TABLE I.
Effective Amounts of Converter Fraction.

2-stage tests		3-stage tests
Activate half of prothrombin in 40 min.	Accelerate prothrombin activation in presence of aged fraction "A"	Accelerate conversion of crude prothrombokinase
2/11 dilution	1/1,000 dilution 1 μg protein-N/ml	1/45,000 dilution 0.02 μg protein-N/ml

Figures refer to final dilution or concentration in the respective activation mixtures. A concentration of 0.02 μg protein-N per ml prothrombokinase mixture becomes 0.002 μg per ml when the mixture is diluted tenfold in prothrombin. The tests were performed as previously described.[1,2]

Previous comparison[1] of prothrombokinase to Factor V was based on Owren's tentative *concept* of V as the precursor of VI.[3] If his V *preparation* did not so function, the comparison does not apply to it. Crude prothrombokinase "may contain an activator complex with more than one significant component."[1] More than one transformation may occur in the complex.

Although the converter fraction is highly active, it may be far from pure, chemically. It *may* represent a thrombokinase which activates not only prothrombin, but also prothrombokinase. Several years ago,[4] it was reported that concentrated prothrombin changed to thrombin in the absence of ionic calcium, without addition of activators; and the possibility was left open that a contaminant was responsible. The possibility must be entertained that the converter contaminates highly purified clotting preparations, and might be responsible for certain effects occasionally attributed to prothrombin or thrombin.

[3] Owren, P. A., *Acta Med. Scand.*, 1947, suppl. 194.

[4] Milstone, J. H., *J. Gen. Physiol.*, 1942, **25**, 679.

Received August 9, 1949. P.S.E.B.M., 1949, **72**.

A Modification of the Hemagglutination Test for Rheumatoid Arthritis.* (17420)

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A diagnostic test for rheumatoid arthritis based upon the agglutination of sheep erythrocytes has been recently reported by Rose, Ragan, Pearce and Lipman.[1] Serum from patients with rheumatoid arthritis agglutinates sheep erythrocytes sensitized with sheep cell hemolysin to a titer at least 16 fold higher than that obtained with normal sheep cells. These are expressed as a differential titer $\left(\frac{\text{titer with sensitized cells}}{\text{titer with normal cells}}\right)$. The test has been confirmed but may be of limited value for diagnostic purposes.[2,3] In the performance of the test as described by Rose, *et al.*,[1] we

* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the authors.

[1] Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 1.

[2] Sulkin, S. E., Pike, R. M., and Coggeshall, H. C., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 475.

[3] Jawitz, E., and Hook, E. V., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 650.

have found that the factor in the serum responsible for the agglutination of normal sheep erythrocytes was present in the sera of both normal and rheumatoid arthritic individuals. This normal agglutinin is a variable factor and is independent of the capacity of the sera of patients with rheumatoid arthritis to agglutinate sensitized cells. It can be selectively absorbed without significantly influencing the titer against sensitized sheep cells. The titer thus obtained reflects the concentration of the factor associated with rheumatoid arthritis. At the same time the need for expressing the results of the test as a differential titer is eliminated. Based upon an absorption technic, a modification of the original test is herein described. A comparison of the results of the test as modified by us, the test as originally described by Rose, *et al.*,[1] and the streptococcus agglutination test is presented.[4-6]

Method and procedure. 1. *Preparation of Normal Sheep Erythrocytes.* One volume of sheep blood is mixed with 1.2 volumes of Alsever solution. Sufficient formaldehyde is added at collection to make a final concentration of 0.2%. This mixture stored at 4-6°C will usually last 4 to 6 weeks. The blood-Alsever mixture should be kept in the refrigerator for 2-3 days before use. The cells are washed 3 times in a graduated centrifuge tube with saline, centrifuging at 2000 rpm for 15 minutes between each washing. The supernatant of the last washing must be water clear. The packed cells are used for absorption. A 2% suspension in saline is also prepared from packed cells. Part of this is further diluted with saline to make a 1% suspension for use in the test.

Preparation of Sensitized Sheep Erythrocytes. Rabbit anti-sheep-erythrocyte hemolysin is prepared in concentrations of 1:4000 (2 units per cc) determined by the complement-fixation technic.[1] To the 2% suspension of erythrocytes as described above an equal volume of a 1:4000 dilution of amboceptor is added. The solution should be allowed to stand for at least 10 minutes before use.

Treatment of patient's serum. Clear serum is obtained from clotted blood and inactivated at 56°C for one-half hour. Serum may be stored in a deep freeze at −20°C for at least 5 months without significant loss of potency.[7] For absorption, 4 volumes of serum are mixed with 1 volume of packed sheep erythrocytes and allowed to remain at room temperature for 40 minutes with gentle shaking at intervals. The mixture is centrifuged and the clear serum is decanted into an additional volume of packed cells for another 40-minute incubation period at room temperature. The mixture is again centrifuged and the clear serum is used for the test. Two absorptions have been adequate to eliminate the normal agglutinating factor for normal sheep cells. Additional absorptive treatment is necessary in cases where the heterophile antibody associated with infectious mononucleosis is present. For this purpose, a 20% boiled ox erythrocyte suspension[8] is centrifuged and the supernatant fluid discarded. Four volumes of the serum is mixed with one volume of the packed sediment, incubated at 37°C for 1 hour, centrifuged, and the serum removed.

Procedure. Two series of two-fold dilutions of absorbed serum are made in 0.5 ml volumes of normal saline. In the first series, consisting of three tubes, the serum dilutions range from 1:3.5 to 1:14. To this series is added an equal volume (0.5 ml) of 1% normal sheep cells; the final dilution range is thus 1:7 to 1:28. The second series, consisting of 10 tubes, is similarly diluted (1:3.5 to 1:1792). To this series, 1% sensitized sheep cells are added in 0.5 ml volume, thus making the final dilutions 1:7, etc. The tubes are shaken to make a homogeneous suspension of the cells, and incubated in a water bath at 37°C for one hour. The tubes are then refrigerated overnight. Readings are made after gentle

4 Cecil, R. L., Nicholls, E. E., and Stainsby, W. J., *Arch. Int. Med.*, 1929, **43**, 571.

5 Cecil, R. L., Nicholls, E. E., and Stainsby, W. J., *Am. J. Med. Sc.*, 1931, **181**, 12.

6 Lipman, M. O., Laboratory of the Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital, New York, personal communication.

7 Unpublished data.

8 Kolmer, J. A., and Boerner, F., Approved Laboratory Technic, D. Appleton-Century Co., N. Y., 4th ed., 1945, p. 644.

TABLE I.
Comparison of Agglutination Titers Obtained with Unabsorbed and Absorbed Sera from Representative Cases.

Diagnosis	Rose test: Unabsorbed serum + Normal RBC	Rose test: Unabsorbed serum + Sensitized RBC	Rose test: Differential titer	Modified test: Absorbed serum + Normal RBC	Modified test: Absorbed serum + Sensitized RBC
Active peripheral rheumatoid arthritis (5 cases)	56	896	16	0	896
	56	1792	32	0	1792
	0	448	448	0	224
	14	112	8	0	224
	56	448	8	0	448
Rheumatoid spondylitis with active peripheral joint involvement (4 cases)	112	224	2	0	112
	0	896	896	0	1792
	14	1792	128	0	896
	0	896	896	0	1792
Rheumatoid spondylitis without peripheral joint involvement (3 cases)	7	7	1	0	0
	14	28	2	0	0
	28	56	2	0	0
Psoriatic arthritis (2 cases)	14	14	1	0	0
	7	14	2	0	0
Cirrhosis of liver	56	112	2	0	0
Hyperglobulinemia (2 cases)	14	14	1	0	0
	28	56	2	0	0
Brucellosis	7	14	2	0	0
Duodenal ulcer	0	14	14	0	0
Gout	14	28	2	0	0
Osteoarthritis	7	28	4	0	0
Rheumatic polyarthritis	28	28	1	0	0

RBC refers to sheep red blood cells.

shaking. For simplicity, 3 values are used: complete agglutination, partial agglutination, and no agglutination perceptible to the naked eye. The test is valid only if no agglutination is observed in the tubes containing the normal cells, thereby indicating complete absorption of the normal sheep cell agglutinin. Both the Rose test and the modified test were run concurrently; the same reagents were always used for both tests. The Rose test was performed as described[1] except that the serial dilutions were the same as those used in our modified test. The streptococcus agglutination test was also performed as described by Lipman.[6]

Results. Agglutinins for normal sheep erythrocytes (Forssman antibody) may be found in the sera of normal individuals as well as in those with rheumatoid arthritis. In our studies, it was found that sera obtained at intervals from the same patient showed variations in the normal agglutinin titer.[7] It is not yet clear whether these variations reflect a fluctuating agglutinin content or are associated with the varying agglutinability of different batches of sheep erythrocytes. Table I demonstrates that this normal agglutinin can be completely absorbed with normal sheep cells. As a result of this procedure, only the sera from patients with active peripheral rheumatoid arthritis retained the capacity to agglutinate sensitized cells. The sera from patients with diseases other than rheumatoid

TABLE II.
Differential Absorption of the Heterophile Factor from Serum of Patients with Infectious Mononucleosis and Patients with Rheumatoid Arthritis.

Diagnosis	Titer of unabsorbed serum +			Titer of serum absorbed with normal sheep cells +		Titer of serum absorbed with sheep and ox cells +	
	Normal RBC	Sensitized RBC	Differential titer	Normal RBC	Sensitized RBC	Normal RBC	Sensitized RBC
1. Infectious mononucleosis	896	1792	2	112	448	0	0
2. Rheumatoid arthritis	14	448	32	0	448	0	448
3. Mixture of equal vol. of No. 1 and 2	448	896	2	224	896	0	224
4. Infectious mononucleosis	896	896	1	112	448	0	0
5. Rheumatoid arthritis	28	3584	128	0	1792	0	1792
6. Mixture of equal vol. of No. 4 and 5	448	896	2	112	1792	0	1792

RBC refers to sheep red blood cells.

arthritis (control group) gave no significant agglutination. Removal of the normal (nonspecific) factor by absorption did not materially affect the titer of the factor associated with rheumatoid arthritis. The sera from cases of infectious mononucleosis could not be completely absorbed by the routine method described. The heterophile antibody continued to give a positive titer with normal sheep cells after absorption of the sera with sheep erythrocytes. However, additional absorption of the sera with boiled ox cells resulted in complete absorption of the heterophile factor. In cases of active peripheral rheumatoid arthritis, such multiple absorptions did not affect the final titer. Mixtures of sera from cases of rheumatoid arthritis and cases of infectious mononucleosis, when treated by this method and allowing for dilutions, retained only the titer of the rheumatoid arthritis factor (Table II).

Table III shows the distribution of agglutination titers using the modified test in 166 cases. Only cases with active peripheral rheumatoid arthritis (with the exception of 2 cases of infectious hepatitis) gave titers of 1:28 or higher with complete agglutination in the first 2 dilutions. For this reason, this value is considered to be the lower limit of a positive reaction. Using this criterion, 35 of 39 cases (90%) of active peripheral rheumatoid arthritis (8 of which also had spondylitis) gave positive values ranging from 1:28 to 1:3584. All 39 were unequivocal cases of active peripheral rheumatoid arthritis with x-ray evidence of small joint involvement. Negative reactions were obtained with the sera of all cases of inactive† peripheral rheumatoid arthritis, rheumatoid spondylitis without peripheral joint involvement or with inactive peripheral joint involvement, and in all cases of psoriatic arthritis. A group of 13 cases of arthritis in which the clinical course was not inconsistent with the picture of early peripheral rheumatoid arthritis but in which x-rays showed no evidence of joint involvement were all negative. All other forms of arthritis, non-rheumatoid in type, and the non-arthritic controls were negative. Ten cases of infectious hepatitis were tested. No determination could be made in one of these cases by our method because complete absorption could not be obtained. After repeated absorptions (10 times), the titer against unsensitized cells remained 1:28. In two other cases the titers were positive (Table III).

† A case was considered inactive if the patient was clinically in symptomatic remission with absence of swelling, pain and tenderness of involved joints and with the sedimentation rate normal.

TABLE III.
Distribution of Cases According to Agglutination Titer, Using the Modified Test.

Diagnosis	No. of cases	Agglutination titer 0	7	14	28	56	112	224	448	896	1792	3584
Active peripheral rheumatoid arthritis												
Without spondylitis	31			1	2(a)	2	3	4	8	5	5	1
With spondylitis	8	2			2		1			1	2	
Peripheral rheumatoid arthritis of questionable activity (1)	7	3		2	1(b)	1						
Inactive peripheral rheumatoid arthritis	8	5	1	1	1(b)							
Rheumatoid spondylitis without peripheral joint involvement	15	12	1	2								
Rheumatoid spondylitis with inactive peripheral joint involvement	7	5	2									
Psoriatic arthritis	9	6	1	2								
Arthritis, type undetermined, possibly rheumatoid (2)	13	10	2	1								
Other arthropathies (3)	30	27	2	1								
Non-arthritic controls except infectious hepatitis (4)	28	23	3	1	1(b)							
Infectious hepatitis	10(c)	3	2	2	1	1						

(1) Definitive evaluation of activity could not be made on the basis of clinical impression and laboratory data.

(2) Clinical course was not inconsistent with early rheumatoid arthritis, but x-ray studies showed no changes in affected joints.

(3) These include 10 cases of rheumatic polyarthritis, 7 cases of gout, 2 cases of osteoarthritis, and 11 cases of arthritis following a variety of infection.

(4) Among these are included 3 cases of hyperglobulinemia and 4 cases of infectious mononucleosis.

(a) One of these is considered negative because the results showed incomplete agglutination in first two tubes.

(b) Negative because of incomplete agglutination in first two tubes.

(c) Determination of titer in 1 case of infectious hepatitis could not be made because of incomplete absorption of the non-specific factor by the standard technic.

Table IV presents a summary of the results obtained with the sera of 166 patients by the modified test, the original Rose technic, and the streptococcus agglutination test. In the group of 39 patients with active peripheral rheumatoid arthritis of varying severity and duration, eight of whom also had spondylitis, the modified test gave a significantly higher percentage of positive reactions than either of the other two tests. Both the modified and the Rose tests were consistently negative in inactive peripheral rheumatoid arthritis, active or inactive spondylitis without peripheral joint involvement, and in psoriatic arthritis. In these groups, the streptococcus agglutination test, however, was found on several occasions to be positive. The group of 13 cases of arthritis in which no definitive diagnosis could be made were negative to all 3 tests employed. In all other arthropathies, including osteoarthritis, rheumatic polyarthritis, gout, *etc.*, the modified test and the Rose test were consistently negative. This was also true of the non-arthritic controls with the exception of infectious hepatitis in which two were positive by our test but negative by the Rose test.

Table V presents the findings in the 11 cases of active peripheral rheumatoid arthritis in which the Rose test was negative whereas the modified test was positive. It is to be noted that in each case the titer against the sensitized sheep red cells was significantly elevated but the differential titer in the Rose test fell below 16 because of the presence of a relatively high non-specific agglutinin titer. This non-specific factor was removed in the

TABLE IV.
Summary of Results Comparing Tests Employed.

Diagnosis	No. of cases	Modified test (1)		Rose test (2)		Streptococcus agglutination	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Active peripheral rheumatoid arthritis							
Without spondylitis	31	29	2	21	10	18	13
With spondylitis	8	6	2	3	5	5	3
Total	39	35	4	24	15	23	16
%	(100)	(90)	(10)	(61)	(39)	(58)	(42)
Peripheral rheumatoid arthritis-questionable activity	7	1	6	1	6	2	5
Inactive peripheral rheumatoid arthritis	8	0	8	0	8	2	6
Rheumatoid spondylitis without peripheral joint involvement	15	0	15	0	15	3	12
Rheumatoid spondylitis with inactive peripheral rheumatoid arthritis	7	0	7	0	7	0	7
Psoriatic arthritis	9	0	9	0	9	1	8
Arthritis, type undetermined, possibly early rheumatoid arthritis	13	0	13	0	13	0	13
Other arthropathies (3)	30	0	30	0	30	2	28
Non-arthritic controls except infectious hepatitis (4)	28	0	28	0	28	Not done	
Infectious hepatitis	9	2	7	0	9	1	8

(1) A titer of 28 or higher with complete agglutination in first 2 tubes is considered positive.
(2) A differential titer of 16 or greater is considered positive.
(3) See Note No. 3—Table III.
(4) See Note No. 4—Table III.

modified test, and the resulting titer was positive in each case. No sera were encountered in which the Rose test was positive and our modified test negative. It is of interest that in the 4 cases of active peripheral rheumatoid arthritis which were negative by our test, both the Rose and the streptococcus agglutination tests were also negative.

Discussion. The agglutination of unsensitized sheep cells by human sera is due to a Forssman antibody (natural amboceptor).[9] This antibody may be present in varying concentrations or may occasionally be absent. It is not related to the hemagglutinin associated with active rheumatoid arthritis nor is it necessary for the reaction with sensitized cells. The factor in the sera of patients with rheumatoid arthritis responsible for the agglutination of sensitized sheep cells behaves like an antibody. This is suggested by its reaction in selective absorption studies and its identity as part of the beta-gamma globulin complex.[1] Our results indicate that this substance is different serologically from the natural amboceptor of human serum as well as from the heterophile antibody of infectious mononucleosis.[7] Evidence of its specificity is found

[9] Kabat, E. A., and Mayer, M. M., Experimental Immuno-chemistry, Charles C. Thomas, Springfield, Ill., 1948, p. 135.

TABLE V.
Comparative Results in Those Cases of Active Rheumatoid Arthritis in Which the Rose Test Was Negative and the Modified Test Positive.

Diagnosis	Rose test			Modified test titer
	Normal RBC	Sensitized RBC	Differential titer	
Active peripheral rheumatoid arthritis (8 cases)	28	112	4	56
	7	56	8	28
	14	56	4	28
	28	224	8	224
	112	224	2	56
	14	112	8	224
	56	448	8	448
	14	112	8	448
Active peripheral rheumatoid arthritis with spondylitis (3 cases)	7	28	4	28
	112	224	2	112
	28	56	2	28

RBC refers to sheep red blood cells.

in the fact that it is present in a high percentage of proven cases of rheumatoid arthritis and cannot be demonstrated in any of the other arthritides or in a wide variety of unrelated medical controls. The occurrence of positive reactions in 2 cases of infectious hepatitis, both in low titer, may be due to the presence of altered globulins or unabsorbed antibodies associated with this disease. The concentration of rabbit anti-sheep amboceptor used in our test is based on its titer as determined by complement-fixation.[1] Our experience has shown that varying the concentration within critical limits will produce false reactions.[7] We believe the present method of determining the sensitizing unit of amboceptor is unsatisfactory. Standardization of the sensitizing unit should be based on the capacity of a particular pool of amboceptor to agglutinate a particular suspension of sheep cells rather than on its lytic capacity. Further studies on standardization along these lines are being pursued.

Natural amboceptor in human sera can contribute to the further sensitization of cells thereby increasing their agglutinability. When the rheumatoid arthritis hemagglutinin is present in high titer, the amount of naturally occurring amboceptor is not of practical significance because of dilution. For this reason there is no essential difference in the titer obtained by absorbed and unabsorbed sera against sensitized cells (Table I). However, when the rheumatoid arthritis hemagglutinin is of relatively low titer, the normal amboceptor in the unabsorbed serum exerts an additive effect with the rabbit anti-sheep amboceptor. In this event, there is relatively little dilution and the titer may then be higher than that obtained by absorbed serum (Table V). Based upon our data, a titer of 1:28 with complete agglutination in the first two tubes can be considered positive. Thus far, we have found that the concentration of the rheumatoid arthritis factor does not necessarily correlate with clinical severity of the disease. On the other hand, in no case of inactive disease was a positive titer obtained. A serious limitation of the test as a diagnostic tool is that it appears to become positive only when x-rays begin to show the joint changes characteristic of rheumatoid arthritis. In the group classified as arthritis of unknown type in which the course was not inconsistent with rheumatoid arthritis but where no x-ray changes characteristic of the disease were present, the test was negative. It will be noted that the streptococcus agglutination test and the Rose test were also negative in all of these cases.

If the hemagglutinin associated with rheumatoid arthritis is indeed an antibody, investigation is required to determine whether this antibody is the result of immunization by an etiological agent or whether it is analogous to the non-specific antibody of the comple-

ment-fixation reaction which is almost always associated with syphilis. If it is a specific antibody, its absence in all cases of active rheumatoid spondylitis without peripheral joint involvement and in psoriatic arthritis is suggestive of different etiologies. The modified test, by eliminating the natural amboceptor and by more closely reflecting the titer of the rheumatoid arthritis factor, is more sensitive than the original hemagglutination test. A comparison of the 3 tests employed indicates that a significantly higher incidence of positive reactions can be obtained by this procedure.

Summary. 1. The hemaggutination test for rheumatoid arthritis is modified by the selective absorption of non-specific factors. The details of this modification are described.

2. Of 39 cases with active peripheral rheumatoid arthritis with x-ray evidence of joint changes, 35 were positive by this test.

3. All cases of inactive peripheral rheumatoid arthritis, rheumatoid spondylitis without peripheral joint involvement and psoriatic arthritis tested were negative.

4. In presumptive cases of early rheumatoid arthritis without x-ray evidence of joint changes, the test was negative.

5. In 67 control cases, including a variety of non-rheumatoid arthropathies, the test was negative except in two cases of infectious hepatitis.

6. The modified test was positive in 90% of the proven cases of active peripheral rheumatoid arthritis tested; the Rose test in 61%; the streptococcus agglutination test in 58%. In all cases in which the modified test was negative, the Rose test and the streptococcus agglutination test were also negative.

The authors express their thanks to Dr. R. Schuman, Miss E. C. Arrowsmith, and Mr. O. Vasquez of the Clinical Laboratory Service for technical assistance.

Received August 12, 1949. P.S.E.B.M., 1949, **72**.

Methyl Alcohol Purification of the Rabbit Papilloma Virus.*† (17421)

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Rabbit papillomatosis is an endemic virus disease of cottontail rabbits readily transmissible to domestic rabbits. Serum from infected cottontail and domestic rabbits contains antibody which is capable of neutralizing the virus *in vitro*.[1] The virus can be obtained easily from cottontail rabbit papillomas, but ordinarily it is not recoverable from domestic rabbit growths.

The demonstration that extravasated fluid from large confluent cottontail papillomatous growths was effective in neutralizing active virus led Kidd[2] to suggest that the neutralizing principle represented antibody derived through escape from altered capillaries. Kidd[2] found no evidence of such "masking" in naturally occurring papillomas of cottontail rabbits.

When Kidd[2] obtained no virus from the growths of 8 of 11 domestic rabbits and little from the other 3, and found that the sera of the 11 rabbits had little or no neutralizing capacity, he concluded that it was improbable that antibody was primarily responsible for the masking of the virus in the growths produced in domestic rabbits.

The method developed by Cox, van der Scheer, Aiston and Bohnel[3] for the purification and concentration of influenza and other

* This investigation was supported by a grant from the American Cancer Society, on recommendation of the Committee on Growth, National Research Council.

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1 Shope, R. E., and Hurst, E. W., *J. Exp. Med.*, 1933, **58**, 607.

2 Kidd, J. G., *J. Exp. Med.*, 1939, **70**, 583.