determinations as a sensitive measure of the biological response to inhalation of toxic vapors encourages further investigation into the nature, extent, and significance of these enzyme changes.

Summary. Serum esterase, xanthine oxidase, and glutamic-oxaloacetic-transaminase (GOT) activities were followed for 9 days after a single 4 hr exposure of male rats to carbon tetrachloride vapors. 1500 ppm of carbon tetrachloride in air produced marked changes in all 3 enzyme systems. 1000 ppm affected only serum GOT and xanthine oxidase activities. At 250 ppm, only serum GOT activity was significantly affected.

1. Ball, W. L., Kay, K., *A.M.A. Arch. Ind. Health,* 1956, v14, 450.

2. Affonso, O. R., Mitidieri, E., Ribeiro, L., Villelo, G., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 527.

3. Wroblewski, F., Jervis, G., LaDue, J., *Ann. Int. Med.,* 1956, v45, 782.

4. Peterson, J. E., Hoyle, H. R., Schneider, E. J., *Am. Ind. Hyg. Assn. Quart.,* 1956, v17, 429.

5. Gomori, G., *J. Lab. and Clin. Med.,* 1949, v34, 275.

6. Westerfeld, W. W., Richert, D. A., Hilfinger, M. F., *Cancer Research,* 1950, v10, 486.

7. Steinberg, D., Baldwin, D., Ostrow, B. H., *J. Lab. and Clin. Med.,* 1956, v48, 144.

8. Bruns, F., Neuhaus, J., *Arch. Biochem. and Biophys.,* 1955, v55, 588.

Received November 1, 1957. P.S.E.B.M., 1958, v97.

Bentonite Flocculation Test for Rheumatoid Arthritis.* (23681)

JOHN BOZICEVICH, JOSEPH J. BUNIM,** JULES FREUND AND STANLEY B. WARD
Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, N.I.H., Bethesda, Md.

The various serological tests for rheumatoid arthritis have attracted increasing attention and interest in recent years, largely because they have been widely recognized as useful diagnostic measures. As an objective diagnostic criterion, the test is helpful in segregating an homogeneous type of rheumatoid arthritis from a general and heterogeneous group of polyarthritides. In addition, the test makes it possible for workers in different parts of the world to agree on the definition of this disease entity. Some basis for such agreement is especially important in determining the prevalence and incidence of rheumatoid arthritis in diverse and widely separated populations and also in critically evaluating therapeutic measures used in the management of this disease. A variety of serological tests for rheumatoid arthritis have been described since the first one was reported(1). A comprehensive review of the methods, principles, relative merits, and clinical correlation of the different tests used to date has been published recently by Ziff(2). All serological tests for rheumatoid arthritis depend on clumping of particulate matter by addition of serum from patients. Some of the tests employ sheep cells; in this type of test, the rbc are either mixed with a subagglutinating amount of immune rabbit serum or with human gamma globulin (HGG); later several dilutions of patient's serum are added to rbc. Other tests used collodion or latex particles coated with HGG. In the present study, bentonite particles were coated with HGG. These particles were employed by Bozicevich *et al.*(3) to adsorb antigenic material from trichina in a serologic test for the presence of antibodies in the serum of patients infected with trichina. The technic used in the present study is a slight modification of that published in 1951. Although useful, most of these tests are complicated, time consuming, and require specialized technics. The bentonite flocculation test, described here, is simple and can be done in 20 minutes once the stock solutions are prepared.

* Read before IXth Internat. Cong. on Rheumatic Diseases, June 23-28, 1957.

** National Institute of Arthritis and Metabolic Diseases.

Bentonite, a colloidal clay of powder consistency, is stable and readily available. It consists of abundant, negatively charged particles whose surface/mass ratio is unusually large. After the particles are "sensitized" with normal human serum gamma globulin (Fraction II), they clump when mixed with serum from patients with rheumatoid arthritis.

Preparation of bentonite suspension. A convenient way to prepare bentonite suspension is as follows: Suspend 0.5 g of bentonite in 100 ml of distilled water and homogenize it with the aid of a blendor. The bentonite suspension is placed in a glass-stoppered graduate and diluted with distilled water to a volume of 500 ml. After shaking the suspension and allowing it to settle for one hour, the supernate is removed and placed in 90 ml centrifuge tubes. The tubes are centrifuged (by tachometer) at 1,300 rpm for 15 minutes, using an International Centrifuge, size 2, with No. 240 head. The supernates are collected and centrifuged at 1,600 rpm for 15 minutes. The accumulated sediment is suspended in 100 ml distilled water with the aid of a blendor. The bentonite suspension may be kept at room temperature for several months, and it should be shaken before removing an aliquot. This technic yields a range of particle size which does not tend to give false positive or false negative reactions.

Preparation of F II solution. One-half g of lyophilized Fraction II† (gamma globulin) derived from pooled normal human sera is dissolved in 50 ml veronal buffer. The veronal buffer is made by dissolving 0.184 g barbituric acid + 1.03 g sodium barbiturate in 100 ml distilled water. This buffer has 0.06 molarity, and its pH is 8.6. The F II solution may be stored at 5°C for one or 2 months. It may be clarified by centrifugation.

Preparation of sensitized bentonite particles. Ten ml of the bentonite suspension is centrifuged and the sediment taken up in 1 ml of distilled water. To this suspension, 2 ml of the F II solution is added and the 2 components gently mixed by tilting and rotating. The suspension is then allowed to stand for 15 to 30 minutes at room temperature to permit adsorption, 15 ml of distilled water is added, and the suspension is gently mixed again. The suspension is centrifuged, and the sediment suspended in 15 ml of water and vigorously shaken for 30 seconds to disperse the particles. This suspension is centrifuged again, and the sediment is taken up in 5 ml water. One ml of 0.1% methylene blue solution is added to the suspension and again vigorously shaken. After 5 minutes, 10 ml of water are added, the suspension is centrifuged, and the sediment is taken up in 10 ml of distilled water. Washing is repeated and the sediment taken up in 4 ml of 0.05 molar phosphate buffer of pH 7.3. It is desirable to add 0.1 ml of 1% Tween 80 to this sensitized bentonite suspension. The serum‡ to be tested is heated for 30 minutes at 56°C prior to use. Microscopic slides (3″ x 2″) with 12 rings, designed for serological flocculation tests, are used to carry out the test. Into each ring on the slide is placed 0.1 ml of serial twofold dilutions of serum in saline. One drop (about 0.025 ml) of sensitized bentonite suspension is added to each ring. (For this purpose, it is convenient to use a capillary pipette which will deliver about 40 drops per ml.) The slide is then placed on a Boerner-type rotating machine and rotated 100 to 120 times per minute for 20 minutes, and read immediately under a microscope at low-power magnification (60 X). Drying is to be avoided. In a 4-plus reaction, all of the sensitized particles are clumped in separate masses. There may be a few large or a number of small clumps, depending upon the titer of the serum. Nevertheless, the fields between the flocs are almost clear. In a 3+ reaction, three-fourths of the bentonite particles are clumped; in a 2+ reaction, half; and in a 1+ reaction, one-fourth. The remaining particles are fairly evenly dispersed. In a negative test, the sensitized bentonite particles remain in suspension. The flocculation test

† Fraction II is obtained by fractional precipitation of pooled normal human serum by the method described by E. J. Cohn, *et al., J. Am. Chem. Soc.*, 1950, v72, 465.

‡ Freshly collected serum is preferable. Serum stored at 5°C for as long as 2 weeks has been found satisfactory.

TABLE I. Bentonite Flocculation Test in Rheumatoid Arthritis (41 Cases).

Age and sex	Duration of arthritis (yr)	Subcut. nodules	ESR (mm/hr)	Titer (flocculation of 2+ or greater)
21 ♀	2½	No	57 (WS)*	8192
43 ♀	6	Yes	127 "	>4096
55 ♀	4	No	104 "	2048
52 ♂	2	Yes	40 "	"
37 ♀	8	No	37 "	"
31 ♀	½	Yes	60 "	1024
35 ♀	6	"	59 "	>1024
37 ♂	7	No	38 "	1024
65 ♀	8	"	93 "	256
51 ♂	2	Yes	74 "	"
47 ♂	6	"	43 "	"
32 ♀	7	No	41 (WN)*	"
34 ♂	10	Yes	36 "	"
66 ♀	39	"	36 "	"
51 ♂	10	"	28 "	"
40 ♂	14	No	25 "	"
44 ♀	13	Yes	16 (WS)	"
18 ♂	5	No	6 "	"
52 ♂	10	Yes	97 "	>128
61 ♂	19	No	38 (WN)	128
46 ♂	15	"	34 "	"
39 ♂	14	Yes	32 "	"
41 ♂	2	No	82 (WS)	64
40 ♂	6	Yes	60 "	"
21 ♀	1	No	57 "	"
30 ♀	4	"	54 "	"
63 ♂	4	Yes	36 (WN)	"
35 ♂	8	No	32 "	"
60 ♀	3	"	45 "	32
48 ♂	1	"	30 "	"
68 ♂	12	"	23 "	"
54 ♂	13	Yes	6 "	"
56 ♂ †	12	"	105 (WS)	0
31 ♀	2	No	77 "	0
34 ♀ †	13	"	40 "	0
53 ♂ †	14	"	38 (WN)	0
69 ♀ †	11	"	30 (WS)	0
34 ♂	7	"	27 (WN)	0
57 ♀ †	8	Yes	10 "	0
25 ♂ †	½	"	2 (WS)	0
57 ♀	7	No		0

* WS = Westergren; WN = Wintrobe corrected.
† Sera from these 6 patients gave negative sheep cell agglutination test, as well as negative BFT. Sera from the remaining 3 patients whose BFT were negative were not tested for sheep cell agglutinins.

is considered positive when a 2+ or stronger clumping occurs in a serum dilution of 1 to 32 or higher. Although a negative serum does not cause any clumping in dilutions up to about 1 to 1,000, occasionally a 1+ or 2+ flocculation may occur in dilutions of 1 to 1,000 or higher. With each test, serial saline dilutions of a known positive and a known negative serum should be included in order to detect any change in the sensitized bentonite particles. Debris in the sensitized bentonite mixture or the control negative serum, may cause clumping of the particles. With experience, the spurious aggregates can readily be distinguished from true flocculation. When the test is positive, the flocs are of uniform density, evenly distributed in the microscopic field and free of "hyaline-like" material or other debris.

Results. In patients with unequivocal rheumatoid arthritis. Sera from 41 adult patients with unquestionable rheumatoid arthritis have been tested. The pertinent clinical and laboratory data and results of the test are listed in Table I. In 32 patients (78%), the tests were positive. None of the patients had ankylosing spondylitis or psoriasis. The BFT in 9 patients with rheumatoid arthritis was negative. The sera of 6 of these patients (including 3 with subcutaneous nodules) were also tested for sensitized sheep erythrocyte agglutination reaction (SSEA) using the euglobulin fraction and all 6 were negative.

In control subjects. Sera from 163 control subjects were tested (Table II). Normal subjects, patients with arthritis and rheumatic diseases other than rheumatoid arthritis, as well as patients with many unrelated diseases were included. None of the sera from the 15 normal persons gave a positive test. Of the sera from 148 control patients, 3 (2%) reacted positively. It is of interest that one of these 3 was from a patient with systemic lupus erythematosus and another from a patient with macroglobulinemia. Of the 160 sera that were negative, 155 failed to show any flocculation at all, even in the first dilution (1:4); 4 sera caused flocculation in dilutions of 1:4 or 1:8; and one, in dilution up to 1:16. None of these 5 sera gave a 4-plus flocculation in any dilution or a 3-plus flocculation in dilutions of 1:8 or higher.

Comparison of BFT and sensitized sheep erythrocyte agglutination (SSEA) test on sera from same patients. A series of 64 sera from 64 patients was subjected to both tests. The SSEA tests were done in the laboratory of Dr. R. R. Williams, Jr., at the Nat. Inst. of Arthritis and Metabolic Diseases by the method described by Ziff and his associates(4).

TABLE II. 163 Cases Included in Control Series.*

Rheumatic diseases other than rheumatoid arthritis		28
Rheumatic heart disease (active and inactive)	10	
Gout	9	
Reiter's	3	
Scleroderma	2	
Dermatomyositis	2	
Systemic lupus erythematosus	1 (1)	
Rheumatic fever	1	
Normal subjects		15
Carcinoma of various organs		41
Hypercholesterolemia		5
Uveitis, brain tumor, and macroglobulinemia		5 (1)
Multiple myeloma		4
Congenital heart disease		4
Undiagnosed disease		4
Muscular dystrophy		4
Osteoporosis		3
Mental defective		3
Stomatitis		3
Arteriosclerotic heart disease		3
Acute leukemia		3 (1)
Diabetes mellitus		2
Pseudohermaphroditism		2
Actinomycosis		2
Viral infections		3
Hypothyroidism		2
Miscellaneous		27
One case of each of the following: Blastomycosis, cerebral degeneration, cirrhosis of the liver, cryoglobulinemia, cryptococcosis, emphysema, gastrointestinal bleeding, hepatitis, hepatosplenomegaly of unknown causes, histoplasmosis, Hodgkin's disease, Huntington's chorea, hyperparathyroidism, hypertensive heart disease, hyperthyroidism, hyperuricemia (without gout), hypoproteinemia, lymphoma, nephrosis, ovarian agenesis, Paget's disease, persistent lactation, sexual precocity, sprue, thrombocytopenia, tuberculous lymphadenitis, and Wilson's disease.		

* No. in parentheses indicates the 3 false positive reactions. The test was negative in all other control cases.

The sera for the latter test were separated from the clot within hours after collection and frozen. Several days later, they were thawed, heated at 56°C for 30 minutes, absorbed with sheep red blood cells, and fractionated. The euglobulin fraction was either tested the next day or frozen and tested days later. The BFT was done on whole, unabsorbed, inactivated sera separated from the clot soon after collection and refrigerated but not frozen. The results are recorded in Table III. In both positive and negative reacting sera, there was agreement between both tests in 63 samples (98%) and discordant results in one (Table III). The titers of sera that gave positive reactions were often higher in the SSEA test than in the BFT. Comparison of the BFT and the Latex test was not possible because Latex particles were not available to the authors.

TABLE III. Comparison of BFT and SSEA Tests in 64 Sera.

BFT+ SSEA+	BFT− SSEA−	BFT− SSEA+	BFT+ SSEA−
23	40	1	0

Summary. 1) When bentonite particles of a selected size are "sensitized" by human gamma globulin (Fraction II) and then mixed with serum from patients with rheumatoid arthritis, they flocculate. This reaction was observed in 78% of adult patients with definite rheumatoid arthritis, in 2% of control patients, and in none of a group of normal subjects. Sera tested by the SSEA test and the BFT showed agreement in 98%. 2) Procedures for doing the BFT are described. The test is simple and rapid. The sera to be tested are not absorbed or fractionated.

We wish to acknowledge with gratitude the cooperation of Dr. Ruth Wichelhausen of Mt. Alto Veterans Admin. Hospital, Washington, D. C., and Dr. Herbert Kayden of Goldwater Memorial Hospital, New York, in permitting us to examine patients and collect blood samples used.

Addendum: The series of cases tested has increased since this paper was submitted for publication. It now includes a total of 82 verified cases of rheumatoid arthritis in adults in which the bentonite flocculation test was positive in 85.5% and 227 controls in which the test was negative in 97.4%.

1. Cecil, R. L., Nicholls, E. E., Stainsby, W. J., *Am. J. Path.*, 1930, v6, 619.

2. Ziff, M., *J. Chronic Dis.*, 1957, v5, 644.

3. Bozicevich, J., Tobie, J. E., Thomas, E. H., Hoyem, H. M., Ward, S. B., *Public Health Rep.*, 1951, v66, 806.

4. Ziff, M., Brown, P., Lospalluto, J., Badin, J., McEwen, C., *Am. J. Med.*, 1956, v20, 500.

Received November 1, 1957. P.S.E.B.M., 1958, v97.