for making available to us procedures for preparing this "antigen."

(11) H. Wieland and W. Kapitel, *Z. physiol. Chem.*, **212**, 269 (1932).

(12) L. F. Fieser and M. Fieser, "Natural Products Related to Phenanthrene," Reinhold Publishing Corp., New York, N. Y., 1949, p. 692.

(13) Compounds mentioned in this series of papers are numbered according to the consecutive order in which they are described in the Experimental Sections of papers III and IV.

(14) Paper IV of this series, THIS JOURNAL, **79**, 2167 (1957).

(14a) Merck and Company, through the kindness of Dr. K. Pfister, has prepared a generous supply of the compound for these tests.

No.	Compound	Source (ref. no.)
	Group A	
28	Ethyl 3β-chloro-11-cholenate	15
27	Methyl 3β-chloro-11-cholenate	15
31	Ethyl 3β-chloro-9(11)-cholenate	15
30	Methyl 3β-chloro-9(11)-cholenate	15
47	Methyl 3,9(11)-choladienate	14
45	Ethyl 3,11-choladienate	14
46	Methyl 3,11-choladienate	14
38	Ethyl 3β-chloro-5-bisnorcholenate[16]	15
17	Ethyl 3α,12α-diacetoxycholanate	15
19	Ethyl 3β-chloro-12α-acetoxycholanate	15
	Group B	
	Ethyl cholanate	17
48	Methyl 3-cholenate	14
49	Ethyl 3-cholenate	14
50	Methyl 9(11)-cholenate	14
51	Methyl 11-cholenate	14
52	Ethyl 11-cholenate	14
	Methyl 3α-acetoxy-9(11)-cholenate	18
12	Ethyl 3α-acetoxy-9(11)-cholenate	15
11	Ethyl 3α-acetoxy-11-cholenate	15
13	Methyl 12α-acetoxy-3-cholenate	15
14	Ethyl 12α-acetoxy-3-cholenate	15
	Methyl 3α,12α-diacetoxycholanate	19
	Ethyl 3β-chlorocholanate	1
21	Ethyl 3α-tosyloxycholanate	15
18	Methyl 3β-chloro-12α-acetoxycholanate	15
	Group C	
1	Methyl lithocholate	15
2	Ethyl lithocholate	15
3	Methyl 12α-hydroxycholanate	15
4	Ethyl 12α-hydroxycholanate	15
5	Ethyl 3α-hydroxy-9(11)-cholenate	15
	Methyl 3α-hydroxy-11-cholenate	20
	Ethyl 3α-hydroxy-11-cholenate	1
6	Methyl 3β-hydroxy-5-bisnorcholenate[21]	15
7	Ethyl 3β-hydroxy-5-bisnorcholenate[21]	15
8	Ethyl 3α-acetoxycholanate	15
9	Methyl 12α-acetoxycholanate	15
10	Ethyl 12α-acetoxycholanate	15
15	Methyl 3β-acetoxy-5-bisnorcholenate	15
16	Ethyl 3β-acetoxy-5-bisnorcholenate	15
20	Methyl 3α-tosyloxycholanate	15
22	Methyl 3α-tosyloxy-9(11)-cholenate	15
23	Methyl 3α-tosyloxy-11-cholenate	15
24	Ethyl 3α-tosyloxy-11-cholenate	15
	Ethyl desoxycholate	22
25	Methyl 3α-tosyloxy-12α-hydroxycholanate	15
26	Ethyl 3α-tosyloxy-12α-hydroxycholanate	15
29	3β-Chloro-11-cholenic acid	15
32	3β-Chloro-9(11)-cholenic acid	15
33	Methyl 3β-chlorocholanate	15
34	3β-Chlorocholanic acid	15
35	Methyl 3β-chloro-12α-hydroxycholanate	15
36	Ethyl 3β-chloro-12α-hydroxycholanate	15
39	Ethyl 12α-benzoxycholanate	15
	Methyl 3α-benzoxy-12α-hydroxycholanate	23
40	Ethyl 3α-benzoxy-12α-hydroxycholanate	15
41	Ethyl 3α-12α-dibenzoxycholanate	15
42	Ethyl 3-ketocholanate	15
	3-Ketocholanic acid	24
43	Methyl 3-keto-9(11)-cholenate	15
44	Methyl 3-keto-11-cholenate	15
	Methyl 3α-carbethoxyoxy-12-keto-9(11)-cholenate	25
53	Methyl 12α-hydroxy-3-cholenate	14
54	Ethyl 12α-hydroxy-3-cholenate	14

Tentative Conclusions.—The compounds reported here are all derivatives of cholanic acid with the exception of several bisnorcholanic acid compounds. Compounds derived from other parent steroids are also being investigated and will be reported in the future. Many of the compounds on these lists were intermediates prepared during the course of synthesis of certain desired structures but were nevertheless included in the testing to provide additional information. Although speculation, at this stage, of the basic nature of the flocculation reaction seems premature and many more structurally different compounds should be examined before profitable conclusions can be drawn, certain empirical deductions already stand out: (1) only esters show activity, free acids are inactive; (2) with one exception (3,11-choladienate) the ethyl esters are more active than the corresponding methyl esters; (3) active groupings are unsaturation, chloro and acetoxy, and two such groups are needed for high activity; (4) inactive groups are hydroxy, tosyloxy, benzoxy and keto, and one such group can counteract the effect of an active group.

Whether a correlation exists between some well-defined physical property of the flocculants and their seroflocculating activity has not been investigated, and obviously this can be done more intelligently when the best flocculants have been discovered. However, from purely qualitative observations made during the handling of these compounds, it can be said that most likely there is no simple relationship between their solubilities and activity.

Acknowledgment.—We wish to acknowledge the able technical assistance of Mrs. Judith Beasley on the work reported in this series of papers, and the generous coöperation of Dr. M. T. Giachino and Mr. E. W. Lard of Grace Chemical Co. in infrared spectrophotometry.

MEMPHIS 3, TENN.

(15) Paper III of this series, THIS JOURNAL, **79**, 2164 (1957).

(16) The corresponding methyl ester 37 is too insoluble in 95% ethanol to test at the concentration (20 mg./ml.) used in the regular test. At a lower concentration (15 mg./ml.), 37 showed some activity.

(17) H. Wieland and E. Boersch, *Z. physiol. Chem.*, **106**, 190 (1919).

(18) L. F. Fieser and S. Rajagopalan, THIS JOURNAL, **73**, 118 (1951).

(19) T. Reichstein and M. Sorkin, *Helv. Chim. Acta*, **25**, 797 (1942).

(20) B. F. McKenzie, W. F. McGurkin and E. C. Kendall, *J. Biol. Chem.*, **162**, 555 (1946).

(21) These compounds were soluble in ethanol at the test concentration of 20 mg. per ml., but immediate precipitation occurred when the suspension was prepared. They could not be tested properly.

(22) H. Wieland and H. Sorge, *Z. physiol. Chem.*, **98**, 59 (1916).

(23) B. F. McKenzie, V. R. Mattox, L. L. Engel and E. C. Kendall, *J. Biol. Chem.*, **173**, 271 (1948).

(24) H. Wieland and P. Weyland, *Z. physiol. Chem.*, **110**, 123 (1920).

(25) L. F. Fieser and S. Rajagopalan, THIS JOURNAL, **72**, 5) (1950).