period, and near the end of this interval an end-expiratory alveolar sample was secured. The inspired gas was then changed to a mixture of 10% CO_2 and 90% O_2, and measurements were continued for another 15 minutes at the end of which another alveolar sample was taken.

Results are summarized in Table II. Substitution of the CO_2-O_2 mixture for pure oxygen produced a rise of 1.3 mm in the alveolar pCO_2 (with lowering of the alveolar pO_2 by the same amount) and a drop of 0.4% in the saturation as determined from the oximeter. The change in % saturation is within the error of the instrument, but the expected change for the change in alveolar gas tensions is about 1%. The minute volume of ventilation was increased by 3 liters per minute or about 21%. These data indicate that no favorable physiological changes occurred from the addition of 10% CO_2 (9.4 mm) to the inspired gas at 40,000 feet. One cannot argue from this that it would not be advantageous to add CO_2 under some conditions, but it must be kept in mind that any rise in alveolar pCO_2 is always accompanied by an equivalent drop in alveolar pO_2 when nitrogen is absent. Probably the only condition in which substitution of a CO_2-O_2 mixture for pure O_2 might be advantageous is that of severe hypocapnia accompanied by a relatively mild hypoxia. In such a case the improvement due to the decreased hypocapnia might be greater than the impairment due to the increased anoxia.[10]

Summary. 1. The addition of 10.5% or 21.7% carbon dioxide to oxygen does not improve the altitude ceiling of mice.

2. Mice taken to altitude in 100% oxygen, with continuous ascent from 30,000 feet at a rate of about 6,000 feet per minute, collapse at a lower ceiling than mice which ascend from 40,000 feet in 2,000-foot steps with 5-minute stops at each stage.

3. In experiments on human subjects no favorable physiological effects were observed when 10% CO_2 was added to the inspired oxygen at 40,000 ft.

[10] Otis, A. B., Rahn, H., Epstein, M. A., and Fenn, W. O., *Am. J. Physiol.*, 1946, **146**, 207.

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Simplified Indirect Complement-Fixation Test Applied to Newcastle Disease Immune Avian Serum.

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(Introduced by Herald R. Cox.)

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The investigation in this laboratory of an avian disease presented a problem of accurate specific diagnosis. Because antigens suitable for the performance of the customary agglutination and precipitin tests were not available, attention was turned to the complement-fixation reaction. The development of the *indirect* method by Rice[1,2,3] made this approach more feasible since this technic apparently overcomes the difficulties which have prevented the use of the regular, or *direct*, method on avian serums.[4]

It also seemed worthwhile to attempt to simplify the *indirect* technic by using the common "serum or antigen-dilution" method and the observation of 100% hemolysis rather than the more detailed quantitative method for the standardization of all test com-

[1] Rice, C. E., *J. Immunol.*, 1947, **59**, 365.

[2] Rice, C. E., *Canadian J. Comp. Med.*, 1948, **12**, 130.

[3] Rice, C. E., *J. Immunol.*, 1948, **60**, 11.

[4] Rice, C. E., *Canadian J. Comp. Med.*, 1947, **11**, 236.

ponents.[5]

The work reported herein describes a study of the Newcastle disease (NCD) virus and immune serum which served as a convenient working model for the evaluation of such a test with avian serum. The hemagglutination-inhibition test was conducted simultaneously to provide a rough estimate of the reliability of the *indirect* complement-fixation results. Since chickens are known to possess an excellent capacity for antibody production, and their serums have been used for the study of various infections (influenza, psittacosis, etc.), a description of a simplified *indirect* complement-fixation test may prove of use to other investigators.

Materials and Methods. NCD immune avian serums. Serum samples were collected from chickens previously vaccinated against, or recovered from, Newcastle disease. *NCD immune guinea pig serum.* A group of guinea pigs received weekly subcutaneous injections of 0.5 ml each for 4 consecutive weeks of the antigen described below, and were bled 1 week after the final inoculation. Before pooling, the individual serum specimens were tested for complement-fixing activity by the *direct* method and only those showing titers of 1:128 or higher were included in the pool. All serum specimens were stored at 4°C.

NCD antigen. Allantoic fluids harvested from infected 11-day-old chick embryos were pooled and the virus concentrated by Sharples centrifugation. The sedimented virus was washed with 0.1 *M* phosphate buffer (pH 7.0) and resuspended in one-half the original volume of buffered saline solution[6] containing 0.05% formalin. A single lot of antigen was used in the complement-fixation and hemagglutination-inhibition tests described below.

Indirect Complement-Fixation Test. Prior to use, all serums were inactivated at 56°C for 30 minutes. Physiological salt solution was added as a diluent for all test components.

In the antigen-immune guinea pig serum titrations and in the tests proper, antigen, immune chicken serum and complement were incubated at 4 to 6°C for 2 hours. Immune guinea pig serum was then added and the tubes returned to the cold room for an additional 16- to 18-hour period (overnight). Sensitized sheep cells were added and hemolytic readings were made after an incubation period of 30 minutes at 37°C. All test components were employed in 0.2 ml amounts, thus making the total volume of 1.2 ml in all titrations and tests.

Sheep Cells. Defibrinated sheep erythrocytes were washed by centrifugation in saline solution, standardized spectrophotometrically, and adjusted to a concentration of 2%.[7]

Amboceptor. Commercial antisheep hemolysin was titrated in the usual manner and 2 units were used in all titrations and tests. A sensitized sheep cell suspension was prepared by mixing equal volumes of 2% cells and amboceptor and allowing the mixture to stand for 15 minutes at room temperature prior to use.

Complement. Serums of normal guinea pigs were pooled, 2 ml amounts were sealed in pyrex ampoules, and stored in the CO_2 icebox. Complement activity remained constant for at least 3 weeks.

For titration, amounts ranging from 0.08 to 0.22 ml (in increments of 0.02 ml) of a 1:30 dilution of complement were mixed with 0.2 ml of antigen (containing the optimal reacting amount) and with saline solution to bring the volumes to 0.8 ml. These mixtures were incubated for 1 hour at 37°C. Four-tenths ml sensitized cells were then added and hemolytic readings were made at the end of an incubation period of 1 hour at 37°C. The smallest amount of complement giving complete hemolysis was taken as 1 unit.

Preliminary titrations of NCD antigen and homologous immune guinea pig serum were carried out by the *direct* complement-fixation method using 2 units of complement. However, in the *indirect* tests, involving the deter-

[5] Wadsworth, A. B., Standard Methods of the Division of Laboratories and Research, New York State Department of Health, Albany, N. Y., Williams and Wilkins, Baltimore, Md., 1947, 3rd ed., pp. 361-465.

[6] Eagle, H., The Diagnosis of Syphilis, Mosby and Co., St. Louis, Mo., 1938.

[7] Kent, J. F., Bukantz, S. C., and Rein, C. R., *J. Immunol.*, 1946, **53**, 37.

TABLE I.
A Protocol Demonstrating the Optimal Proportions of Antigen and Immune Guinea Pig Serum to Be Used in the Indirect Complement Fixation Procedure.

Dilutions of antigen	Dilutions of immune guinea pig serum	Dilutions of immune chicken serum								
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Control*
1:8	1:70	|	|	|	|	++ +	+++ +++ +	+++ ++ +	+++++++ +++++++ ++++ ++++	+++++ +++++ ++++ ++
	1:80	|	|	|	|	|	|	|	+++++++ +++++ +++++ ++++	+++++ +++++ ++++ +
	1:90	|	|	|	|	|	|	|	+++++++ ++++ +++ +++	+++++ ++ +
	1:100	|	|	|	|	|	+	|	+++++++	+++++ +
	1:110	|	|	|	|	|	|	|	+++++++	+++++
	1:120	|	|	|	|	|	|	|	+++++++	+++++
	1:130	|	|	|	|	|	|	|	+++++++	+++++
1:16	1:20	|	|	|	|	+	+	|	+++++++	+++++
	1:30	|	|	|	|	|	|	|	+++++++	+++++
	1:40	|	|	|	|	|	|	|	+++++++	+++++
	1:50	|	|	|	|	|	|	|	+++++++	+++++
	1:60	|	|	|	|	|	|	|	+++++++	+++++

* Immune chicken serum replaced by saline solution.

mination of optimal amounts of antigen and immune guinea pig serum to be used in the titration of an immune chicken serum, it was found that 1.5 units of complement gave more uniform and consistent results than the larger quantity. This amount is approximately equivalent to the three 50% units employed by Rice.[2,3]

Titration of Antigen. A preliminary antigen titer of 1:8 was obtained by the simultaneous "cross titration" of both antigen and immune guinea pig serum by the *direct* method. It will be seen that this titration furnished a fairly reliable basic titer for use in the *indirect* method. The titration by the latter method is described in the following section.

Titration of Immune Guinea Pig Serum. That the antigen and known immune serum (guinea pig) be present in proportions which would permit maximal inhibition of fixation by the test serum (chicken), has been stressed by Rice.[1,2] A simplified procedure for the titration of these components was developed by simultaneously testing varying amounts of antigen and immune guinea pig serum in the presence of varying amounts of a known immune chicken serum, as illustrated in Table I.

The amounts of antigen and immune guinea pig serum giving complete inhibition of hemolysis in the control tube (*i.e.*, in the *absence* of immune chicken serum), and complete hemolysis in the presence of the smallest quantity of immune chicken serum, were selected as the optimal amounts to be used in subsequent tests. In this case an antigen dilution of 1:8 and an immune guinea pig serum dilution of 1:100 were found to be the properly reacting quantities of these components.

Test. The test technic was similar to that described by Rice[2] in which serial twofold dilutions of the suspected immune chicken serum were prepared in triplicate. Antigen was added to sets No. 1 and No. 2, saline solution to set No. 3. All tubes then received complement. After the preliminary cold incubation, immune guinea pig serum was added to set No. 1 and saline solution to sets No. 2

TABLE II.
Results Obtained by the Indirect Complement-Fixation Test of 59 Newcastle Disease Immune Chicken Serums Compared with Hemagglutination-Inhibition Test.*

Indirect complement fixation test	Hemagglutination-inhibition test									
	0	1:16	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
0	3	1		2		1				
1:2			2							
1:8			2	3	4	2				
1:16			1	2	6	2				
1:32				1	2	4	2			
1:64				1	1	1	1			
1:128					1	2	1			
1:256					3		1	3	1	
1:512								1		
1:2048									1	1

* The numbers in the body of the table refer to the number of serums giving the endpoint titers indicated.

and No. 3. Thus, set No. 1 represented the *indirect* test, No. 2 served as the *direct* test, and No. 3 as the control for anticomplementary activity of the test serum. Additional controls were included daily for hemolytic activity of antigen, immune guinea pig serum, and antigen plus immune guinea pig serum, as well as the titration of a standard NCD immune chicken serum.

The titer of the test serum was expressed in terms of the highest dilution which inhibited fixation of complement by exhibiting ++ hemolysis or more.

Hemagglutination-Inhibition Test. Titration of Antigen. Serial twofold dilutions of virus were prepared in buffered saline solution, pH 7.1.[6] To 0.5 ml amounts of each dilution was added an equal volume of 0.25% chicken red blood cells and the mixture incubated at room temperature for 1 hour. The highest final dilution* of virus giving complete agglutination of the red cells was designated as 1 virus unit.

Test. On the basis of the above titration, a dilution of antigen containing 16 units per ml was made and used as diluent in the preparation of twofold dilutions of the test serum. To 0.5 ml amounts of the serum-virus mixtures was added an equal volume of 0.25% chicken red blood cells. Tests were read after an incubation period of 1 hour at room temperature. The endpoint of serum-inhibition was that final dilution* of serum which completely inhibited agglutination, as indicated by the flowing of the red cell deposit to a degree comparable to the red cell saline control when the test tube rack was tilted and held at approximately 70 degrees from horizontal.

Discussion of Results. The data presented in Table II show that a good, general agreement exists between the titers obtained by the *indirect* complement-fixation and by the hemagglutination-inhibition tests. No anticomplimentary serums were encountered. Four serums were negative when examined by the *indirect* complement-fixation test, yet gave positive results ranging from 1:16 to 1:512 with the other method. Nevertheless, those serums which exhibited high titers by one method also did with the other. The same holds true for specimens of intermediate and low antibody titer.

No effort was made to determine whether or not the two tests were measuring the same serum component. It is quite possible that different antibodies are involved, hence too much emphasis should not be placed on the observed agreement of test results until further information has been obtained.

However, the results of this study suggest the possibility that the described simplified *indirect* complement-fixation test should pro-

* This term refers to the dilution of the original amount of a particular component in terms of its subsequent dilution by the addition of other components. Thus, an *initial* serum dilution of 1:8, in this case, has a *final* dilution of 1:16.

vide a useful tool in investigations involving the examination of serums of certain species in which the *direct* complement-fixation procedures are not feasible.

Summary. 1. A simplified procedure for the *indirect* complement-fixation test is described.

2. The serums of 59 Newcastle disease immune chickens were tested by the simplified *indirect* complement-fixation and by the hemagglutination-inhibition tests.

3. Good general agreement was observed between the titers obtained by the two methods.

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Preparation and Amino Acid Composition of Salmin and Clupein.

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Salmin, on acid hydrolysis, was shown to yield arginine, proline, serine, valine, alanine, and isoleucine.[1] This observation was confirmed and extended by Tristram[2] who also identified glycine. This paper confirms the presence of glycine in salmin and also indicates that clupein, from *Clupea pallasii*, differs from salmin in that it contains threonine but no glycine.

1. *Preparation.* The procedure to be described gives approximately 10 times the yield of protamine from frozen milt than was obtained by any of the older methods.[3] One thousand grams of frozen milt are thawed and rinsed with cold tap water, mixed with an equal volume of a 1% solution of citric acid, finely ground, and 2.5 liters of 1% of citric acid are added. After standing in the refrigerator over night, the insoluble material is removed by centrifugation and the residue is washed with citric acid. This precipitate is suspended in 4 liters of 0.2 *N* HCl for 18-24 hours at room temperature with occasional mixing. The precipitate is removed and washed with 0.2 *N* HCl. The combined filtrate and washings are heated to boiling and adjusted to pH 7-8 with dilute ammonia. The resulting precipitate is removed by centrifugation and ammonia is added to the filtrate, at 30°C, to bring the pH to 9.4-9.6. The protein precipitate is removed by filtration. The contaminating proteins may also be removed by precipitation with 14% ammonia at room temperature at pH 10.4-10.6. In contrast to the first method, the histones (?) so prepared are completely soluble in 0.2 *N* HCl.

The filtrate is cooled to 12-15°C. Aliquots of the cold solution are removed and graded quantities of freshly prepared 33% of HPO_3 are added. The oily precipitates of protamine metaphosphate are thrown down by centrifuging and are washed with cold water. The quantity of HPO_3 required for optimal precipitation is thus ascertained.

The calculated quantity of 33% HPO_3 is then added slowly with stirring to the main protamine solution and the resulting oil is removed and washed with cold water until the washings are free from ammonia. The protamine metaphosphate precipitate is dissolved in a minimum quantity of hot *N* sulfuric acid. The solution is boiled until a small aliquot no longer gives a white cloud when cooled below 5°C, *i.e.*, when the metaphosphate has been completely converted to the sulfate but no longer. Protamine sulfate is precipitated and dried by the addition of alcohol or acetone. The yield is 2.0-2.5% of the weight of the original milt.

More than 30 preparations of salmin sulfate or hydrochloride and 8 preparations of clupein sulfate have been prepared from samples of

[1] Block, R. J., and Bolling, D., *Arch. Biochem.*, 1945, **6**, 419.

[2] Tristram, G. R., *Nature*, 1947, **160**, 637.

[3] Kossel, A., Protamine und Histone; Leipsig, Wien, 1929.