Test Based on Normal Serum Component Implementing Fixation of Complement by Turkey Antiserum.*† (25219)

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Because heated turkey and chicken antiserum failed to fix complement in the presence of homologous antigen, Bushnell and Hudson (1) and later Rice(2) reported a direct complement fixation test on unheated fowl serum. However, they discarded this method because of anticomplementary quality of avian serum. In a direct complement fixation (CF) test for ornithosis antibody in turkey serum, Benedict and McFarland(3) were successful in using heated serum with a purified detergent extracted antigen or fresh unheated serum with the anticomplementary effect removed by adsorption of serum with kaolin. Brumfield and Pomeroy(4) first developed a direct test that used sufficient complement to overcome the anticomplementary effect. After publication of this method it was found that titers could be increased 2 to 8 fold in many sera by addition of fresh normal chicken (or turkey) serum to heated or unheated serum antibody. The antigen was not purified nor was the use of this normal labile factor restricted to the ornithosis system. Although the greater part of this work was performed with turkey antiserum and crude yolk sac ornithosis antigen, pilot experiments showed the principle applied to bacterial and viral systems where antigens were made in a variety of ways.

Materials and methods. Test for ornithosis antibody in turkey serum. 1) Veronal buffered saline, pH 7.3 containing 0.00015 M Ca Cl_2 and 0.0005 M Mg Cl_2 was used for diluting all reagents. 2) The suspension of sheep erythrocytes was stored at 4°C in Alsever's solution. Cells were washed at least 3 times or until supernate was clear, made up to 4% suspension and sensitized with equal volume of hemolysin containing 2 units hemolysin/ml. Final suspension 2% sensitized with 2 units hemolysin. 3) Bacto-Antisheep Hemolysin. Difco Lab. 4) Frozen guinea pig serum pre-tested for lack of reaction with psittacosis antigen. For each new pool of complement, dilution was determined by incubating 0.1 ml (half portion) at dilutions 1/20, 1/22, 1/24, with 0.2 ml of 10 different unheated turkey sera diluted 1/8 and 0.3 ml diluent. The hemolytic system was added after 1 hour (0.2 ml). Readings were made 30 minutes later. The highest dilution of complement in 0.1 ml (usually 2 units) causing hemolysis of indicator system was considered half portion. This amount was used in both serum and antigen controls. Twice that amount (usually 4 units) was used in the test. 5) More than 2 units of antigen, preferably 4 units was used. Antigen was titrated with 4 units of unheated fresh or frozen turkey serum. Antiserum of other species seldom gave the same titer. 6) For enhancement and stabilization of the test, normal chicken (N-ch) serum was incorporated in the system. N-ch should be from a pool. It was collected and stored as quickly as possible at –20°C to preserve the labile substance. This serum was tested for lack of reaction with antigen. 7) Usually test sera were not heated, they were tested fresh or after storage at –20°C. The order of addition of reagents was *mandatory*. (a) First, 0.2 ml of test serum dilution was dispensed, (b) followed by approximately 0.01 ml N-ch delivered from a 0.2 ml pipette. Because pools of N-ch vary, amount of N-ch was determined by following titration. From one set of immune serum dilutions, inactivated 30 min at 56°C, multiple titrations were made with increasing amounts of unheated N-ch in each set. The amount that gave maximal titer without causing anticomplementary antigen control was optimum. Amount of N-ch was usually 5% in relation to antigen aliquot (Table I). (c) This was followed by 3 to 4 units antigen contained in 0.2 ml. N-ch was also added to antigen control. (d) Complement was added last. After 1 hour at 37°C,

* Approved for publication as Scientific Journal Series Paper, Minn. Agric. Exp. Sta.

† This work was supported in part by grants from U. S. Dept. of Agric., U.S.P.H.S.

TABLE I. Titration for Optimal Amount of Normal Factor Required to Restore Titer of Inactivated Ornithosis Turkey Antiserum.

N-ch, %*	Serum dilution 1/32	1/64	1/128	1/256	Ag. control
0	0				Neg
2	4	4			"
3	4	4	3		"
5	4	4	4		"
7	4	4	4		"
10	4	4	4	2	2

* Calculated in relation to antigen aliquot.

the indicator system was added. Readings were made 30 minutes later or sooner if both serum and antigen controls were hemolyzed.

Results. Effect of temperature and kaolin treatment. The normal factor in chicken serum (N-ch) was destroyed at 56°C in 30 minutes but tolerated 45°C for 2 hours. It deteriorated at about the same rate as complement at room or refrigerator temperatures. It could be stored at –20°C for several weeks. The factor could be adsorbed out of diluted serum on kaolin and was found in water insoluble fraction of serum. The same substance was also present in immune serum and because of it, made titration of unheated serum possible. However, during serum dilution procedure, the factor was diluted out and fixation ceased even though antibody still might be present at higher dilutions (Table II).

Effect of serum dilution. Although addition of N-ch to antigen mixture has been used by us for some time, its lability to dilution was recognized only recently. For example, in procedure using 0.2 ml aliquots of serum dilution, antigen, and complement, 0.01 ml of N-ch, undiluted added separately gave a slightly higher titer than 0.1 ml 1/10 dilution and in turn both gave higher titers than when 0.2 ml of 1/20 dilution is added. The separate addition of 1/20 dilution gave titers comparable to antigen fortified with 5% N-ch. In each case the amount of N-ch was the same although activity was lost through dilution. The effect of dilution was also seen when serum was treated with kaolin. Very little of the factor was adsorbed from whole serum but at dilution of about 1/8 it was removed. The diluted adsorbed serum failed to fix complement as completely as heated serum. However, as was true with heated serum, the titer could be restored by replacing the labile factor (Table III).

TABLE III. Effect of Normal Serum on Heated or Kaolin Treated Antiserum and Ornithosis Antigen.

	Serum antibody titers			
	Unheated	Unheated + N-ch	Heated	Heated + N-ch
Serum				
Untreated	1/32	1/128	Neg	1/128
+ kaolin	1/16	"	"	"
½ + kaolin	1/16	"	"	"
¼ + "	1/ 8	"	"	"
⅛ + "	Neg	"	"	"

Replacement of labile factor in chicken antiserum was not as uniform as in turkey. There was evidence of zoning in certain sera even when unheated, which precludes use of this procedure for screening chicken sera until zoning can be blocked satisfactorily.

Discussion. The use of normal serum to induce or enhance immune reactions has been noted many times. Mathos and Kidd(5) observed lymphoma cells exposed to heated anti-lymphoma serum were not destroyed. However, if normal unheated serum was added, the cells failed to grow when transplanted. In

TABLE II. Effect of Heated or Kaolin Treated Normal Chicken Serum on Ornithosis Antibody Titrations.

Serum No.	Antigen treated	Serum dilutions 1/8	1/16	1/32	1/64	1/128	AgC
89*	Ag alone	4†					Neg
	+ N-ch untreated	4	4	4	4	4	"
	heated	4					"
	kaolin treated	4					"
23*	Ag alone	4	4				
	+ N-ch untreated	4	4	4	1		
	heated	4	4				
	kaolin treated	4	4				

* Turkey test serum, unheated. † 4 = complete inhibition; 1 = 25% hemolysis.

the review by Lamanna(6) on adhesion phenomenon, he pointed out the necessity for adding unheated normal serum to bring about adhesion reaction. Ross(7) discussing enhancement by normal serum of the cytotoxic action of antiserum on certain cancer cells, referred to normal serum factor as complement.

If this normal chicken serum factor is a component of complement, indications are that it is C′ 1 or associated with C′ 1. It is heat labile and found in water insoluble fraction of serum. Perhaps turkey antibody-antigen complexes cannot activate guinea pig C′ 1(8). However, it is difficult to understand why this labile factor must be added before the heated antibody and antigen combine. If chicken C′ 1, is substituting for guinea pig C′ 1, one would not anticipate the order of addition to be of such importance.

The normal factor also had some characteristics of properdin(9). It was adsorbed on kaolin, was destroyed at 56°C, tolerated 45°C for 2 hours and was found in water insoluble fraction of serum. Pillemer *et al.*(10) state that properdin was not destroyed at zero or −20°C. In contrast, this factor was inactivated after a few days at zero to 4°C and was greatly reduced in titer after several weeks at −20°C. Nor did this substance appear to form a complex with inulin(11). Neither whole serum nor serum diluted 1/10 lost activity after treatment with inulin in the range of 25 to 200 mg/ml of serum.

The N-ch factor appeared to be specific for systems in which turkey and chicken antiserum were used. It did not alter the titer of heated human, bovine, sheep, or in fact, pigeon antiserum nor did it always prevent zoning in all chicken sera.

Studies in progress will attempt to identify this normal component and to investigate a similar relationship in other serum species.

Summary. A factor in normal chicken serum when added to heated or unheated immune turkey serum-antigen complexes caused fixation of guinea pig complement. The factor was labile at 56°, unstable at room or refrigerator temperatures but remained active for several weeks at −20°C. It was found in the water insoluble fraction of serum and could be removed from diluted serum on kaolin.

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Received March 19, 1959. P.S.E.B.M., 1959, v102.

Antibody Response to Homografts. I. Technic of Lymphoagglutination and Detection of Lymphoagglutinins upon Spleen Injection.* (25220)

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There is considerable evidence that serum antibody responses of various types occur following transplantation of homologous tissues

* This investigation was supported by research grant from NIH, PHS and Cancer Research Co-ordinating Comm., Univ. of Calif.